EDITED BY
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SECOND EDITION

ACCURATE RESULTS IN THE CLINICAL LABORATORY

A GUIDE TO ERROR DETECTION AND CORRECTION



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Edited by

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Foreword (from the first edition)

Clinicians must make decisions from information presented to them, both by the patient and ancillary resources available to the physician. Laboratory data generally provide quantitative information, which may be more helpful to physicians than the subjective information from a patient's history or physical examination. Indeed, with the prevalent pressure for physicians to see more patients in a limited timeframe, laboratory testing has become a more essential component of a patient's diagnostic work-up, partly as a time-saving measure but also because it does provide information against which prior or subsequent test results, and hence patients' health, may be compared. Tests should be ordered if they could be expected to provide additional information beyond that obtained from a physician's first encounter with a patient and if the results could be expected to influence a patient's care. Typically, clinicians use clinical laboratory testing as an adjunct to their history taking and physical examination to help confirm a preliminary diagnosis, although some testing may establish a diagnosis, for example molecular tests for inborn errors of metabolism. Microbiological cultures of body fluids may not only establish the identity of an infecting organism, but also establish the treatment of the associated medical condition. In outpatient practice clinicians primarily order tests to assist them in their diagnostic practice, whereas for hospitalized patients, in whom a diagnosis has typically been established, laboratory tests are primarily used to monitor a patient's status and response to treatment. Tests of organ function are used to look for drug toxicity and the measurement of the circulating concentrations of drugs with narrow therapeutic windows is done to ensure that optimal drug dosing is achieved and maintained. The importance of laboratory testing is evident when some physicians rely more on laboratory data than a patient's own assessment as to how he or she feels, opening them to the criticism of treating the laboratory data rather than the patient.

In the modern, tightly regulated, clinical laboratory in a developed country few errors are likely to be made, with the majority labeled as laboratory errors occurring outside the laboratory itself. One study from 1995 showed that when errors were made 75% still produced results that fell within the reference interval (when perhaps they should not) [1]. Half of the other errors were associated with results that were so absurd that they were discounted clinically. Such results clearly should not have been released to a physician by the laboratory and could largely be avoided by a simple review by human or computer before being verified. However, the remaining 12.5% of errors produced results that could have impacted patient management. The prevalence of errors may be less now than previously, since the quality of analytical testing has improved, but the ramifications of each error are not likely to be less. The consequences of an error vary depending on the analyte or analytes affected and whether the patient involved is an inpatient or outpatient. If the patient is an inpatient a physician, if suspicious about the result, will likely have the opportunity to verify the result by repeating the test or other tests addressing the same physiological functions, before taking action. However, if the error occurs with a specimen from an outpatient causing an abnormal result to appear normal, that patient may be lost to follow-up and present later with advanced disease. Despite the great preponderance of accurate results clinicians should always be wary of any result that does not seem to fit with the patient's clinical picture. It is, of course, equally important for physicians not to dismiss any result that they do not like as a "laboratory error". The unexpected result should always prompt an appropriate follow-up. The laboratory has a responsibility to ensure that physicians have confidence in its test results while still retaining a healthy skepticism about unexpected results.

Normal laboratory data may provide some assurance to worried patients who believe that they might have a medical problem, an issue seemingly more prevalent now with the ready accessibility of medical information available through computer search engines. Yet both patients and physicians tend to become over-reliant on laboratory information, either not knowing or ignoring the weakness of laboratory tests, in general. A culture has arisen of physicians and patients believing that the published upper and lower limits of

the reference range (or interval) of a test define normality. They do not realize that such a range has probably been derived from 95% of a group of presumed healthy individuals, not necessarily selected with respect to all demographic factors or habits that were an appropriate comparative reference for a particular patient. Even if appropriate, 1 in 20 individuals would be expected to have an abnormal result for a single test. In the usual situation in which many tests are ordered together the probability of abnormal results in a healthy individual increases in proportion to the number of tests ordered. Studies have hypothesized that the likelihood of all of 20 tests ordered at the same time falling within their respective reference intervals is only 36%. The studies performed to derive the reference limits are usually conducted under optimized conditions such as the time since the volunteer last ate, his or her posture during blood collection and, often the time of day. Such idealized conditions are rarely likely to be attained in an office or hospital practice.

Factors affecting the usefulness of laboratory data may arise in any of the preanalytical, analytical or postanalytical phase of the testing cycle. Failures to consider these factors do constitute errors. If these errors occur prior to collection of blood or after results have been produced, while still likely to be labeled as laboratory errors because they involve laboratory tests, the laboratory staffs are typically not liable for them. Yet the staff does have the responsibility to educate those individuals who may have caused them to ensure that such errors do not recur. If practicing clinicians were able to use the knowledge that experienced laboratorians have about the strengths and weaknesses of tests it is likely that much more clinically useful information could be extracted from existing tests. Outside the laboratory, physicians rarely are knowledgeable about the intra- and interindividual variation observed when serial studies are performed on the same individuals. For some tests a significant change for an individual may occur when his/her test values shift from toward one end of the reference interval toward the other. Thus a test value does not necessarily have to exceed the reference limits for it to be abnormal for a given patient. If the preanalytical steps are not standardized when repeated testing is done on the same person, it is more likely that trends in laboratory data may be missed. There is an onus on everyone involved in test ordering and test performance to standardize the processes to facilitate the maximal extraction of information from the laboratory data. The combined goal should be of pursuit of information rather than just data. Laboratory information systems provide the potential to integrate all laboratory data that can then be integrated with clinical and other diagnostic information by hospital information systems.

Laboratory actions to highlight values outside the reference interval on their comprehensive reports of test results to physicians with codes such as “H” or “L” for high and low values exceeding the reference interval have tended to obscure the actual numerical result and to cement the concept that the upper and lower reference limits define normality and that the presence of one of these symbols necessitates further testing. The use of the reference limits as published decision limits for national programs for renal function, lipid or glucose screening has again placed a greater burden on the values than they deserve. Every measurement is subject to analytical error, such that repeated determinations will not always yield the same result, even under optimal testing conditions. Would it then be more appropriate to make multiple measurements and use an average to establish the number to be acted upon by a clinician?

Much of the opportunity to reduce errors (in the broadest sense) rests with the physicians who use test results. Over-ordering leads to the possibility of more errors. Inappropriate ordering, for example repetitive ordering of tests whose previous results have been normal, or ordering the wrong test or wrong sequence of tests to elucidate a problem should be minimized by careful supervision by attending physicians of their trainees involved in the direct management of their patients. Laboratorians need to be more involved in teaching medical students so that when they become residents their test ordering practices are not learned from senior residents who had learned their habits from the previous generation of residents. Blanket application of clinical guidelines or test order-sets has probably led to much misuse of clinical laboratory tests. Many clinicians and laboratorians have attempted to reduce inappropriate test ordering, but the overall conclusion seems to be that education is the most effective means. Unfortunately, the education needs to be continuously reinforced to have a lasting effect. The education needs to address the clinical sensitivity of diagnostic tests, the context in which they are ordered and their half-lives. Above all education needs to address issues of biological variation and preanalytical factors that may affect test values, possibly masking trends or making the abnormal result appear normal and *vice versa.*

This book provides a comprehensive review of the factors leading to errors in all the areas of clinical laboratory testing. As such it will be of great value to all laboratory directors and trainees in laboratory medicine and the technical staff who perform the tests in daily practice. By clearly identifying problem areas, the book lays out the opportunities for improvement. This book should be of equal value to clinicians, as to laboratorians, as they seek the optimal outcome from their care of their patients.

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Preface

Clinical laboratory tests have significant impact on patient safety and patient management because more than 70% of all medical diagnosis are based on laboratory test results. Physicians rely on hospital laboratories for obtaining accurate results and a falsely elevated or falsely lower value due to interference or pre-analytical errors may have significant influence on diagnosis and management of patients. Usually, a clinician questions the validity of a test result if the result does not match with clinical evaluation of the patient and calls laboratory professionals for interpretation. However, clinically significant inaccuracies in laboratory results may go unnoticed and mislead the clinicians into inappropriate diagnostic and therapeutic approaches, sometimes with very adverse outcomes. The first edition of "Accurate Results in the Clinical Laboratory: A Guide to Error Detection and Correction" was published by Elsevier in 2013 and was intended as a guide to increase awareness of both clinicians and laboratory professionals about the various sources of errors in clinical laboratory tests and what can be done to minimize or eliminate such errors. The first edition of the book had 22 chapters and was well received by readers. Due to success of the first edition, Elsevier requested a second edition of the book. In this edition, we not only updated all chapters of the first edition, but also added 9 new chapters so that the second book could be a concise but comprehensive guide for both clinicians and laboratory professionals to detect errors and sources of misinterpretation in the clinical laboratory and to prevent or correct such results.

Recently, biotin interferences in immunoassays that utilize biotinylated antibodies have been described which may lead to wrong diagnosis of Grave's disease due to falsely low TSH (sandwich assay that shows negative interference due to biotin) but falsely elevated T3, T4 and FT4 (competitive immunoassays showing positive biotin interferences). The Food and Drug Administration reported a fatal outcome due to a falsely low troponin value as a result of negative interference of biotin in the troponin assay. Because people take megadoses of biotin, this is a serious public health concern. Therefore, we added a new chapter (Chapter 8). Another new chapter (Chapter 16) is also added to discuss issues of false negative results in toxicology due to the difficulty in detecting certain drugs such as synthetic cathinone (bath salts) and synthetic cannabinoids (spices). Chapter 27 is also added to discuss sources of errors in flow cytometry. Moreover, Chapters 29–31 are also newly added chapters in the second edition.

The objective of this second edition book is to provide a comprehensive guide for laboratory professionals and clinicians regarding sources of errors and misinterpretation in the clinical laboratory and how to resolve such errors and identify discordant specimens. Accurate laboratory result interpretation is essential for patient safety. This book is intended as a practical guide to laboratory professionals and clinicians who deal with erroneous results on a regular basis. We hope this book will help them to be aware of such sources of errors and empower them to eliminate such errors when feasible or to account for known sources of variability when interpreting changes in laboratory results.

We would like to thank all contributors for taking time from their busy professional demands to write chapters. Without their dedicated contributions this project would never materialize. We also thank our families for putting up with us for the last year when we spent many hours during weekends and evenings writing chapters and editing this book. Finally our readers will be the judges of the success of this project. If our readers find this book useful, all the hard work of contributors and editors will be rewarded.

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CHAPTER

1

Variation, errors, and quality in the clinical laboratory

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INTRODUCTION

Recent studies demonstrated that *in vitro* diagnostic tests are performed in up to 96% of patients and that up to 80% of clinical decisions involve consideration of laboratory results [1]. In addition, approximately 40–94% of all objective health record data are laboratory results [2–4]. Diagnostic errors accounted for 26–78% of identified medical errors [5] and nearly 60% of malpractice claims [6], and were involved in 17% of adverse effects due to medical errors in one large study [7]. Undoubtedly, appropriate ordering and interpretation of accurate test results are essential for major clinical decisions involving disease identification, classification, treatment, and monitoring. Factors that constitute an accurate laboratory result involve more than analytical accuracy and can be summarized as follows:

1. The right test, with the right costs and right method, was ordered for the right patient, at the right time, for the right reason [8]: the importance of appropriate test selection cannot be minimized as studies have shown that at least 20% of all test orders are inappropriate [9], up to 68% of tests ordered do not contribute to improve patient management [10] and conversely tests were not ordered when needed in nearly 50% of patients [9].
2. The right sample was collected on the right patient, at the correct time, with appropriate patient preparation.
3. The right technique was used collecting the sample to avoid contamination with intravenous fluids, tissue damage, prolonged venous stasis, or hemolysis.
4. The sample was properly transported to the laboratory, stored at the right temperature, processed for analysis, and analyzed in a manner that avoids artifactual changes in the measured analyte levels.
5. The analytical assay measured the concentration of the analyte corresponding to its "true" level (compared to a "gold standard" measurement) within a clinically acceptable margin of error (the total acceptable analytical error (TAAE)).
6. The report reaching the clinician contained the right result, together with interpretative information, such as a reference range and other comments, aiding clinicians in the decision-making process.

Failure at any of these steps can result in an erroneous or misleading laboratory result, sometimes with adverse outcomes. For example, interferences with point-of-care glucose testing due to treatment with maltose containing fluids have led to failure to recognize significant hypoglycemia and to mortality or severe morbidity [11].

ERRORS IN CLINICAL LABORATORY

Errors can occur in all the steps in the laboratory testing process, and such errors can be classified as follows (see Table 1.1):

1. Pre-analytical steps, encompassing the decision to test, transmission of the order to the laboratory for analysis, patient preparation and identification, sample collection, and specimen processing.
2. Analytical assay, which produces a laboratory result.
3. Post-analytical steps, involving the transmission of the laboratory data to the clinical provider, who uses the information for decision making.

Although minimization of analytical errors has been the main focus of developments in laboratory medicine, the other steps are more frequent sources of erroneous

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TABLE 1.1 Types of error in the clinical laboratory.

PRE-ANALYTICAL	
Test ordering	
• Duplicate Order • Ordering provider not identified • Ordered test not performed (include add-ons)	• Order misinterpreted (test ordered <> intended test) • Inappropriate/outmoded test ordered • Order not pulled by specimen collector
Sample collection	
• Unsuccessful phlebotomy • Traumatic phlebotomy • Patient complaint about phlebotomy	• Check-in not performed (in the LIS) • Wrong patient preparation (e.g., non-fasting) • Therapeutic drug monitoring test timing error
Specimen transport	
• Inappropriate sample transport conditions • Specimen leaked in transit	• Specimen damaged during transport • Specimen damaged during centrifugation/analysis
Specimen identification	
• Specimen unlabeled • Specimen mislabeled: No Name or ID on tube • Specimen mislabeled: No Name on tube • Specimen mislabeled: Incomplete ID on tube • Wrong specimen label • Wrong name on tube • Wrong ID on tube • Wrong blood type	• Date/time missing • Collector's initials missing • Label illegible • Two contradictory labels • Overlapping labels • Mismatch requisition/label • Specimen information misread by automated reader
High pre-analytical turnaround time	
• Delay in receiving specimen in lab • Delay in performing test	• STAT not processed urgently
Specimen quality	
• Specimen contaminated with infusion fluid • Specimen contaminated with microbes • Specimen too old for analysis	• Hemolyzed • Clotted or platelet clumps
Specimen containers	
• No specimens received/ Missing tube • Specimen lost in laboratory • Wrong specimen type • Inappropriate container/tube type • Wrong tube collection instructions	• Wrong preservative/ anticoagulant • Insufficient specimen quantity for analysis • Tube filling error (too much anticoagulant) • Tube filing error (too little anticoagulant) • Empty tube

TABLE 1.1 Types of error in the clinical laboratory.—cont'd

ANALYTICAL	
• High analytical turnaround time • Instrument caused random error • Instrument malfunction • QC failure • QC not completed	• Test perform by unauthorized personnel • Results discrepant with other clinical or laboratory data • Testing not completed • Wrong test performed (different from test ordered)
POST-ANALYTICAL	
• Report not completed • Delay in reporting results • Critical results not called • Delay in calling critical results • Results reported incorrectly • Results reported incorrectly from outside laboratory • Results reported to wrong provider	• Reported questionable results, detected by laboratory • Reported questionable results, detected by clinician • Failure to append proper comment • Read back not done • Results misinterpreted • Failure to act on results of tests
OTHER	
• Proficiency test failure • Product wastage • Product not delivered timely • Product recall	• Employee injury • Safety failure • Environmental failure • Damage to equipment

results. An analysis indicated that pre-analytical errors accounted for 62% of all errors, with post-analytical representing 23% and analytical 15% of all laboratory errors [12]. The most common pre-analytical errors included incorrect order transmission (at a frequency of approximately 3% of all orders) and hemolysis (approximately 0.3% of all samples) [13]. Other frequent causes of pre-analytical errors include the following:

- Patient identification error
- Tube filling error, empty tubes, missing tubes, or wrong sample container
- Sample contamination or collected from infusion route
- Inadequate sample temperature

Particular attention should be paid to patient identification because errors in this critical step can have severe consequences, including fatal outcomes, for example, due to transfusion reactions or misguided therapeutic decisions. To minimize identification errors, health care systems are using point-of-care identification systems, which typically involve the following:

1. Handheld devices connected to the laboratory information systems (LIS) that can objectively identify the patient by scanning a patient-attached bar code, typically a wrist band.

2. Current laboratory orders can be retrieved from the LIS.
3. Ideally, collection information, such as correct tube types, is displayed in the device.
4. Bar-coded labels are printed at the patient's side, minimizing the possibility of misplacing the labels on the wrong patient samples.
5. After attaching to containers with the patient samples, bar-coded labels should be scanned to confirm that they were applied to the right patient, especially if any significant delay has occurred between label printing and sample collection. In this case, rescanning of patient-attached identifiers should be done in close temporal proximity to sample scanning.

Analytical errors are mostly due to interference or other unrecognized causes of inaccuracy, whereas instrument random errors accounted for only 2% of all laboratory errors in one study [12]. According to that study, most common post-analytical errors were due to communication breakdown between the laboratory and the clinicians, whereas only 1% were due to miscommunication within the laboratory, and 1% of the results had excessive turnaround time for reporting [12]. Post-analytical errors due to incorrect transcription of laboratory data have been greatly reduced because of the availability of automated analyzers and bidirectional interfaces with the LIS [12]. However, transcription errors and calculation errors remain a major area of concern in those testing areas without automated interfaces between the instrument and the LIS. Further developments to reduce reporting errors and minimize the testing turnaround time include auto-validation of test results falling within pre-established rule-based parameters and systems for automatic paging of critical results to providers.

When classifying sources of error, it is important to distinguish between *cognitive errors*, or mistakes, which are due to poor knowledge or judgment, and *noncognitive errors*, commonly known as slips and lapses, due to interruptions in a process that is routine or relatively automatic. Whereas the first type can be prevented by increased training, competency evaluation, and process aids such as checklists or "cheat sheets" summarizing important steps in a procedure, noncognitive errors are best addressed by process improvement and environment re-engineering to minimize distractions and fatigue. Furthermore, it is useful to classify adverse occurrences as *active*—that is, the immediate result of an action by the person performing a task—or as *latent* or *system errors*, which are system deficiencies due to poor design or implementation that enable or amplify active errors. In one study, only approximately 11% of the errors were cognitive, all in the pre-analytical phase, and approximately 33% of the errors were latent [12]. Therefore, the vast majority of errors are noncognitive slips and lapses performed by the personnel directly involved in the process. Importantly, 92% of the pre-analytical, 88% of analytical, and 14% of post-analytical errors were preventable. Undoubtedly, human factors, engineering, and ergonomics—optimization of systems and process redesigning to include increased automation and user-friendly, simple, and rule-based functions, alerts, barriers, and visual feedback—are more effective than education and personnel-specific solutions to consistently increase laboratory quality and minimize errors.

Immediate reporting of errors to a database accessible to all the personnel in the health care system, followed by automatic alerts to quality management personnel, is important for accurate tracking and timely correction of latent errors. In our experience, reporting is improved by using an online form that includes checkboxes for the most common types of errors together with free-text for additional information (Fig. 1.1). Reviewers can subsequently classify errors as cognitive/noncognitive, latent/active, and internal to laboratory/internal to institution/external to institution; determine and classify root causes as involving human factors (e.g., communication and training or judgment), software, or physical factors (environment, instrument, hardware, etc.); and perform outcome analysis. Outcomes of errors can be classified as follows:

1. Target of error (patient, staff, visitors, or equipment).
2. Actual outcome on a severity scale (from unnoticed to fatal).
3. Worst outcome likelihood if error was not intercepted on the same severity scale, since many errors are corrected before they cause injury.

Errors with significant outcomes or likelihoods of adverse outcomes should be discussed by quality management staff and laboratory directors to determine appropriate corrective actions and process improvement initiatives.

Clearly, efforts to improve accuracy of laboratory results should encompass all of the steps of the testing cycle, a concept expressed as "total testing process" or "brain-to-brain testing loop" [14]. Approaches to achieve error minimization derived from industrial processes include total quality management (TQM) [15]; lean dynamics and Toyota production systems [16]; root cause analysis (RCA) [17]; health care failure modes and effects analysis (HFMEA) [18,19]; failure review analysis and corrective action system (FRACAS) [20]; and Six Sigma [21,22], which aims at minimizing the variability of products such that the statistical frequency of errors is below 3.4 per million. A detailed description

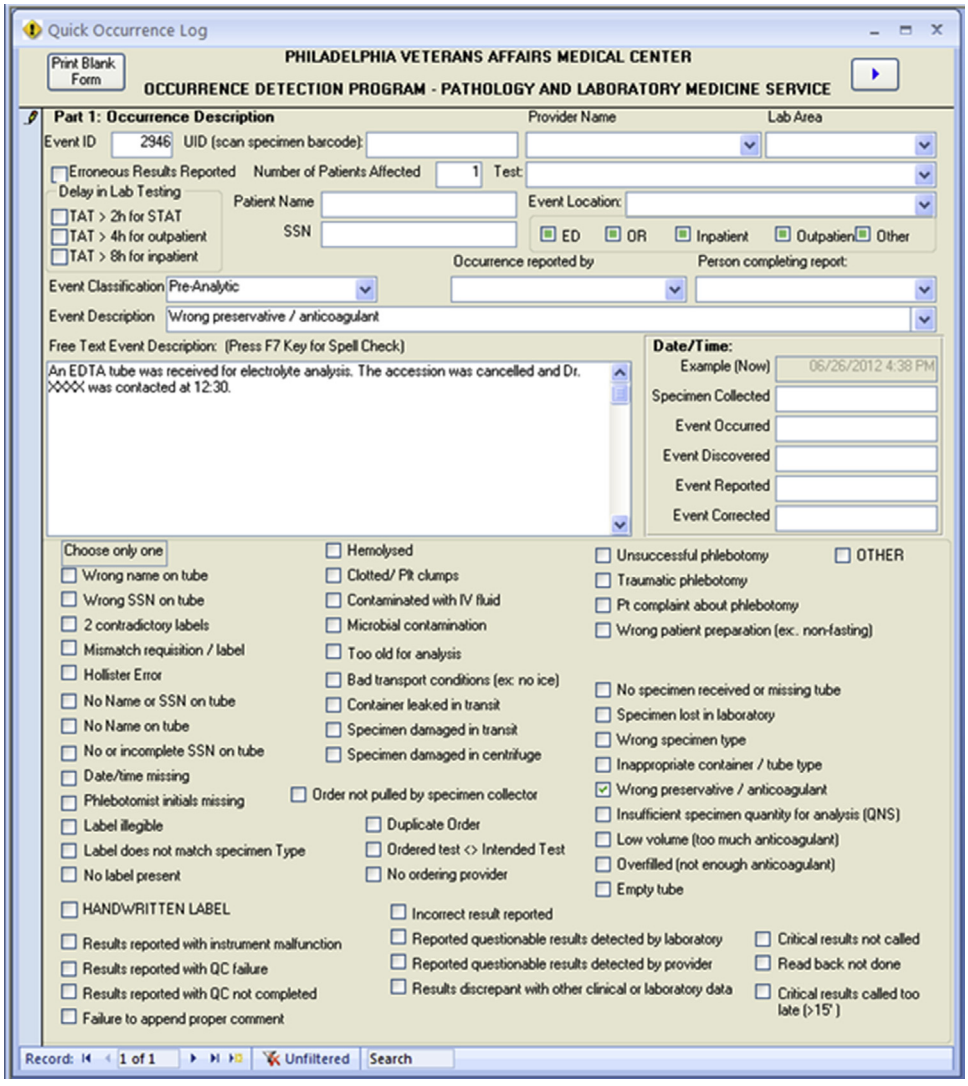


FIG. 1.1 Example of an error reporting form for the clinical laboratory.

of these approaches is beyond the scope of this book, but laboratorians and quality management specialists should be familiar with these principles for error prevention, error detection, and error management to achieve efficient, high-quality laboratory operation and patient care [15].

QUALITY IMPROVEMENT IN CLINICAL LABORATORY

Quality is defined as all the features of a product that meet the requirements of the customers and the health care system. Many approaches are used to improve and ensure the quality of laboratory operations. The concept of TQM involves a philosophy of excellence concerned with all aspects of laboratory operations that impact on the quality of the results. Specifically, TQM approaches apply a system of statistical process control tools to monitor quality and productivity (*quality assurance*) and encourage efforts to continuously improve the quality of the products, a concept known as *continuous quality improvement*. A major component of a quality assurance program is *quality control* (QC), which involves the use of periodic measurements of product quality, thresholds for acceptable performance, and rejection of products that do not meet acceptability criteria. Most notably, QC is applied to all clinical laboratory testing processes and equipment, including testing reagents, analytical instruments, centrifuges, and refrigerators. Typically, for each clinical test, external QC materials with known performance, also known as *controls*, are run two or three times daily in parallel with patient specimens. Controls usually have preassigned analyte concentrations covering important medical decision levels, often at low, medium, and

high concentrations. Good laboratory QC practice involves establishment of a laboratory- and instrument-specific mean and standard deviation for each lot of each control and also a set of rules intended to maximize error detection while minimizing false rejections, such as Westgard rules [23]. Another important component of quality assurance for clinical laboratories is participation in proficiency testing (or external quality assessment programs such as proficiency surveys sent by the College of American Pathologists), which involves the sharing of samples with a large number of other laboratories and comparison of the results from each laboratory with its peers, usually with reporting of the mean and standard deviation (SD) of all the laboratories running the same analyzer/reagent combination. Criteria for QC rules and proficiency testing acceptability should take into consideration the concept of total acceptable analytical error because deviations smaller than the total analytical errors are unlikely to be clinically significant and therefore do not need to be detected.

Total analytical error (TAE) is usually considered to combine the following (Fig. 1.2): (1) systematic error (SE), or bias, as defined by deviation between the average values obtained from a large series of test results and an accepted reference or gold standard value, and (2) random error (RE), or imprecision, represented by the coefficient of variation of multiple independent test results obtained under stipulated conditions (CV_a). Assuming a normal distribution of repeated test results, at the 95% confidence level, the RE is equal to 1.65 times the CV_a for the method; consequently.

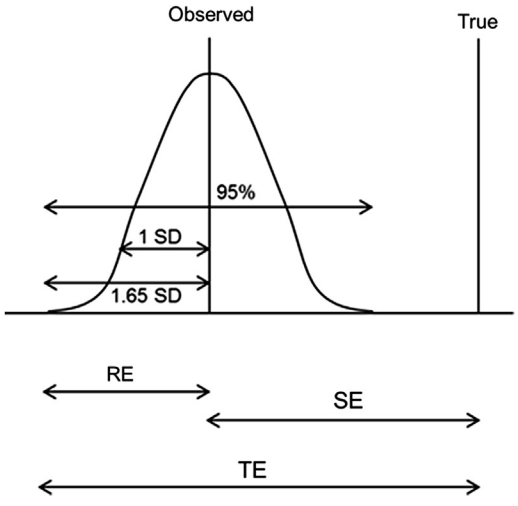


FIG. 1.2 Total analytical error (TE) components: random error (RE), or imprecision and systematic error (SE), or bias, which cause the difference between the true value and the measured value. Random error can increase or decrease the difference from the true value. Because in a normal distribution, 95% of the observations are contained within the mean ± 1.65 standard deviations (SDs), the total error will not exceed bias + 1.65 × SD in 95% of the observations.

$$TAE = 1.65 \times CV_a + bias$$

Clinical laboratories frequently evaluate imprecision by performing repeated measurements on control materials, preferably using runs performed on different days (between-day precision), whereas bias (or trueness) is assessed by comparison with standard reference materials with assigned values and also by peer comparison, where either the peer mean or median are considered the reference values.

One important concept that some clinicians disregard is that no laboratory measurement is exempt of error; that is, it is impossible to produce a laboratory result with 0% bias and 0% imprecision. The role of technologic developments, good manufacturing practices, proficiency testing, and QC is to identify and minimize the magnitude of the TAE. A practical approach is to consider the clinically acceptable total analytical error or TAAE for each test. Clinical acceptability has been defined by legislation (e.g., the Clinical Laboratory Improvement Act (CLIA)), by clinical expert opinion, and by scientific and statistical principles that take into consideration expected sources of variation. For example, Callum Fraser proposed that clinically acceptable imprecision, or random error, should be less than half of the intraindividual biologic variation for the analyte and less than 25% of the total analytical error [24]. The systematic error, or bias, should be less than 25% of the combined intraindividual (CV_w) and interindividual biological (CV_g) variation:

$$TAAE_{95\%} < 1.65 \times 0.5 \times CV_w + 0.25 \times \sqrt{CV_w^2 + CV_g^2}$$

Tables of intra- and interindividual biological variation, with corresponding allowable errors, are available and frequently updated [25]. See Table 1.2 for examples. Importantly, the allowable errors may be different at specific medical decision levels because analytical imprecision tends to vary with the analyte concentration, with higher imprecision at lower levels. Also, biological variation may be different in the various clinical conditions, and available databases are starting to incorporate studies of biologic variation in different diseases [25].

A related concept is the *reference change value* (RCV), also called *significant change value* (SCV)—that is, the variability around a measurement that is a consequence of analytical imprecision, within-subject biologic variability, and the number of repeated tests performed [24,26,27]. Assuming a normal distribution, at the 95% confidence level, RCV can be calculated as follows:

$$RCV_{95\%} = 1.96 \times \sqrt{2} \times \sqrt{CV_a^2 + CV_w^2}$$

Because multiple repeats decrease imprecision errors, if the change is determined from the mean of repeated

tests, the formula can be modified to take into consideration the number of repeats in each measurement (n_1 and n_2) [27]:

$$RCV_{95\%} = 1.96 \times \sqrt{\frac{2}{n_1 \times n_2}} \times \sqrt{CV_{a^2} + CV_{w^2}}$$

For example, for a serum creatinine measurement with an analytical imprecision (CV_a) of 7.6% and within-subject biologic variation of 5.95%, the RCV at 95% confidence is 26.8% with one measurement for each sample. With two measurements for each sample, the RCV is 18.9%. Therefore, a change between two results that does not exceed the RCV has a greater than 95% probability that it is due to the combined analytical and intraindividual biological variation; in other words, the difference between the two creatinine results (measured without repeats) should exceed 26.8% to be 95% confident that the change is due to a pathological condition. Conversely, for any change in laboratory values, the RCV formula can be used to calculate the probability that it is due to analytical and biological variation [24,26,27]. See Table 1.2 for examples of RCV at the 95% confidence limit, using published intraindividual variation and typical laboratory imprecision for each test. Ideally, future LIS should integrate available knowledge and patient-specific information and automatically provide estimates of expected variation based

TABLE 1.2 Allowable errors and reference change values for selected tests.

Test	CV_a	CV_w	CV_g	CLIA TAAE	Bio TAAE	Allowable imprecision	Allowable bias	RCV_{95}
Amylase	5.3	8.7	28.3	30	14.6	4.4	7.4	28.2
Alanine aminotransferase	2.8	19.4	41.6	20	27.48	9.7	11.48	54.3
Albumin	2.6	3.2	4.75	10	4.07	1.6	1.43	11.4
Alkaline phosphatase	4.2	6.45	26.1	30	12.04	3.23	6.72	21.3
Aspartate aminotransferase	2.2	12.3	23.1	20	16.69	6.15	6.54	34.6
Bilirubin total	10.0	21.8	28.4	20	26.94	10.9	8.95	66.5
Chloride	2.4	1.2	1.5	5	1.5	0.6	0.5	7.4
Cholesterol	2.7	5.95	15.3	10	9.01	2.98	4.1	18.1
Cortisol	5.3	21.7	46.2	25	30.66	10.85	12.76	61.9
Creatine kinase	3.6	22.8	40	30	30.3	11.4	11.5	64.0
Creatinine	7.6	5.95	14.7	15	8.87	2.98	3.96	26.8
Glucose	3.4	4.5	5.8	10	5.5	2.3	1.8	15.6
HDL cholesterol	3.3	7.3	21.2	30	11.63	3.65	5.61	22.2
Iron	2.5	26.5	23.2	20	30.7	13.3	8.8	73.8
Lactate dehydrogenase (LDH)	2.5	8.6	14.7	20	11.4	4.3	4.3	24.8
Magnesium	2.8	5.6	11.3	25	7.8	2.8	3.2	17.4
pCO_2	1.5	4.8	5.3	8	5.7	2.4	1.8	13.9
Protein, total	2.6	2.75	4.7	10	3.63	1.38	1.36	10.5
Thyroxine (T4)	4.8	4.9	10.9	20	7	2.5	3	19.0
Triglyceride	3.9	19.9	32.7	25	25.99	9.95	9.57	56.2
Urate	2.9	8.6	17.5	17	11.97	4.3	4.87	25.2
Urea nitrogen	6.2	12.1	18.7	9	15.55	6.05	5.57	37.7

All values are percentages. Bio TAAE, total allowable analytical error based on interindividual and intraindividual variation; CLIATAAE, total allowable analytical error based on Clinical Laboratory Improvement Act (CLIA); CV_a, analytical variability in a typical clinical laboratory; CV_g, interindividual variability; CV_w, intraindividual variability. Allowable imprecision = 50% of CV_w. Allowable bias = $0.25 \times \sqrt{CV_w^2 \times CV_g^2}$. RCV_{95}, reference change value at 95% confidence based on CV_w and CV_a.

Based on Westgard J. Desirable specifications for total error, imprecision, and bias, derived from intra- and inter-individual biologic variation. 2014. Available from: http://www.westgard.com/biodatabase1.htm.

on the previous formulas to facilitate interpretation of changes in laboratory values and guide laboratory staff regarding the meaning of deviations from expected results. In summary, the use of TAAE and RCV brings objectivity to error evaluation, QC and proficiency testing practices, and clinical decision making based on changes in laboratory values.

CONCLUSIONS

As in other areas of medicine, errors are unavoidable in the whole diagnostic process involving laboratory testing. A good understanding of the sources of error, frequently involving pre-analytical factors, together with a quantitative evaluation of the clinical significance of the magnitude of analytical errors, aided by the establishment of limits of acceptability based on statistical principles of analytical and intraindividual biological variation, are critical to design a quality program to minimize the clinical impact of errors in the clinical laboratory.

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CHAPTER

2

Errors in patient preparation, specimen collection, anticoagulant and preservative use: how to avoid such pre-analytical errors

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INTRODUCTION

Patient preparation and the specimen type are important pre-analytical factors to consider for laboratory assessment. Although the clinical laboratory has limited capabilities in controlling for the physiological state of the patient, such as biological rhythms and nutritional status, these variables as well as the effect of patient posture, tourniquets, and serum/plasma indices (hemolysis, icterus, lipemia) on measurement of analytes must be understood by both the clinical team and laboratory personnel. The most accessible specimen types include blood, urine, and oral fluid. The numerous functions associated with blood make it an ideal specimen to measure biomarkers corresponding to various physiological and pathophysiological processes. Blood can be collected by skin puncture (capillary), which is preferred when blood conservation and minimal invasiveness is stressed, such as in the pediatric population. Other modes of collection include venipuncture and arterial puncture, where issues to consider include the physical state of the site of collection and patient safety. Blood can also be taken from catheters and other intravascular lines, but care must be taken to eliminate contamination and dilution effects associated with heparin and other drugs. Clinical laboratory specimens derived from blood include whole blood, plasma, and serum. However, noticeable differences between these specimen types need to be considered when choosing the optimal specimen type for laboratory analysis. Such important factors include the presence of anticoagulants in plasma and in whole blood, hematocrit variability, and the differences in serum characteristics associated with blood coagulation. Anticoagulants for plasma and/or whole blood collection include ethylenediaminetetraacetic acid (EDTA), heparin, hirudin, oxalate, and citrate, which are available in solid or liquid form. Optimal anticoagulant-to-blood ratios are crucial to prevent clot formation while avoiding interference with analyte measurement, including dilution effects associated with liquid anticoagulants. Given the availability of multiple anticoagulants and additives, blood collection tubes should be filled according to a specified order to minimize contamination and carryover. Other factors to consider regarding blood collection tubes include differences between plastic and glass surfaces, surfactants, tube stopper lubricants, and gel separators, which all affect analyte measurement.

The second most popular clinical specimen is urine, which is essentially an ultrafiltrate of blood before elimination from the body and is the preferred specimen to detect metabolic activity as well as urinary tract infections. Proper timing must be ensured for urine collections depending on the need for routine tests, patient convenience, clinical sensitivity, or quantitation. Furthermore, proper technique is required for clean catch samples for subsequent microbiological examination. Certain urine specimens require additives to preserve cellular integrity for cytological analysis and to prevent bacterial overgrowth. It is important to recognize the pre-analytical variables that affect analyte measurement in patient specimens so that properly informed decisions can be made regarding assay selection and development as well as troubleshooting unexpected outcomes from laboratory analysis.

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BIOLOGICAL RHYTHMS AND LABORATORY TEST RESULTS

Predictable patterns in the temporal variation of certain analytes, reflecting patterns in human needs, constitute biological rhythms. Different analytes have different rhythms, ranging from a few hours to monthly changes. Awareness of such changes can be relevant to proper interpretation of laboratory results. These changes can be divided into circadian, ultradian, and infradian rhythms according to the time interval of their completion.

During a 24-h period of human metabolic activity, programming of metabolic needs may cause certain laboratory tests to fluctuate between a maximum and a minimum value. The amplitude of change of these circadian rhythms is defined as one-half of the difference between the maximum and the minimum values. Although, in general, these variations occur consistently, alteration in these natural circadian rhythms may be induced by artificial changes in sleep/wake cycles such as those induced by different work shifts. Therefore, in someone working an overnight ("graveyard") shift, an elevated blood iron level taken at midnight would be normal for that individual; however, the norm is for high iron levels to be seen only in early morning.

Patterns of biological variation occurring on cycles less than 24 h are known as ultradian rhythms. Analytes that are secreted in a pulsatile manner throughout the day show this pattern. Testosterone, which usually peaks between 10:00 a.m. and 5 p.m., is an example of an analyte showing this pattern.

The final pattern of biological variation is infradian. This involves cycles greater than 24 h. The example most commonly cited is the monthly menstrual cycle, which takes approximately 28–32 days to complete. Constituents such as pituitary gonadotropin, ovarian hormones, and prostaglandins are significantly affected by this cycle.

PATIENT PREPARATION

There are certain important issues regarding patient preparation for obtaining meaningful clinical laboratory test results. For example, glucose testing must be done after the patient has fasted overnight. These issues are discussed in this section.

Fasting

The effects of meals on blood test results have been known for some time. Increases in serum glucose, triglycerides, bilirubin, and aspartate aminotransferase are commonly observed after meal consumption. On the other hand, fasting will increase fat metabolism and increase the formation of acetone, β-hydroxybutyric acid, and acetoacetate both in serum and in urine. Longer periods of fasting (more than 48 h) may result in up to a 30-fold increase in these ketone bodies. Glucose is primarily affected by fasting because insulin keeps the serum concentration in a tight range (70–110 mg/dL). Diabetes mellitus, which results from either a deficiency of insulin or an increase in tissue resistance to its effects, manifests as an increase in blood glucose levels. In normal individuals, after an average of 2 h of fasting, the blood glucose level should be below 7.0 mmol/L (126 mg/dL). However, in diabetic individuals, fasting serum levels are elevated and thus constitute one criterion for making the diagnosis of diabetes. Other well-known examples of analytes showing variation with fasting interval include serum bilirubin, lipids, and serum iron.

Body position

Physiologically, blood distribution differs significantly in relation to body posture. Gravity pulls the blood into various parts of the body when recumbent, and the blood moves back into the circulation, away from tissues, when standing or ambulatory. These shifts directly affect certain analytes due to dilution effects. This process is differential, meaning that only constituents of the blood that are non-diffusible will rise because there is a reduction in plasma volume upon standing from a supine position. This includes, but is not limited to, cells, proteins, enzymes, and protein-bound analytes (e.g., thyroid-stimulating hormone, cholesterol, T4, and medications such as warfarin). The reverse will take place when shifting from erect to supine because there will be a hemodilution effect involving the same previously mentioned analytes. Postural changes affect some groups of analytes in a much more profound way—at times up to a twofold increase or decrease depending on whether the sample was obtained from a supine or an erect patient. Most affected are factors directly influencing homeostasis, including renin, aldosterone, and catecholamines. It is vital for laboratory requisitions to specify the need for supine samples when these analytes are requested.

WHOLE BLOOD, PLASMA, AND SERUM SPECIMENS FOR CLINICAL LABORATORY ANALYSIS

Approximately 8% of total human body weight is represented by blood, with an average volume in females and males of 5 and 5.5 L, respectively [1]. Whole

blood consists of a cellular fraction (~45%) composed of erythrocytes (red blood cells), leukocytes (white blood cells), and thrombocytes (platelets), and a liquid fraction (plasma) (~55%) that transports these elements throughout the body. Blood vessels interconnect all the organ systems in the body and play a vital role in communication and transportation between tissue compartments. Blood serves numerous functions, including delivery of nutrients to tissues; gas exchange; transport of waste products such as metabolic by-products for disposal; communication to target tissues through hormones, proteins and other mediators; and cellular protection against invading organisms and foreign material. Given these myriad roles, blood is an ideal specimen for measuring biomarkers associated with various physiological conditions, whether it is direct measurement of cellular material and surface markers or measurement of soluble factors associated with certain physiological conditions.

Plasma consists of approximately 93% water, with the remaining 7% composed of electrolytes, small organic molecules, and proteins. Various constituents of plasma are summarized in Table 2.1. These analytes are in transit between cells in the body and are present in varying concentrations depending on the physiological state of the various organs. Therefore, accurate analysis of the plasma is crucial for obtaining information regarding diagnosis and treatment of diseases. In clinical laboratory analysis, plasma can be obtained from whole blood through the use of anticoagulants followed by centrifugation. Consequently, plasma specimens for the clinical laboratory contain anticoagulants such as heparin, citrate, EDTA, oxalate, and fluoride. The relative roles of these anticoagulants in affecting analyte measurements are discussed later in this chapter. In contrast to anticoagulated plasma specimens, serum is the clear liquid that separates from blood when it is allowed to clot. Further separation of the clear serum from the clotted blood can be achieved through centrifugation. Given that fibrinogen is converted to fibrin in clot formation during the coagulation cascade, serum contains no fibrinogen and no anticoagulants. In the clinical laboratory, suitable blood specimens include whole blood, plasma, and serum. Key differences in these sample matrices influence their suitability for certain laboratory tests Table 2.2.

TABLE 2.1 Principal components of plasma.

Component	Reference range	Units
Sodium	136–145	mmol/L
Potassium	3.5–5.1	mmol/L
Bicarbonate	17–25	mmol/L
Chloride	98–107	mmol/L
Hydrogen ions	40	mmol/L
Calcium	8.6–10.2	mg/dL
Magnesium	1.6–2.6	mg/dL
Inorganic phosphate	2.5–4.5	mg/dL
Glucose	70–99	mg/dL
Cholesterol	<200 (Desirable)	mg/dL
Fatty acids	3.0	g/L
Total protein	6.4–8.3	g/dL
Albumin	3.2–4.6	g/dL
α-Globulins	0.1–0.3	g/dL
β-Globulins	0.7–1.2	g/dL
γ-Globulins	0.7–1.6	g/dL
Fibrinogen	145–348	mg/dL
Prothrombin	1	g/L
Transferrin	200–360	mg/dL

Whole blood

In addition to the obvious advantage of whole blood for the analysis of cellular elements, these specimens are also preferred for analytes that are concentrated within the cellular compartment. Erythrocytes can be considered to be a readily accessible tissue with minimal invasive procedures and may more accurately reflect tissue distribution of certain analytes. Examples of such analytes, including vitamins, trace elements, and certain drugs, are listed in Table 2.3. Erythrocytes are the most abundant cell type in the blood. In adults, 1 μL of blood contains approximately 4–6 million erythrocytes, 4000–11,000 leukocytes and 150,000–450,000 platelets [2]. (The ratio of erythrocytes: platelets: leukocytes is on the order of 900:60:1.) The hematocrit is the volumetric fraction of erythrocytes expressed as a percentage of packed erythrocytes in a blood sample after centrifugation. The normal range for adult males is 41–51%, and that for adult females is 36–45% [2]. Clearly, alterations in hematocrit will directly alter the available plasma water concentration, which in turn affects the measurement of water-soluble factors in whole blood.

A major use for whole blood specimens is for point-of-care analysis. Although point-of-care meters can be located in the clinical laboratory, the primary advantage of this technology is near-patient testing offering rapid and convenient analysis and using small sample volumes while the clinician is examining the patient. The most common point-of-care specimens are taken by skin puncture. Commonly called capillary blood, these samples are composed of a mixture of blood from the arterioles, venules and capillaries with interstitial and intracellular fluids. Furthermore, the extent of dilution with interstitial and intracellular fluid is also affected

TABLE 2.2 Components of whole blood, plasma, and serum matrices.

Whole blood	Plasma	Serum
Cellular elements		
Erythrocytes		
Leukocytes		
Thrombocytes		
Proteins	Proteins	Proteins (excluding fibrinogen)
Electrolytes	Electrolytes	Electrolytes
Nutrients	Nutrients	Nutrients
Waste (metabolites)	Waste (metabolites)	Waste (metabolites)
Hormones	Hormones	Hormones
Gases	Gases	Gases
May contain anticoagulants	*Contains* anticoagulants	Contains *no* anticoagulants
Patient on therapeutics		
Specimen additive		

TABLE 2.3 Examples of analytes measured in blood cell lysates.

Hematology	Vitamins	Trace elements	Drugs	Toxic elements
Hemoglobin	**Direct measurement**	Chromium	Cyclosporine	Cyanide
Red cell indices	Vitamin E	Selenium	Sirolimus (rapamycin)	Lead
Porphyrias	Vitamin B_1 (thiamine)	Zinc	Tacrolimus (FK506, Prograf)	Mercury
Cytoplasmic porphyrin metabolic enzyme activity	Vitamin B_2 (riboflavin) Also FMN, FAD			
	Vitamin B_6 (pyridoxine, pyridoxamine, pyridoxal) PLP			
	Biotin			
	Folic acid (folate)[a]			
	Pantothenic acid			
	Functional activity			
	Vitamin B_1 (thiamine) Transketolase			
	Vitamin B_2 (riboflavin) FAD-dependent glutathione reductase			
	Vitamin B_6 (pyridoxine, pyridoxamine, pyridoxal) AST, ALT activity			
	Vitamin B_{12} (cyanocobalamin) Deoxyuridine suppression test			
	Niacin NAD/NADP ratio			

ALT, alanine aminotransferase; *AST*, aspartate aminotransferase; *FAD*, flavin adenine dinucleotide; *FMN*, riboflavin-5′-phosphate; *NAD*, nicotinamide adenine dinucleotide; *NADP*, nicotinamide adenine dinucleotide phosphate; *PLP*, pyridoxal-5′-phosphate.
[a]*Plasma or serum folate measurements are usually preferred.*

by the hematocrit. Because arteriolar pressure is greater than that of capillaries and venules, arterial blood will predominate in these samples [2,3]. Given these physiological differences, analytes measured in whole blood do not exactly match results obtained from analysis of plasma or serum samples. Indeed, there is less water inside erythrocytes compared to the plasma; therefore, levels of hydrophilic analytes such as glucose, electrolytes, and water-soluble drugs will be lower in the capillary whole blood [4].

As mentioned previously, it is apparent that changes in both hematocrit and plasma water levels contribute to the discrepancy in analyte measurements between whole blood and plasma methods. However, in the case of point-of-care glucose meters, it was proposed and later adopted by the International Federation of Clinical Chemistry that a general conversion factor of 1.11 be applied to obtain plasma-equivalent glucose molarity [5,6]. Although this was an attempt to produce more harmonized results regarding glucose measurement and reduce clinical misinterpretations, the application of a general conversion factor does not take into account the wide variations in both hematocrit and plasma water levels exhibited by some patient subpopulations. Indeed, the proportion of total errors exceeding 10 and 15% in glucose measurements has been found to increase with patient acuity [7]. For this reason, interpretation of analyte measurements in whole blood should be sensitive to the hemodynamic status of the patient.

Plasma versus serum specimens

Although it is clear that there are certain advantages to whole blood specimens in point-of-care and hematological testing, plasma and serum are the preferred blood specimens for measuring soluble factors in the clinical laboratory. In addition to their previously mentioned discrepancies in composition, plasma and serum exhibit variations in the concentration of certain analytes. Certainly, the coagulation cascade contributes to consumption of some substances (e.g., fibrinogen, platelets, and glucose) and to the release of others (e.g., potassium, lactate, lactate dehydrogenase (LD), phosphate, and ammonia). For example, the presence of fibrinogen in plasma contributes significantly to the higher levels (±5%) of total protein compared to serum [8]. Conversely, the release of elements or cell lysis associated with the coagulation cascade is responsible for the increase in potassium (±6%), inorganic phosphate (±11%), ammonia (±38%), and lactate (±22%) in serum compared to plasma [8]. Furthermore, anticoagulants, preservatives, and other additives that aid or inhibit coagulation may interfere with the assay, as discussed later. Also, the presence of fibrinogen may interfere with chromatic detection or binding in immunoassays or the appearance of a peak that may simulate a false monoclonal protein in the gamma region during protein electrophoresis [8,9].

There are many advantages to using plasma over serum for clinical laboratory analysis; however, for some analytes, serum is preferred over plasma. Characteristics of serum and plasma specimens are compared below.

Sample volume

If blood is allowed to clot and is then centrifuged, approximately 30–50% of the original specimen volume is collected as serum. Conversely, plasma constitutes approximately 55% of the volume of uncoagulated blood after centrifugation. Therefore, the higher yield associated with plasma samples is generally preferred, especially when sample volume may be critical as in the case of the pediatric population, smaller patients, or in special cases in which blood volume needs to be conserved.

Sample preparation time

The process of clotting requires at least 30 min under normal conditions without coagulation accelerators. Furthermore, coagulation may still occur post-centrifugation in serum samples. Therefore, another advantage of plasma is that analyte determinations can be achieved in whole blood prior to plasma separation provided that a suitable anticoagulant has been used. For example, an anticoagulated whole blood specimen may be used for point-of-care measurements followed by plasma separation, which would avoid the delay associated with obtaining an additional specimen for laboratory analysis.

In vitro hemolysis

In addition to the time delay associated with blood clotting, there is an increased risk of lysis and consequent false increases in many intracellular analytes such as potassium, iron, and hemoglobin (hgb) released from erythrocytes in serum specimens. Therefore, it is advised to separate the serum as quickly as possible. Conversely, plasma separation can be achieved at higher centrifugal speeds without risking the initiation of hemolysis and thrombocytolysis [8].

Specimen composition

Anticoagulants and additives in plasma specimens can directly interfere with the analytical characteristics of the assay, protein binding with the analyte of interest, and sample stability. Furthermore, liquid anticoagulants may lead to improper dilution of the sample. For example, blood drawn in tubes with sodium citrate is diluted by 10%, but this may increase depending on whether the draw is complete. Moreover, incomplete mixing with anticoagulants can lead to the risk of clot formation. Also, the choice of anticoagulant will depend on their respective influences on the various assays offered by the clinical laboratory, and tubes with anticoagulants and additives are often more expensive.

ANTICOAGULANTS AND PRESERVATIVES, ORDER OF DRAW, SEPARATOR TUBE GEL INTERFERENCE AND VOLUME

Selection of evacuated blood collection tubes (BCT) for specimen collection has a major impact on clinical laboratory results. Appropriate quality processes must be maintained to ensure accurate and reliable laboratory results. Kricka et al. [10] demonstrated how laboratories can establish their own processes using control materials to verify interferences in the BCT they use. Bowen et al. [11] provided a comprehensive review of the different BCT components that can contribute to analytical errors in the clinical laboratory. Table 2.4 summarizes the components of BCT and indications for use. The inclusion of additives is necessary for reducing pre-analytical variability and for faster turnaround times in the laboratory. BCT are available with a variety of labeling options and stopper colors as well as a range of draw volumes. Additives are designated to preserve or stabilize analytes by inhibiting metabolic enzymes. Additives such as clot activators are added to plastic BCT to facilitate clotting. Stoppers and stopper lubricants are generally manufactured to facilitate capping, de-capping, and blood flow during collection and to minimize adsorption. Separator gels are used to separate packed blood cells from serum or plasma. Anticoagulants are used to prevent coagulation of blood or blood proteins. For a summary of collection additives, refer to Table 2.5.

Plastic and glass tubes

Most clinical laboratories have routinely moved away from glass to plastic BCT to comply with occupational health and safety standards because plastic BCT are safer to use and reduce potential exposure to blood [12]. Compared to glass, the polyethylene terephthalate materials used in BCT are generally unbreakable, can withstand high centrifugation speeds, are inert to adsorption of analytes, are lighter, and can be easily incinerated for disposal. In contrast to plastic surfaces, the relatively hydrophilic surface of glass allows for clot adherence and is ideal for blood flow. Also, the silica surface of glass greatly facilitates the clotting of blood, allowing for a cleaner separation of the clot from serum during centrifugation. For these reasons, the interior surface of plastic collection tubes and stoppers is now routinely spray-coated with surfactants and silicate polymers to make the surface properties similar to those of glass. A few clinically significant differences between glass and plastic exist for a variety of different tests ranging from general chemistry to special chemistry, molecular testing, and hematology.

TABLE 2.4 Components in evacuated blood collection tubes and indications for use.

Components	Indications
Tube wall	Plastic or glass: Plastic preferable for safety reasons
Stopper	Inert plasticizers
Surfactants	Silicone minimizes adsorption of analytes, cells
Stopper lubricants	Ease of capping, de-capping
Clot activator	Promote clotting to obtain serum in plastic collection tubes

Surfactants

Commercially available BCT may contain different types of surfactants that are often not listed in the manufacturer's package insert but are commonly silicone-based polymers. Although these are considered to be inert, there have been reports of interferences in clinical assays. Generally, surfactants can bind nonspecifically, displace from solid matrix and complex with or mask detection of signal antibodies in immunoassay reagents, contributing to increases in absorbance and turbidity to cause interferences [13]. Bowen et al. [14,15] showed that the surfactant Silwet L-720 used in BD SST collection tubes gave falsely elevated total triiodothyronine (TT3) by the Immulite 2000/2500 immunoassay. One mechanism shown was that increasing surfactant concentrations dose dependently desorbed the capture antibody from the solid phase among other nonspecific effects. However, this was method-dependent because TT3 levels were unaffected by the AxSYM immunoassay, in which antibodies are adsorbed onto the solid phase with more robust binding [14,15]. This BCT manufacturer confirmed similar interferences for a variety of other immunoassays (folate, vitamin B_{12}, follicle-stimulating hormone, hepatitis B surface antigen, cancer antigen 27, and cortisol) on a variety of different instrument platforms and has since decreased the surfactant content to reduce this interference [16,17].

Stoppers and stopper lubricants

Stopper lubricants containing glycerol or silicone make capping and de-capping of tubes easier, as well as minimize adherence of cells and clots to stoppers. Standard red-topped BCT are contaminated with zinc, aluminum and magnesium; and all contain varying amounts of heavy metals [18]. For this reason, most labs have specific requirements for collection of specimens for heavy metal assay. Components such as tris

TABLE 2.5 Blood collection tubes, additives, and general applications.

Tube type	Additive	Stopper color	Application
SERUM			
Glass	None	Red	Tests that cannot be collected into SST tubes
Plastic (acid washed)	None	Royal blue (red band label)	Trace elements serum (copper, zinc, aluminum, chromium, nickel)
Plastic	Clot activator	Red/black	Tests that cannot be collected with gel; some therapeutic drugs (antidepressants)
Plastic also called SST	Clot activator + gel separator	Gold	Many chemistry and immunochemistry tests; hepatitis tests
Plastic also called RST	Thrombin + gel separator	Orange	Rapid clotting (5 min); general chemistry tests for acute care requiring urgent turnaround time
PLASMA OR WHOLE BLOOD			
Plastic also acalled PST	Lithium-heparin + gel separator	Light mint green	Most chemistry tests for acute care requiring fast turnaround time, ammonia
Heavy metal free	Na_2EDTA or K_2EDTA	Royal blue (with lavender or blue band on label)	Trace elements blood (lead, arsenic, cadmium, cobalt, manganese, mercury, molybdenum, thallium)
Plastic	Acid citrate dextrose solution "A" (ACDA)	Pale yellow	Flow cytometry (CD3, CD4, CD8); HLA typing
Plastic	Sodium fluoride, potassium oxalate, iodoacetate	Gray	Preserves glucose up to 5 days; lactate, glucose (tolerance)
Plastic	Sodium, lithium, or ammonium heparin (no gel)	Dark green	Amino acids, blood gases, glucose phosphate dehydrogenase (G6PD)
Plastic	Sodium citrate	Light blue	PT (INR), PTT, other coagulation tests
Glass	Sodium citrate	Black	Erythrocyte sediment rate
Glass	Sodium heparin	Dark green	Toxicology and nutritional tests
	K_2EDTA or K_3EDTA	Lavender/Purple	Routine hematology, pretransfusion (blood bank), HbA_{1c}, antirejection drugs, parathyroid hormone

(2-butoxyethyl) phosphate from rubber stopper tubes leaching into the blood during collection have been shown to displace drugs from binding proteins in blood [19]. Manufacturers have reformulated stopper plasticizer content to minimize this effect. Triglyceride assays that measure glycerol can be falsely elevated by such effect. Stopper components and stopper lubricants can also be a potential source of interference with mass spectrometry-based assays [20].

Serum separator gel tubes (SST)

Separator gels are thixotropic materials that form a physicochemical barrier after centrifugation in BCT to separate packed cells from plasma or serum. Delay in separating clot results in intracellular leakage of potassium, phosphate, magnesium, and LD into serum, plasma, or whole blood [21]. Ease of use of a single centrifugation step, improved specimen analyte stability, reduced need for aliquoting, convenient storage, and transport in a single primary tube are reasons for preferential usage of SST in the clinical laboratory. It is very important to follow manufacturer protocols for laboratory conditions such as proper tube mixing after collection, centrifugation acceleration and deceleration speeds, temperature, and storage conditions. Noncompliance may result in unexpected degradation of separator gel or release of gel components. Gel and oil droplets interfere with accuracy and liquid-level sensing of instrument pipettes, coat cuvettes, and bind

to solid phase in heterogeneous immunoassays. Re-centrifugation, inadequate blood-draw volume, storage time, temperature, and drug adsorption may affect laboratory results. Hydrophobicity of the drug, length of time on separator gel, storage temperature, and methodology sensitivity are important considerations with regard to the stability of therapeutic drugs in BCT. Dasgupta et al. [22] demonstrated that hydrophobic drugs such as phenytoin, phenobarbital, carbamazepine, quinidine, and lidocaine are adsorbed onto the gel with significant decreases (ranging from 5.9 to 64.5%) in serum Vacutainer SST tubes. Reformulation of the separator gel in SST II tubes significantly reduced absorption and improved performance [23]. Schouwers et al. [24] demonstrated minimal effect of separator gel in Starstedt S-Monovette serum tubes for the collection of four therapeutic drugs (amikacin, vancomycin, valproic acid, and acetaminophen) and eight hormones and proteins.

Anticoagulants

Anticoagulants are used to prevent coagulation of blood or blood proteins to obtain plasma or whole blood specimens. The most routinely used anticoagulants are EDTA, heparin (sodium, ammonium, or lithium salts), and citrates (trisodium and acid citrate dextrose). Anticoagulants can be powdered, crystallized, solids, or lyophilized liquids. The optimal anticoagulant: blood ratio is essential to preserve analytes and prevent clot or fibrin formation via various differing mechanisms.

In most clinical laboratories, potassium EDTA is the anticoagulant of choice for the complete blood count, as recommended by the International Council of Standardization in Hematology [25] and the Clinical and Laboratory Standards Institute [26]. Dipotassium, tripotassium, or disodium salts of EDTA are used as dry or liquid additives in final concentrations ranging from 1.5 to 2.2 mg/mL blood when the evacuated BCT is filled correctly to its stated draw volume. EDTA acts as a chelating agent to bind cofactor divalent cations (mainly calcium) to inhibit enzyme reactions in the clotting cascade—particularly the conversion of prothrombin to thrombin and subsequent inhibition of the thrombolytic action on fibrinogen to fibrin necessary for clot formation [27]. For this reason, EDTA plasma is not recommended for coagulation tests such as prothrombin time (PT) and activated partial thromboplastin time (aPTT) [28]. EDTA is an excellent preservative of blood cells and morphology parameters. Stability is 48 h for hgb and 24 h for erythrocytes. Because the hypertonic activity by EDTA can alter erythrocytic indices and hematocrit, smears should be made within 2 or 3 h of the blood draw. The white blood cell count remains stable for at least 3 days in EDTA anticoagulated blood stored at room temperature. EDTA is adequate for platelet preservation; however, morphological changes occur over time [29]. Clotting can result if there is insufficient EDTA relative to blood. This is usually caused by overfilling the vacuum tube or poor solubility of EDTA (most commonly with disodium salts) [30]. EDTA draws water from cells to artifactually dilute plasma and is generally not recommended for general chemistry tests. EDTA chelates other metallic ions such as copper, zinc, or magnesium and alters cofactor-dependent activity of many enzymes, such as alkaline phosphatase and creatine kinase, and hence is not used for these chemistry assays. EDTA is also used for blood bank pretransfusion testing; flow cytometry; Hemoglobin A_{1c} (HbA_{1c}); and most common immunosuppressive anti-rejection drugs, such as cyclosporine, tacrolimus, sirolimus, and everolimus. Whole blood EDTA in BCT transported on ice is preferable for the collection of unstable hormones susceptible to proteolysis *in vitro*, such as corticotropin, parathyroid hormone, C-peptide, vasoactive peptide (VIP), antidiuretic hormone, carboxy-terminal collagen cross-links, calcitonin, renin, procalcitonin, and unstable peptides such as cytokines. Spray-dried potassium EDTA is the preferred anticoagulant for quantitative proteomic and molecular assay protocols such as viral nucleic acid extraction, gene amplification, or sequencing [27].

Heparin, a heterogeneous mixture of anionic glycosaminoglycans, inactivates serine proteases in the coagulation cascade—primarily thrombin and factors II (prothrombin) and Xa—through an antithrombin-dependent mechanism. For this reason, heparinized plasma is not used for coagulation tests. Typically, lyophilized or solid lithium, sodium, or ammonium salts of heparin are added to BCT at varying final concentrations of 10–30 USP units/mL of blood [26]. Hygroscopic heparin formulations are used instead of solutions to avoid dilution effects. Heparin is the recommended anticoagulant for many chemistry tests requiring whole blood or plasma because chelating properties and effects on water shifts in cells are minimal. Heparinized plasma is useful for tests requiring faster turnaround times because it does not require clotting, minimizing the risk of sample pipetting interference due to fibrin microclots [31]. Heparin is the only anticoagulant recommended for the determination of pH blood gases, electrolytes, and ionized calcium [32]. Lithium heparin is commonly used instead of sodium heparin for general chemistry tests [33]. Obviously, additives may directly affect the measurement of certain analytes. For example, lithium heparin plasma can have lithium levels in the toxic range, greater than 1.0 mmol/L; when filled correctly, sodium heparin tubes can elevate sodium levels 1–2 mmol/L; and ammonium heparin increases measured ammonia levels [28].

Heparin should not be used for coagulation tests and is not recommended for protein electrophoresis and cryoglobulin testing because of the presence of fibrinogen, which co-migrates with β_2 monoclonal proteins. Information on tube type performance on many analytical tests as well as specimen stability characteristics is available from manufacturers upon request. The BD Diagnostic Preanalytical Division publishes clinical "white papers" for most of their various BCT products [34].

The BCT recommended by the Clinical Laboratory Standards Institute (CLSI H21-A5-2008) for coagulation testing is trisodium citrate buffered (to maintain the pH) or unbuffered, available as 3.2% (or 3.8%) concentrations [35]. Because the different concentrations produce different results, it is necessary that only one concentration (preferably 3.2%) be used consistently by a laboratory. The combination of sodium citrate and citric acid is called buffered sodium citrate. The recommended preservative ratio is 9:1 (blood: citrate). Citric acid and dextrose should not be used because this combination will dilute the plasma and cause hemolysis. Different citrate concentrations can have significant effects on PTT and PT assays, resulting in variable reagent responsiveness. It is necessary for labs using the international normalization ratio (INR) to ensure the same citrate concentration is used for the determination of the International Sensitivity Index. Sodium citrate acts primarily to chelate calcium, and coagulation factors are unaffected. The binding effect of citrate can be reversed by recalcifying the blood or derived plasma to its normal state. This reversible action makes it highly desirable for clotting and factor assay studies. Citrate also has minimal effects on cells and platelets and is used for platelet aggregation studies.

Hirudin is a single-chain, carbohydrate-free polypeptide derived from the leech (*Hirudo medicinalis*). Hirudin binds irreversibly to the fibrinogen recognition site of thrombin without the involvements of cofactors to prevent transformation of fibrinogen to fibrin. The use of hirudin as a more potent anticoagulant is promising for general chemistry, hematology, and molecular testing, although it is not readily commercially available [36].

Thrombin initiates clotting in the presence of calcium. Rapid 5-min Clot Serum Tube (RST) containing thrombin as an additive is now commercially available from BD. Laboratories find these ideal for obtaining serum for assays requiring a fast turnaround time [37]. Strathmann et al. [38] showed that RST tubes have fewer false-positive results and better reproducibility compared to lithium heparin PST tubes for 28 general chemistry tests and immunoassays.

Potassium oxalate is used in combination with sodium fluoride and sodium iodoacetate to inhibit enzymes involved in the glycolytic pathway. Potassium oxalate chelates calcium and calcium-dependent enzymes and reactions to act as an anticoagulant. Sodium fluoride inhibits enolase and iodoacetate inhibits glyceraldehyde-3-phosphate dehydrogenase enzyme activity to prevent metabolism of glucose and ethanol. Oxalate BCT is useful specifically for glucose, lactate, and ethanol tests. Glycolysis enzyme inhibition is not immediate and may be delayed up to 4 h after collection, allowing glucose levels to fall by 5–7% per hour at room temperature. For this reason, the use of fluoride anticoagulants is undesirable for the collection of neonatal glucose specimens in capillary whole blood unless the specimens are transported on ice.

ORDER OF DRAW OF VARIOUS BLOOD COLLECTION TUBES

To avoid erroneous results, BCT must be filled or used during phlebotomy in a specified order. A standardized order of draw (OFD) minimizes carryover contamination of additives between tubes. Table 2.6 shows an example of the OFD for blood collection as used at Calgary Laboratory Services. Many laboratories have established their own protocols for the OFD for multiple tube collections, with slight variations based on CLSI recommendations. The general order of draw is as follows:

1. Microbiological blood culture tubes
2. Trace element tubes (nonadditive)
3. Citrated coagulation tubes
4. Non-anticoagulant tubes for serum (clot activator, gel or no gel)
5. Anticoagulant: heparin tubes (with or without gel)
6. Anticoagulant: EDTA tubes
7. Acid citrate dextrose tubes
8. Glycolytic inhibitor tubes

Tubes with additives must be thoroughly mixed by gentle inversion as per manufacturer recommended protocols. Erroneous test results may be obtained when the blood is not thoroughly mixed with the additive. A discard tube (plastic/no additive) is sometimes used to remove air and prime the tubing when a winged blood collection kit is used. Tubes for microbiological blood cultures are filled first to avoid bacterial contamination from epidermal flora. When trace metal testing on serum is ordered, it is advisable to use trace element tubes. Royal-blue Monoject trace element BCT are available for this purpose. These tubes are free from trace and heavy metals; however, it is advisable to consult the manufacturer's package insert to determine upper tolerable limits of trace and heavy metal contamination to determine acceptability for clinical diagnostic use.

TABLE 2.6 Order of blood draw.

Order of draw	Color of stopper	Invert	Additive	Comments/common tests[a]
1	Clear	Not required	No additive	Tube used *only* as a discard tube.
2	Blood culture bottle	Invert gently to mix	Bacterial growth medium and activated charcoal	When a culture is ordered along with any other blood work, the blood cultures *must* be drawn first.
3	Yellow	8–10 times	Sodium polyanethol sulfonate (SPS)	Tube used for mycobacteria (AFB) blood culture.
4	Royal blue (with red band on label)	Not required	No additive	Tube used for copper and zinc.
5	Red glass	Not required	No additive	Tube used for serum tests that *cannot* be collected in serum separator tubes (SST), such as tests performed by tissue typing. *Note*: Red plastic tubes are preferable for lab tests.
6	Light blue	3–4 times	Sodium citrate anticoagulant	Tube used mainly for PT (INR), PTT, and other coagulation studies.
7	Black glass	3–4 times	Sodium citrate anticoagulant	Tube used for ESR *only*.
8	Red	5 times	Clot activator, and no anticoagulant	Tube used for serum tests that *cannot* be collected in SST tubes, such as tests performed by tissue typing.
9	Gold/Orange	5 times	Gel separator and clot activator	Gold top tubes are usually referred to as "SST" (serum separator tube) and orange top tubes are referred to "RST" (rapid separator tube). After centrifugation, the gel forms a barrier between the clot and the serum.
10	Dark green Glass (with rubber stopper)	8–10 times	Sodium heparin anticoagulant	Tube used for antimony.
11	Dark green	8–10 times	Sodium heparin anticoagulant	Tube used mainly for amino acids and cytogenetics tests.
12	Light green (mint)	8–10 times	Lithium heparin anticoagulant and gel separator	Usually referred to as "PST" (plasma separator tube). After centrifugation, the gel forms a barrier between the blood cells and the plasma. Tube used mainly for chemistry tests on acute care patients.
13	Royal blue (with blue band on label)	8–10 times	K_2EDTA anticoagulant	Tube used for trace elements.
14	Royal blue (with lavender band on label)	8–10 times	Na_2EDTA anticoagulant	Tube used for lead.
15	Lavender	8–10 times	EDTA anticoagulant	Tube used mainly for complete blood count, pretransfusion testing, HbA_{1c}, and antirejection drugs.
16	Pale yellow	8–10 times	Acid citrate dextrose solution "A" (ACDA)	Tube used for flow cytometry testing.
17	Gray	8–10 times	Sodium fluoride and potassium oxalate anticoagulant	Tube used for lactate.

[a]*Blood collection tubes must be filled in a specific sequence to minimize contamination of sterile specimens, avoid possible test result error caused by carryover of additives between tubes, and reduce the effect of microclot formation in tubes. When collecting blood samples, allow the tube to fill completely to ensure the blood: additive ratio necessary for accurate results. Gently invert each tube the required number of times immediately after collection to adequately mix the blood and additive. Never pour blood from one tube into another tube. See http://www.calgarylabservices.com/files/HealthcareProfessionals/Specimen_Collection/BloodCollectionTubes.pdf for the most current version of this document.*

Citrated tubes are used next because plastic tubes contain a silica clot activator that may cause interference with coagulation clotting factors. Serum BCT without and with gel is drawn next before plasma anticoagulant non-gel or gel tubes to reduce anticoagulant contamination. The OFD for tubes with anticoagulants is heparin, EDTA, and, lastly, glycolytic inhibitors. With potassium EDTA, contamination is postulated to occur by direct transfer of blood to other tubes, backflow for potassium EDTA-containing tubes to other tubes (incorrect OFD), or syringe needle contamination. Calam and Cooper [39] originally reported that incorrect OFD results in hyperkalemia and hypocalcemia. Cornes et al. [40] also showed that *in vitro* contamination by potassium EDTA is a relatively common but often unrecognized cause of spurious hyperkalemia, hypomagnesaemia, hypocalcemia, hypophosphatemia, and hyperferritinemia. However, Sulaiman et al. [41] reported that when using the Sarsted S-Monovette system, there are no effects of EDTA contamination from an incorrect OFD, and they suggested that the ease of use of the phlebotomy venesection system or the experience of the phlebotomists are more important considerations. Likewise, a study concluded that collection for coagulation tests after serum collection using clot activators in collection tubes (silica particles and thrombin) showed minimal effects [42]. The recommended CLSI OFD for microcollection tubes to prevent microclot formation and platelet clumping is blood gases, EDTA tubes, and other additive tubes for plasma or whole blood and serum [43]. The combined effects of fluoride and oxalate may inhibit enzyme activity in some immunoassays or interfere with sodium, potassium, chloride, lactic acid, and alkaline phosphatase measurements. EDTA plasma yields potassium levels greater than 14 mmol/L and total calcium levels less than 0.1 mmol/L [28]. Manufacturers use different tube colors for easy visual identification. The universal colors are lavender for EDTA, blue for citrate, red for serum, green for heparin, and gray for fluoride.

Inadequate blood volume is a common cause of sample rejection in the laboratory. CLSI recommends that the draw volume be no more than 10% below the stated draw volume. The excess amount of additive to blood volume has the potential to adversely affect the accuracy of test results. Some studies have shown that underfilled coagulation tubes have excess citrate that can neutralize calcium in the reagent to give falsely prolonged PT and hence inaccurate INR [30]. However, for automated hematology, underfilled tubes containing powdered potassium EDTA had no effect on blood counts [44]. Inappropriately high heparin: blood ratios can cause prolonged clotting times and increased fibrin microclots. Gerhardt et al. [45] suggest that binding of heparin to troponins decreases immunoreactivity and hence lowers cardiac troponin levels by 15% compared to serum. In contrast, Daves et al. [46], using tubes from a different manufacturer, demonstrated no interference from heparin but, rather, from the separator gel in Terumo Venosafe tubes.

COLLECTION SITES; ARTERIAL, CAPILLARY, AND VENOUS BLOOD SAMPLES; COLLECTIONS FROM CATHETERS AND INTRAVENOUS LINES

Although a wide range of specimens is occasionally analyzed in the clinically laboratory, the primary specimen is blood in one form or another. Arteries are blood vessels that carry blood away from the heart so arterial blood, with the exception of that in the pulmonary artery, has uniform high oxygen content. Similarly, because veins carry blood toward the heart, venous blood, except that in the pulmonary vein, has decreased oxygen and glucose as well as increased carbon dioxide (CO_2), lactic acid, ammonia, and acidity (lower pH). The exact composition of venous blood varies throughout the body and depends on the metabolic activity of the tissues that a specific vein drains [2]. Because the source of blood has no consequence to most analyses, venous blood is usually preferred because of its ease of collection. Arterial blood is necessary only for the measurement of arterial blood gases (pO_2, pCO_2, and pH) in order to assess oxygenation status in critically ill patients and those with pulmonary disorders. Although venous blood may yield an adequate assessment of pH status, it does not accurately reflect the arterial pO_2 and alveolar pCO_2 status but, rather, that of the extremity from which it is drawn [2].

Laboratory analysis of blood specimens may be affected by the technique used to collect the sample. Multiple sites may be used for the collection of venous blood, but the sites chosen usually depend on age and condition of the individual, amount of blood needed, and the analyses to be performed.

Skin puncture

Skin puncture is the usual method for collection of blood from infants as well as some older pediatric patients (<2 years of age). In some specific situations, such as obesity, severe burns, thrombotic tendencies, and point-of-care testing, skin puncture may also be used on adults [3]. In infants, the heel is the primary site of collection, with the earlobe or finger often being used in older patients [3]. As mentioned previously, it is essentially mildly diluted arterial blood, but it will suffice for most analyses. Due to its similarity to arterial blood, in most patients it may be used to assess pH and

pCO_2 regardless of site of collection, but for measurement of pO_2, only blood collected from the earlobe is recommended [2]. If used for blood gas measurement, care must be taken to minimize exposure to ambient air while collecting the specimen [3,47]. A few assays cannot be performed on these specimens, including erythrocyte sedimentation rate and coagulation studies and blood cultures [47].

Venipuncture

The median cubital vein in the antecubital fossa is the most commonly used site due to its accessibility and size, followed by the neighboring cephalic and basilic veins [2,3,12,48]. Veins on the dorsal surface of the hand and wrist, radial aspect of the wrist, followed by dorsal and lateral aspects of the ankle are also used, but these should only be used if one can demonstrate good circulation [3,48]. Sites to be avoided include arms ipsilateral to a mastectomy; scarred skin and veins; fistulas; sites proximal to (above) intravenous (IV) lines; and edematous, obese, and bruised areas [12].

Arterial puncture

In order, the radial, brachial, and femoral arteries are the preferred sites for arterial puncture [2,3,12]. Sites to be avoided include those that are irritated, edematous, and inflamed or infected. Although skin puncture provides a similar specimen to arterial blood, for neonates, specimens for blood gas analysis are best collected from an umbilical artery catheter [48].

Indwelling catheters and intravenous lines

For single phlebotomy, it is generally better to avoid an area near an IV line [12]. A site in a different extremity or distal to the IV line is preferred. However, for patients periodically requiring numerous specimens, collecting blood through IV lines and indwelling catheters, including central venous lines or arterial catheters, offers the advantage of facilitating this without phlebotomy [2]. This allows staff without extensive phlebotomy skills to collect blood, thereby freeing experienced phlebotomists to concentrate on other patients. Unfortunately, this creates an inherent risk for improper specimen collection. In order to avoid contamination and dilution with IV fluid; it is recommended that the valve be closed for at least 3 min prior to specimen collection [48]. In order to clear the IV fluid from the line, approximately 6–10 mL should be withdrawn and discarded [2,48]. Because heparin is often in a line to maintain patency, larger volumes may need to be discarded for coagulation studies [2]. Blood drawn from these lines should not be cultured due to the high risk of contamination from bacteria growing in the line [2]. Some drugs such as cyclosporine adhere so tightly to these lines that specimens for drug monitoring should always be obtained from a site unrelated to drug administration [49].

Contamination

Intravenous (IV) fluid is typically composed of water containing various electrolytes, glucose, and occasionally other substances. Therefore, contamination of a specimen with this fluid falsely elevates concentrations of these analytes, but at the same time, contamination causes dilution of the specimen. Thus, values of analytes that are not present in the IV fluid should be decreased [47]. Skin antisepsis is typically accomplished with isopropanol or iodine compounds [3,48]. Isopropanol is generally recommended. The site should be allowed to dry for 30–60 s to minimize the risk of interference with alcohol assays. Iodine compounds have been noted to affect some assays and probably should be avoided for chemistry studies. In particular, povidone iodine can falsely elevate potassium, phosphorous, and uric acid in specimens collected by skin puncture [3].

Tourniquet effect

Application of a tourniquet to approximately 60 mmHg pressure causes anaerobic metabolism and thus may elevate the lactate and ammonia and lower the pH [48]. Tissue destruction may cause the release of intracellular components such as potassium and enzymes. Venous stasis due to prolonged tourniquet application (>3 min) may cause significant hemoconcentration with an 8–10% increase in several enzymes, proteins, protein-bound substances, and cellular components [3]. Prolongation of venous occlusion from 1 to 3 min was documented to increase total protein (+4.9%), iron (+6.7%), lipids (+4.7%), cholesterol (+5.1%), aspartate aminotransferase (AST) (+9.3%), and bilirubin (+8.4%) and to decrease potassium (+6.2%) [50]. In addition, stress, hyperventilation, and muscle contractions (e.g., repeated fist clenching) may elevate analytes such as glucose, cortisol, muscle enzymes, potassium, and free fatty acids. For these reasons, it is important to limit venous occlusion to less than 1 min if possible [48].

Hemolysis

Hemolysis will elevate the concentration of any constituent of erythrocytes and may slightly dilute constituents present in low levels in erythrocytes. It becomes significant when the serum concentration of hgb

surpasses 20 mg/dL. Typically, hemolysis elevates aldolase, acid phosphatase, isocitrate dehydrogenase, LD, potassium, magnesium, alanine aminotransferase (ALT), hgb, and phosphate [2,48]. AST is elevated slightly [48]. Colorimetric assays are also affected by the increased absorbance of light by hgb in the 500- to 600-nm range [2].

URINE COLLECTION, TIMING, AND TECHNIQUES

The examination of urine for diagnostic purposes dates back at least to Roman times [51], when it formed one of the cornerstones of the ancient "diagnostic laboratory" (in essence, the practitioners' senses of sight, smell, and taste). Urinalysis remains one of the key diagnostic tests in the modern clinical laboratory, and as such, proper timing and collection techniques are important.

Urine is essentially an ultrafiltrate of blood, which is supplied to the kidneys at a rate of approximately 120−125 mL/min. As a result, approximately 1−2 L of urine are produced daily. The kidneys are responsible for many homeostatic functions, including acid−base balance, electrolyte balance, fluid balance, and the elimination of nitrogenous waste products. In addition to these regulatory functions, the kidneys produce the hormones erythropoietin and calcitriol [1,25(OH)2 vitamin D_3] as well as the enzyme renin. Not surprisingly, the complexity of the kidneys makes them susceptible to a number of toxic, infectious, hypoxic, and autoimmune insults. Fortunately, there is a considerable amount of redundancy built into the renal apparatus such that individuals can function normally with only a single kidney.

Examination of urine may take several forms: microscopic, chemical (including immunochemical), and electrophoretic. Each of these may yield important diagnostic information about the health of the genitourinary system or about substances (e.g., drugs of abuse) that are found in the blood.

Timing of urine collection

Three different timings of collection are commonly encountered. The most common is the random or "spot" urine collection. This collection is performed at the patient's convenience and is generally acceptable for most routine urinalysis and culture. However, if it would not unduly delay diagnosis, the first voided urine in the morning is generally the best sample. This is because the first voided urine is generally the most concentrated and contains the highest concentration of sediment [2,52]. The third timing of collection is the 12- or 24-h collection. This is the preferred technique for quantitative measurements such as creatinine, electrolytes, steroids, and total protein. The usefulness of these collections is limited, however, by poor patient compliance and/or inaccurate recording of collection times.

Specimen labeling

As with all laboratory specimens, proper labeling is essential. This should include labeling both the requisition and the specimen container with the patient's name and additional unique identifiers as required by the receiving laboratory, the ordering practitioner's name, and the date and time of collection. This is especially important when specimens are aliquoted from 24 h collection containers for distribution to different analytical areas of the laboratory.

Clean catch specimen

For most urine testing, a clean catch specimen is optimal. The technique as described for males is to retract the foreskin (if present) and clean the glans with a mild antiseptic solution. With the foreskin still retracted, allow the bladder to partially empty. This will serve to flush bacteria and other material from the urethra. Finally, collect a resulting "midstream" sample for testing. The technique for females is similar: Separate the labia minora and clean the urethral meatus with mild soap followed by rinsing. Sterile cotton balls are commonly used for this purpose. Continue holding the labia minora apart and partially empty the bladder. Finally, collect a midstream sample for testing. Practically, the cleansing step is often poorly performed or skipped altogether. Indeed, even without prior cleansing, contamination rates in one study were not increased relative to a clean catch control group (23 and 29%, respectively) [53]. For reasons other than bacteriologic examination (e.g., pregnancy testing), the cleansing step may be omitted.

Catheterization

In situations in which the patient cannot provide a clean catch specimen, catheterization represents another option. Due to the attendant risks (infection and mechanical damage), this technique is performed only when necessary and only by trained personnel. Attention must be paid to sterile technique and the selection of a properly sized catheter.

Suprapubic aspiration

More invasive still is the technique of suprapubic puncture of the bladder and needle aspiration of the

urine sample. This technique has been advocated for infants and young children to confirm a positive test from an adhesive bag or container sample [54]. In the experience of the author, this is rarely done. A suprapubic aspiration sample may also be obtained in conjunction with the placement of a suprapubic catheter for the relief of urethral obstruction.

Adhesive bags

Urine collection from infants and young children prior to toilet training can be facilitated through the use of disposable plastic bags with adhesive surrounding the opening. When used in an outpatient setting, these are checked regularly by the parents, and the urine is transferred to a sterile container as soon as it is noticed in the bag.

Specimen handling, containers, and preservatives

For point-of-care urinalysis (e.g., urine dipstick and pregnancy testing), any clean and dry container is acceptable. Disposable sterile plastic cups and even clean waxed paper cups are often employed. If the sample is to be sent for culture, the specimen should be collected in a sterile container. For routine urinalysis and culture, the containers should not contain preservative. For specific analyses, some preservatives are acceptable. A list of these is given in Schumann and Friedman [54]. The exception to this is for timed collections in which hydrochloric acid, boric acid, or glacial acetic acid is used as a preservative.

Storage of the sample at room temperature is generally acceptable for up to 2 h. After this time, the degradation of cellular and some chemical elements becomes a concern. Likewise, bacterial overgrowth of both pathologic and contaminating bacteria may occur with prolonged storage at room temperature. Therefore, if more than 2 h will elapse between collections and testing of the urine specimen, it must be refrigerated. Storage in refrigeration for up to 12 h is acceptable for urine samples destined for bacterial culture. Cellular degradation in cytologic urine specimens may be inhibited by refrigeration for up to 48 h [54]. However, the optimal technique is timely mixture of the cytologic urine sample with equal parts of a preservative: 50–70% ethanol has traditionally been used for this purpose. With the advent of liquid-based cytology platforms, ethanol has been largely replaced by proprietary preservative solutions and collection containers designed for the specific liquid-based cytology platform used. Consultation with the laboratory processing the sample is necessary to ensure the proper preservative is used. In all cases, formalin is an unsuitable preservative for cytology specimens.

CONCLUSIONS

Many pre-analytical variables affect clinical laboratory test results. Therefore, it is very important to be aware of such factors in order to investigate potentially erroneous test results before specimens arrive at the laboratory. Because blood and urine are the two major specimens analyzed in clinical laboratories, this chapter focused on pre-analytical issues that affect laboratory test results for these specimens. This chapter has described important pre-analytical factors such as patient preparation, specimen type (whole blood, plasma, serum, or urine), type of collection container, and site of collection. A proper understanding of these pre-analytical factors by healthcare providers and the clinical laboratory is important to ensure that the most accurate results are provided for proper patient care.

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CHAPTER

3

Sample processing and specimen misidentification issues: major sources of pre-analytical errors

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INTRODUCTION

Every step of handling and processing laboratory specimens should be closely monitored to ensure ideal conditions for testing and producing meaningful test results. Like all areas of healthcare, the laboratory is subject to error, especially in the pre-analytical phase of testing, which includes specimen collection, labeling, transportation, storage, and processing [1] The Institute of Medicine report in 2000, "To Err is Human: Building a Safer Health System" estimated an alarming 44,000–98,000 deaths occur in the United States each year as the result of medical errors [2]. While most of those deaths were due to medication errors, laboratory errors do account for some mortality, either directly or indirectly [3,4]. Advances in information systems, automation, and instrumentation have vastly reduced the analytical error rate; however there is still much room for improvement, particularly in pre-analytical processes, which are the source of the majority of laboratory errors [2]. Laboratory errors can be difficult to quantify due to poor reporting and recognition of events. Studies of laboratory errors show error rates from 1 error in every 33–50 events in one laboratory to as low as 1 error in every 283 laboratory results [5]. Mistakes occurring prior to specimen analysis accounted for the majority of laboratory errors. Estimates of pre-analytical error rates among all laboratory errors range from 31.6% to as high as 84.5%, with over 90% of those due to mistakes originating in patient care units (e.g., phlebotomy, see Chapter 2) [5,6]. This chapter reviews errors in sample processing and identification, from immediately after sample collection to just before testing, and examine strategies to prevent such errors.

TRANSPORTATION

All specimens must travel from the collection site to processing and/or testing sites. This ranges from short trips down the hall, to long cross-country trips to reference laboratories. No matter the nature of transportation, laboratory staff must be cognizant of time, temperature, and turbulence, which can all influence specimen integrity.

Transportation time

Many obstacles prevent timely transportation from collection site to laboratory. Frequently, samples are collected at remote off-site locations and transported to a main laboratory for processing. Specimens can get lost or misplaced for hours. Tubes can get stuck or delayed in pneumatic tubes systems. Couriers may get stuck in traffic while samples sit in the car. A nurse or phlebotomist may slip a tube into his or her pocket. No matter the reason, sample processing is often delayed, and this can be a source of pre-analytical error. Many compounds are subject to variation with time, especially prior to separation of serum/plasma from cells. Every reasonable effort should be made to reduce transport time between drawing and processing the sample.

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Certain analytes are particularly susceptible to change with time, particularly if a sample is exposed to cellular components (Table 3.1). Blood cells retain *in vitro* metabolic activity, especially when maintained at room temperature (RT) or higher, which can alter the chemical constituents of a sample. In uncentrifuged, whole blood samples kept at RT, glucose concentrations fall at 5–7% per hour due to on-going glycolysis by the blood cells [26–28]. Even in samples that have been centrifuged, but not separated, glucose in serum or

TABLE 3.1 Stability of common chemistry and immunochemistry analytes with varying time, temperature, and tube types.

Analyte	Serum	Heparin plasma	EDTA plasma	Urine	<24 h	24 h	48–72 h	≥14 days	References
ACTH			X		4°, RT				[7]
Alpha-fetoprotein	X							4°, RT	[8]
Albumin	X	X	X				4°, RT		[9–13]
Aldosterone			X			4°, RT			[7]
Alkaline Phosphatase	X	X					4°, RT		[9,11,12]
ALT	X	X	X				4°, RT		[9,12–14]
Amylase	X						4°, RT		[10–12]
Apo A1 and B			X				RT		[13]
AST	X	X					4°, RT		[9,11,12,14]
Bilirubin, total	X						4°, RT		[12]
BNP			X			RT	4°	−20°	[15–17]
BUN	X	X			RT[a]		4°, RT		[7,12]
Calcium	X	X					4°, RT		[9,12]
Catecholamines				X				4°, −20°	[18]
Cholesterol, total	X	X	X				RT		[9,11,13,15]
hCG	X							4°, RT	[8]
Creatine Kinase	X		X				4°, RT		[11,13]
Carbon Dioxide	X	X				RT			[9,15]
Creatinine	X	X	X			RT[a]	4°, RT		[12,13,15]
				X				4°, −20°	[19]
Cortisol	X	X				RT[a]	RT		[11,20]
C-Reactive Protein			X				4°, RT		[21]
Estradiol			X			4°, RT			[7]
Estriol, unconjugated	X							4°, RT	[8]
Ferritin	X	X				RT			[20]
GGT	X	X					4°, RT		[9,11,12]
Growth Hormone						4°, RT			[7]
Glucagon						4°, RT			[7]
Glucose	X	X			4°, RT[a]	4°			[9,12,22]
Hemoglobin A1C			X				RT		[23]
HDL	X	X				RT			[9,15]
Homocysteine		X					4°		[24]
Lactate	X	X			RT				[9]

TABLE 3.1 Stability of common chemistry and immunochemistry analytes with varying time, temperature, and tube types.—cont'd

Analyte	Serum	Heparin plasma	EDTA plasma	Urine	<24 h	24 h	48–72 h	≥14 days	References
LDH	X	X			RT	4°			[9,12]
Microalbumin				X				4°, −20°	[25]
Metanephrines				X			RT		[18]
Phosphorus	X	X				RT	4°		[9,12]
Potassium	X	X			4°	RT[a]	RT		[15]
Protein, Total	X	X	X				4°, RT		[9,12,13]
Sodium	X	X			4°		RT		[9,12,15]
Triglycerides	X	X					RT		[9,11,12,15]
TSH	X	X				RT			[7]
Free T4	X	X				RT			[7]
Uric Acid	X	X					4°, RT		[9,12]
Vitamin B12	X	X				RT			[7]

ACTH, adrenocorticotropic hormone; *ALT*, alanine aminotransferase; *AST*, aspartate aminotransferase; *BNP*, brain natriuretic peptide; *BUN*, blood urea nitrogen; *GGT*, gamma-glutamyl transferase; *hCG*, human chorionic gonadotropin; *HDL*, high density lipoprotein; *LDH*, lactate dehydrogenase; *RT*, room temperature (21–25 °C); *TSH*, thyroid stimulating hormone. Note: This list is not exhaustive, but shows the results from several studies using different times, temperatures, and specimen types. Most data shown represents the ending time point for the respective studies, not necessarily the time at which the analyte stability fails.
[a]*Denotes different stability in un-separated samples.*

plasma will continue to drop, falling outside a clinically significant change range in less than 4 h [9]. Likewise, lactate increases concurrently with the fall in glucose, as pyruvate is reduced to lactate during glycolysis [29]. Samples with bacterial growth or leukocytosis undergo more glycolysis, rapidly decreasing glucose and elevating lactate [10,29,30]. Care should be taken to avoid prolonged time to processing, especially in patients with bacteremia and/or leukocytosis, as artifactual hypoglycemia may otherwise be misinterpreted and prompt an unnecessary workup for hypoglycemia [31,32]. The same phenomenon is well known in another setting: low cerebrospinal fluid (CSF) glucose (hypoglycorrhachia) is a hallmark of meningitis with bacteria and white cells in the CSF [33].

Elevations in intracellular erythrocyte components can occur via trans-membrane diffusion of cellular constituents when serum or plasma is maintained on the clot or cells. Phosphate is about seven-times more concentrated in red cells than plasma, and leaks from the red cell into plasma with extended storage time [10]. The same is true for potassium, though the intracellular gradient may be maintained if the cells are kept at RT and can consume glucose to generate the adenosine triphosphate (ATP) required to feed the Na^+/K^+ATPase [10]. Chloride and carbon dioxide concentrations fall over time, likely due to the chloride-bicarbonate shift into red cells (see Chapter 8) [9]. Other components that are concentrated inside cells, such as lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) may leak out; however, as long as the red cells remain intact, a significant rise in enzyme concentration is not observed [9,10]. While infrequently caused during handling and transportation, hemolysis can also significantly affect laboratory tests, such as potassium, AST, and LDH [34]. See Chapter 5 for more information on hemolysis.

In addition to depletion via metabolism, many analytes are simply unstable *in vivo* and *in vitro*, and remain intact for a relatively short time after specimen collection. Often, these are short peptide hormones, such as adrenocorticotropic hormone (ACTH) and brain natriuretic peptide (BNP), which are rapidly degraded [35–37]. Other hormones, such as insulin and parathyroid hormone, are subject to degradation, though at a slower rate [10,38]. Finally, insignificant increases may be observed in several analytes, such as sodium, due to hemoconcentration, as water moves into the cells as the samples stand [9].

The Clinical Laboratory Standards Institute (CLSI) guidelines for specimen handling and processing recommend separating plasma or serum from the cells within 2 h of sample collection [28,39]. Most analytes are stable for longer than 2 h (Table 3.1), so rejection of all specimens received 2 h or more after draw is unnecessary. If laboratories were to strictly follow guidelines, specimen processing stations, including centrifuges, should be placed at every location where blood is drawn, so samples could be immediately processed and serum or plasma mechanically separated from the

cells. This is not feasible in most large medical centers and clinical practices. Therefore, laboratories need sound strategies for identifying specimens of poor integrity and policies for accepting and rejecting those samples. Specimen integrity must be maintained from draw to analysis. Tactics to optimize specimen integrity include: implementation of strategies to reduce transport time, use of appropriate tubes, and development of strict guidelines for specimen storage conditions during transport. Reducing transit time preserves sample stability and shortens turnaround time (TAT). For inpatient specimens, robotic couriers and pneumatic tube systems can cut-down on staffing and decrease transport time to the laboratory. These solutions may provide an alternative to opening a satellite lab in far sites from a medical center. One study estimated that robotic couriers could decrease turnaround time by 34% in a 591 bed hospital [40]. Pneumatic tube systems can send carriers containing laboratory specimens, paperwork, pharmaceuticals, and more throughout a hospital at high speeds. Thus, pneumatic tube systems are in wide use in medical centers around the world [41–43].

Gel separator tubes were introduced to provide a single, closed system to draw, process, test, and store samples. The tubes contain a thixotropic gel, which has a density intermediate between plasma or serum and cells. Upon centrifugation, the gel moves to the interface between the liquid and cellular components of the sample [44]. Use of serum and plasma separator tubes extends the stability of most chemistry analytes [15,39,45,46]. However, many drugs, such as phenytoin, phenobarbital, carbamazepine, lidocaine, and tricyclic antidepressants absorb into the gel, so these tubes should not be used for therapeutic drug monitoring [39,47,48]. Gel separator tubes offer a practical option for rapidly separating plasma or serum from cells, and slow many of the time-dependent processes that reduce specimen integrity [20]. These tubes have the added advantage of helping to prevent labeling errors. Since samples can be drawn, tested, and stored all in the same tube, there is less need to aliquot and re-label, reducing labeling and misidentification errors.

When highly accurate glucose results are necessary, such as for glucose tolerance tests, samples should always be drawn into tubes containing sodium fluoride and potassium oxalate ("gray-top tubes") [49]. Fluoride and oxalate inhibit glycolysis, preventing artifactually low glucose results [27]. Prevention of glycolysis ensures that lactate concentrations also remain stable, making the gray-top tube the preferred tube type for lactate samples. It takes up to 2 h for fluoride and oxalate to completely inhibit glycolysis, so some glucose loss can still occur [50]. After 2 h of collection in fluoride and oxalate containing blood collection tube, glucose is stable for 72 h [27]. Since the anti-glycolytics come as salts with sodium and potassium, these tubes are not suitable for electrolyte determinations; they may also interfere with certain enzymatic assays, such as the urease method for blood urea nitrogen (BUN) [39]. Regardless of the benefit of glycolysis inhibition, immediate separation of plasma or serum from the cells remains the best means for stabilizing glucose [49]. It is important to ensure proper procedure to maintain specimen integrity, especially during long periods in transit, for preventing pre-analytical errors and providing optimal test results.

Effects of temperature

Maintenance of a well-controlled transport environment is essential to reduce pre-analytical error. Manipulation of transport temperature may increase analyte stability. Lower temperatures generally enhance analyte stability, but care must be taken, as one temperature does not fit all analytes. Extreme heat denatures proteins and can diminish enzyme activity [51]. Lower temperatures inhibit metabolic processes, like glycolysis. Thus, most analytes are more stable at 4 °C than RT (Table 3.1). Some analytes must be chilled because they rapidly degrade at RT including: ammonia, lactate, pyruvate, parathyroid hormone-related protein, and gastrin. These, specimens should be chilled immediately after collection, and this temperature should be maintained throughout the pre-analytical phase [39,52].

Generally, most analytes are more stable at lower temperatures; however, there are several notable exceptions. Cold inhibits glycolysis, which provides the ATP required for cell surface Na^+/K^+ pumps. Without ATP, intracellular potassium accumulates and begins to leak out of cells over time. Thus, extracellular potassium may be artificially elevated in specimens stored in the cold for longer than 6 h (see Chapter 9 for a case report) [9,39]. Likewise, if a sample is maintained at RT, glucose is consumed to sustain glycolysis. This process maintains the appropriate potassium concentration, but falsely decreases glucose [10]. This makes transporting specimens collected for a basic metabolic panel difficult, but the problem is solved by separating the plasma from the cells before transporting.

Testing is often intentionally delayed, for example for batch analyses or to send specimens to reference laboratories and specimens may be frozen to preserve sample integrity until they are tested. Some enzymes are sensitive to freezing temperatures. When stored in liquid nitrogen, AST, alanine aminotransferase (ALT), alkaline phosphates, gamma-glutamyl transferase (GGT), and LDH remain stable, while amylase increases and creatinine kinase (CK) activity decreases [53]. CK activity decreases significantly when frozen to −20 °C for even short periods [54]. Certain analytes, such as cryoglobulins, must be maintained at body temperature,

and require transit in a warm water bath, kept around 37 °C[55].

CLSI guidelines recommend using an ice bath to rapidly and effectively cool a specimen. Using a mixture of ice and water will ensure good contact between the tube and the cooling medium [39]. Care should be taken to prevent direct contact of the sample with ice, especially dry ice, as hemolysis can occur with temperature extremes [39]. When transporting samples outside, special considerations should be made for the weather. For example, on very hot days, samples should be sealed in a plastic bag, and then immersed in ice slurry, to provide better cooling than ice packs alone. On the other hand, in very cold climates, coolers may not require additional ice packs, or may need extra insulation to keep the sample from freezing [56,57].

Humidity is also a consideration, particularly for samples that will be exposed to outside air, such as dried blood spots. High humidity speeds the degradation of some analytes in dried blood spots, including biotinidase, galactose-1-phosphate uridyltransferase, glucose-6phosphate dehydrogenase and T4 [58–60]. High humidity may also cause inaccurate results on self-monitoring blood glucose meters [39]. Humidity can be prevented by transporting samples in plastic bags with desiccant packets, and can be monitored with humidity indicator cards [61,62].

Effects of specimen handling and turbulence

Moving a specimen from the draw site to the laboratory for processing may involve one or more forms of transportation. Outreach specimens are often brought to the main laboratory by courier; although not perfect, courier services are the preferred method for transporting samples from remote locations [63]. Compared to mailing specimens, couriers are faster and can provide better control of the sample environment, both temperature and jostling [63]. Inpatient samples, however, are frequently delivered to the laboratory via pneumatic tube system or robots. Robotic couriers replace full time employees, and transport samples efficiently and safely [40]. Pneumatic tube systems are widely used because they swiftly transport samples within hospitals, thereby reducing TAT [64,65]. Pneumatic tube systems can move at speeds upwards of 7.5 m/s (over 17 miles/h), and often go through rapid accelerations, sharp corners, and abrupt decelerations. The biomechanical forces experienced by the samples in pneumatic tubes can affect the quality of transported specimens. There is an increased risk of container breakage or leakage, particularly when they are glass and/or difficult to close (e.g., urine collection cups). The mechanical forces imposed by a pneumatic tube system can also directly damage blood cells, leading to hemolysis.

TABLE 3.2 Analytes affected by agitation with transportation.

Analyte	Effect of transportation	References
Lactate dehydrogenase	++	[41,42]
Potassium	+	[41–43]
AST	+	[41]
Phosphorus	+	[41]
Plasma hemoglobin	+	[42]

This is particularly true of specimens from patients with hematologic disorders or on chemotherapy and other conditions which cause red and white cell fragility [41,65]. Smartphones equipped with accelerometers have been suggested for use in monitoring pneumatic tube system performance [66].

Lactate dehydrogenase (LDH) is a good marker for evaluating exposure to turbulence because it is quickly released from traumatized red cells. LDH activity may rise in a sample, even without significant hemolysis [43]. Along with elevating LDH, pneumatic tube systems induce elevations in plasma hemoglobin, AST, and potassium (Table 3.2) [9,41–43]. Full tubes are less subject to damage by agitation compared to partially-filled tubes [42]. In particular, pneumatic tube-induced changes in LDH ranged from −1.0 to 13.9% for full tubes, compared to 8.6–30.7% for half-filled tubes [10].

Considerations must be made for the proper use of pneumatic tube systems to prevent spills and specimen damage. Samples should be placed in sealed plastic bags prior to transport. Chilled specimens must be double sealed to prevent ice or water leakage. Tubes should not be placed directly into the ice bag, since the water may cause labels to come off, and may leak into the tubes. Finally, container seals should be evaluated prior to transport. Poorly sealed containers should not be sent through pneumatic tube systems. Since a risk of spilling remains, some institutions chose to restrict types of specimens sent in pneumatic tubes. For example, specimens soaking in formalin may not be permitted in the carriers.

Specimen damage due to turbulence in a pneumatic tube system can be prevented in numerous ways. First, when new systems are designed, care should be taken to avoid rapid acceleration and deceleration [41,65]. Second, tubes should be completely filled [10]. Third, samples should be well packed into pneumatic tubes prior to transporting. Towels, foam pads, bubble wrap, and other means have been used to wrap specimens, to prevent jostling. In a small quality improvement study, five conditions were examined: control (remained in laboratory with no transport), no padding, or wrapped in bubble wrap, a towel, or a sheet of foam ($n = 5$, each condition), and LDH was measured for all the samples. LDH increased significantly for samples transported

without any padding, as well as for those with either bubble wrap or foam in the carrier. Only when the specimens were tightly wrapped in a towel, was their only minimal increase in LDH [67].

Case Report: A glomerular filtration rate study was performed, in which the patient was administered Technetium 99 m (6 h half-life), and blood was sent via pneumatic tube system at ½ h intervals to the laboratory to measure the circulating radioactivity using a gamma counter. The laboratory was notified in advance that the procedure would be done and to prepare for the testing. When no samples arrived 1 h after expected, the technologist called the floor to inquire about the samples. He was told the samples were sent via pneumatic tube to the laboratory, however they were never received. An investigation was launched to find the missing pneumatic tube carrier, including looking for carriers trapped in the system. Eventually, a runner was sent to each tube station in the hospital, until the missing carrier was found in a clinic that was closed at the time the carrier arrived. Unfortunately, the samples were found too late to be used, and the procedure had to be repeated. This case is an example of a pitfall of pneumatic tube systems and human error: sometimes carriers get sent to the wrong tube stations. Risks like this may be avoided by restricting which stations carriers can be sent between, as well as clearly posting station numbers at each tube station.

Shipping to reference laboratory

Shipping samples is inevitable, especially for specialized tests which are not performed in most clinical laboratories. Therefore, systems should be in place for sending specimens to distant reference labs. As with transporting samples from nearby locations, time, temperature, and handling must be considered when shipping samples over greater distances. In most cases, plasma or serum samples should be separated from cells and aliquot into a separate tube, rather than shipping the primary tube. Certain tests may have special specimen requirements. Always consult the reference laboratory's test directory for appropriate specimen type and storage conditions prior to collection of send out samples. Specimens may also be placed into a secondary container or packing to reduce turbulence. Generally, samples are sent either frozen or refrigerated in a Styrofoam container, with walls at least 1 inch thick, to ensure adequate insulation. Refrigerated specimens should be sent with sufficient frozen packs to keep the interior of the container between 0° and 10 °C [68]. Frozen specimens should be sent with dry ice. One solid chunk of dry ice (1″ × 3″ × 4″) should be enough to keep a sample frozen for 48 h [52]. Staff must be properly trained for the shipping of biological specimens and dry ice. Biological specimens should be treated as infectious agents and therefore are subject to specific laws and regulations; dry ice is considered a hazardous material to ship and thus requires special considerations. Overnight or next-day shipping reduces transit time and preserves specimen integrity.

Special case: blood gases and ionized calcium

Specimens collected for blood gas determinations require special care, as the analytes are very sensitive to time, temperature, and handling. In standing whole blood samples, pH falls at a rate of 0.04−0.08/h at 37 °C, 0.02−0.03/h at 22 °C, and <0.01/h at 4 °C. This drop in pH is concordant with decreased glucose and increased lactate. In addition, pCO_2 increases around 5.0 mm Hg/h at 37 °C, 1.0 mm Hg/h at 22 °C, and 0.5 mm Hg/h at 4 °C. At 37 °C, pO_2 decreases by 5−10 mm Hg/h, but only 2 mm Hg/h at 22 °C. Metabolic activity in the specimen is affected by temperature as well as the number of metabolically active cells present: specimens with leukocytosis and/or thrombocytosis may demonstrate a spurious hypoxemia, as leukocytes and platelets consume oxygen *in vitro* [69,70]. pO_2 loss is best prevented by immediate analysis, as even rapid cooling may not sufficiently reduce metabolism[70,71].

Ideally, all blood gas specimens should be measured immediately and never stored [71]. A plastic syringe, transported at RT, is recommended if analysis will occur within 30 min of collection. If testing is delayed for more than 30 min, specimens should be collected in a glass syringe and immediately immersed and kept in a mixture of water and crushed ice to chill the specimen [72]. Plastic contracts with cooling, making pores large enough for atmospheric oxygen to cross into the tube, but not carbon dioxide [73]. Glass syringes are recommended for delayed analysis because glass does not allow the diffusion of oxygen or carbon dioxide [73−76].

Blood gas analyzers re-heat samples to 37 °C for analysis to recapitulate physiological temperature. However, for patients with abnormal body temperature, either hyperthermia due to fever, or induced hypothermia in patients undergoing cardiopulmonary bypass, a temperature correction should be made to determine accurate pH, pO_2, and pCO_2 results [71,77]. This adjustment is particularly important in hypothermia, in which the temperature change has marked impact on pH, pO_2, and pCO_2 [71]. Blood gas instrument manufacturers use various but similar equations. Eqs. (3.1)−(3.3) are recommended by CLSI for temperature corrections [71,77]:

$$\mathrm{pH} = \mathrm{pH_m} - [0.0147 + 0.0065 \times (\mathrm{pH_m} - 7.40)](T - 37^{\circ}) \tag{3.1}$$

Where pH is patient's adjusted pH, T is patient's temperature, and pH_m is measured pH

$$pCO_2 = pCO_{2m} \times 10^{0.019(T-37^\circ)} \quad (3.2)$$

Where pCO_2 is patient's adjusted pCO_2, T is patient's temperature, and pCO_{2m} is measured pCO_2

$$pO_2 = pO_{2m} \times 10^{\left[\frac{\left(5.49\times10^{-11}pO_2^{3.88}\right)+0.071}{\left(9.72\times10^{-9}pO_2^{3.88}\right)+2.30}\right](T-37^\circ)} \quad (3.3)$$

Where pO_2 is patient's adjusted pO_2, T is patient's temperature, and pO_{2m} is measured pO_2

Blood gas specimen exposure to air should be minimized, as gases in the sample will rapidly equilibrate with atmospheric air. Anaerobic technique should be used to draw all blood gas samples. However, even with the most careful collection, air bubbles can arise from the syringe hub dead space. Bubbles must be completely expelled from the specimen prior to transport, as the pO_2 will be significantly increased and pCO_2 decreased within 2 min [78].

Rapid turnaround of blood gas results is important for patient care, and requires swift specimen transport to the laboratory. However, blood gas specimens are particularly sensitive to handling and transport. Transit through a pneumatic tube system has been shown to significantly change pO_2, especially when there are air bubbles in the sample, though only very small changes have been noted for pCO_2 and pH [79]. Therefore, it is recommended that if specimens must be transported by pneumatic tube, all air bubbles by completely removed prior to transport [77].

Ionized calcium is often measured in by ion sensitive electrodes in blood gas analyzers. Ionized calcium is inversely related to pH: decreasing pH decreases albumin-binding to calcium, thereby increasing free, ionized calcium. Therefore, specimens sent to the lab for ionized calcium determinations should be handled with the same caution as other blood gas samples, since pre-analytical errors in pH will impact ionized calcium results [80–82].

EFFECT OF CENTRIFUGATION ON TEST RESULTS

Due to instability of certain analytes in unprocessed serum or plasma, CLSI recommend that plasma or serum be separated from cells as soon as possible, but definitely within 2 h of collection [39]. Centrifugation is an integral part of specimen processing. Improper centrifugation techniques may lead to erroneous results for several laboratory tests.

Appropriate preparation of specimens prior to centrifugation is required to ensure accurate laboratory results. Serum specimens must be allowed ample time to clot prior to centrifugation. Tubes with clot activators require sufficient mixing and at least 30 min of clotting time, while serum tubes may require up to 60 min. Patients taking anticoagulants may require longer clotting times [39,83–85]. Plasma specimens must be mixed gently according to manufacturer's instruction to ensure efficient release of additive/anticoagulant [84,85]. Insufficient mixing leads to inefficient clotting in serum tubes and platelet clumping/and or clotting in plasma tubes, leading to inadequate separation of plasma and/or serum from cellular material during the centrifugation phase (see below) [84,85]. Specimens should not be opened prior to or during centrifugation in order to avoid evaporation [83]. Serum specimens requiring anaerobic conditions, like ionized calcium and pH should not be exposed to air prior to centrifugation. Exposure to air causes loss of CO_2, leading to increased pH and decreased ionized calcium [82,83,86].

Case Report: A 40 year old man was admitted to the hospital with a serum potassium of 8.0 mmol/L (mEq/L) obtained on a specimen with no detectable hemolysis. The patient was given potassium lowering drugs and another serum specimen was collected for potassium analysis. After treatment the serum potassium concentration was 7.5 mmol/L, with no hemolysis. The patient became confused, developed muscle cramps and vomiting. The doctor requested a point of care whole blood potassium, the result of this assay was 2.7 mmol/L. The potassium lowering therapy was promptly discontinued. On examination of the complete blood count, the white blood count was 20×10^9/liter (normal $4.5–11.0 \times 10^9$/liter), and the platelet count was 480×10^9/liter (normal $150–350 \times 10^9$/liter). The elevated serum potassium results were due to an error in the centrifugation process. The laboratory scientists determined that the g-force used for centrifugation was too high causing platelet lysis, particularly in a patient with elevated counts. The normal potassium results from whole blood were accurate [87].

Laboratories should select appropriate centrifugation speed, time, and temperature for each analyte. Inadequate centrifugation speed may lead to interferences with numerous assays in the clinical laboratory. Specimens spun at speeds too slow for the tube or for not enough time, may not have adequate separation of serum from the clot or plasma from cellular components. Centrifugation is required to remove all microclots and fibrin strands from serum. Erroneous results may occur for numerous chemistry and immunochemical assays in the presence of such materials. Invisible microfibers or other particulate matter in incompletely centrifuged serum or plasma interferes with assays such as Troponin [88,89]. Platelet contamination of the plasma in inadequately separated specimens leads to

inaccurate results in coagulation studies [90] Further, centrifugation at slow speeds leads to increased "trapped plasma" in the red cell fraction, leading to abnormal results for analytes found in red cells (i.e., potassium and glucose-6-phosphate dehydrogenase: G6PD). Specimens spun too fast are subject to *in vitro* hemolysis and/or cell lysis and release of intracellular constituents like potassium [91,92].

In order to avoid abnormal results due to improper centrifugation, all laboratories should establish appropriate centrifugation time and speed for tube type, centrifuge, and rotor [39]. Calculate relative centrifugal force (RCF), not revolutions per minute (RPM), for each centrifuge model, rotor, head and the radius of the rotor in order to determine appropriate speed [39]. The equation (Eq. 3.4) for RCF, expressed as multiples of gravitational force (g), is listed below.

$$\mathrm{RCF(g)} = 1.118 \times 10^{-5} * \text{radius of the rotor (cm)} * (\mathrm{RPM})^2 \tag{3.4}$$

Manufacturers provide recommendations for appropriate RCF and spin times for individual tube types [84,85].

Specimens should be centrifuged at appropriate temperatures to ensure specimen integrity prior to analysis. The internal temperature of a centrifuge may become hot with activity, leading to degradation of temperature sensitive compounds like ACTH. High temperature during centrifugation may also induce hemolysis. Centrifugation at refrigerated temperatures is not appropriate for all specimens. Laboratories should only perform potassium measurements on specimens stored and/or centrifuged in the cold for less than 2 h, as potassium leaks from cells with prolonged cold exposure [39,93]. Gel polymers in plasma and serum separator tubes are often temperature dependent, thus centrifugation of these tubes in a chilled centrifuge may result in inefficient barrier formation. Laboratories should consult manufacturer's guidelines for spin temperatures for any barrier tubes [84]. Ionized calcium (iCa) and pH in serum are affected by centrifugation temperatures, where iCa results change by 0.006 mmol/L per 1 °C [82]. Tight control of internal temperature is critical during the centrifugation phase. Centrifuge temperature should not deviate more than ± 2.5 °C for ionized calcium or pH [82]. Unless specimens are heat labile (in which case they should be maintained at even cooler temperatures), CLSI recommends fixing centrifuge temperatures at 20–22° [39,83]. Heat sensitive and specimens arriving to the laboratory chilled should be spun in the cold [39].

Laboratories should consult tube manufacturer's literature when deciding which centrifuge is appropriate for their specimen. No matter the type of centrifuge, they should always be balanced prior to processing specimens. Improper balancing can lead to tube cracking, breakage and/or inadequate separation of serum or plasma from cells, as well as centrifuge damage. The use of fixed angle rotors, particularly with separator tubes will cause the gel to form at an angle. Angled gel formation may indicate an inadequate barrier seal, leading to mixing of serum or plasma with cells; numerous abnormalities are associated with storage on the clot or cells. Further, centrifugation in fixed angled rotors for prolonged times may induce hemolysis [93]. Swinging bucket rotors allow for a more reliable barrier seal and will not cause hemolysis at appropriate speed and temperatures and are recommend by most tube manufacturers [39,84,85].

Case Report: Physicians from a community hospital alerted the central laboratory about numerous cases of hyperkalemia in otherwise healthy patients. None of the specimens had significant hemolysis. At the time, phlebotomy and processing practices were to collect potassium requests into serum separator tubes, allow them to clot for 20–60 min, and then centrifuge the tubes according to manufacturer's instructions. The processed serum separator tubes (SSTs) were then delivered to the central laboratory the following day, recentrifuged and analyzed. The laboratory decided to discontinue re-centrifugation and the hyperkalemia phenomenon was no longer observed. Further, in a large timed study, the investigators demonstrated that re-centrifugation after prolonged storage in processed SSTs resulted in falsely elevated potassium [87].

Re-centrifugation of tubes is not recommended by CLSI and tube manufacturers [39,84,85]. As in the case report, several other studies demonstrated falsely elevated potassium after re-centrifugation [87,94,95]. According to manufacturers, re-centrifugation of gel separator tubes disrupts the barrier and may allow mixing of cell components with separated plasma. Release of potassium may occur as the result of cell lysis. Hira et al., proposed that a small portion of plasma remains with the cell fraction after centrifugation of gel separator tubes [87]. Extended exposure to cells allows leakage of potassium into that plasma fraction. Re-centrifugation causes this potassium rich fraction to mix with the plasma layer. Longer initial centrifugation times reduce this effect, presumably by reducing the amount of plasma in the cell fraction [94].

Until recently, no study had examined the effect of re-centrifugation on other common chemical and immunochemical analytes. In-house studies by the authors demonstrated that re-centrifugation of plasma separator tubes (PSTs) not only caused spurious changes in potassium, but other analytes including creatinine, glucose, bilirubin, and AST (Table 3.3) [95]. Interestingly, in this study potassium was not increased in recentrifuged tubes stored at RT. Concentrations increased with

TABLE 3.3 Stability of common chemical and immunochemical analytes in serum or heparin plasma separator tubes after re-centrifugation.

	Specimen		Time before re-centrifugation			
Analyte	**Serum**	**Heparin plasma**	**<4 h**	**<12 h**	**Up to 24 h**	**References**
Potassium		X			RT	[94]
	X			4 °C		[87,94]
Sodium		X			RT, 4 °C	[94]
Chloride		X			RT, 4 °C	[94]
CO2		X			RT, 4 °C	[94]
BUN		X			RT, 4 °C	[94]
Creatinine		X	RT		4 °C	[94]
Glucose		X	RT		4 °C	[94]
Calcium		X			RT, 4 °C	[94]
Tprot		X			RT, 4 °C	[94]
Albumin		X		4 °C	RT	[94]
Tbili		X	RT, 4 °C			[94]
AlkP		X			RT, 4 °C	[94]
AST		X	RT, 4 °C			[94]
ALT		X	RT		4 °C	[94]
HDL-C		X			RT, 4 °C	[94]
Chol		X			RT, 4 °C	[94]
Trig		X			RT, 4 °C	[94]
LDL-C		X			RT, 4 °C	[94]
TSH		X			RT, 4 °C	[94]
fT4		X		4 °C	RT	[94]
Ferritin		X			RT, 4 °C	[94]
VitB12		X			RT, 4 °C	[94]

Stability was assessed based upon values outside the significant change limits after recentrifugation compared to values immediately before recentrifugation [95]. *RT*, room temperature (21–25 °C) *AlkP*, alkaline phosphatase; *ALT*, alanine aminotransferase; *AST*, aspartate aminotransferase; *Chol*: Cholesterol; *fT*4, Free Thyroxine; *HDL-C*, high density lipoprotein cholesterol; *LDL-C*, low density lipoprotein cholesterol; *Li-Hep*, lithium heparin plasma separator tube; *PST*, plasma separator tube; *SST*, serum separator tube; *tBili*, total bilirubin; *tProt*, total protein; *Trig*, triglycerides; *TSH*, thyroid stimulating hormone; *VitB*12, Vitamin B 12.

increasing time at 4 °C, supporting the claim that a small amount of plasma remains in the cell fraction of PSTs and becomes K+ rich with increasing refrigeration time. Glucose is significantly lower in recentrifuged tubes, but only in those stored at room temperature. Glycolysis occurs in the plasma fraction exposed to the cells and upon mixing with pre-processed cells, significantly lowers glucose concentrations. Total bilirubin and AST increase in fractions stored at room temperature and 4 °C, and this is likely due to hemolysis with centrifugation. Creatinine increases in specimens stored at room temperature after re-centrifugation are likely due to an analytical interference with the Jaffe reaction [91,92]. These findings were supported by a study from van Balveren et al., which found no analytically relevant changes in 30 analytes following a second centrifugation [96].

Nevertheless, due to the conflicting data on re-centrifugation, to avoid erroneous results, re-centrifugation should be avoided. This may be difficult for laboratories with large outreach programs and automation. Such laboratories might consider using a code in their laboratory information system (LIS) directing these tubes to bypass the centrifuge or dedicate a lane in the input/output buffer for pre-spun tubes. Should laboratories want to clarify pre-spun specimens, serum or plasma can be aliquot into a separate tube and centrifuged.

EFFECT OF STORAGE CONDITIONS ON LABORATORY RESULTS

Delays in specimen analysis and physician requests for additional testing to a specimen make it important for laboratories to establish specific in-laboratory storage conditions. There are numerous types of interferences that result from inappropriate storage of specimens in the laboratory. These include, inappropriate storage temperatures/times, interfering substances, and light sensitivity. Inappropriate storage temperatures or times most often result in specimen degradation in whole blood and unseparated serum/plasma specimens. CLSI guidelines recommend the following as "general" guidelines for in-lab specimen storage. Serum/plasma should be separated from cellular components immediately after centrifugation, either by transferring to a new tube, or by use of physical separators, such as gel [39]. Separated specimens can be stored, tightly capped to avoid evaporation and concentration, up to 8 h at RT (preferably 20–25 °C), up to 48 h at −4 °C, and after 48 h specimens should be frozen at −20 °C [39,97]. Samples should be snap frozen on dry ice or liquid nitrogen to avoid gradient formation [98]. Prior to analysis, specimens should be thawed at room temperature because heating may denature analytes. Gentle inversion can remove gradients formed with freezing. Centrifugation will sediment cellular material and/or fibrin strands that form upon freezing [99]. Although repeat freeze/thaw cycles are not recommended by CLSI [39], very few analytes are affected by this process [100].

There are several analytes that cannot be stored according to the CLSI "general" recommendations. Whole blood specimens should not be centrifuged. Storage of whole blood depends on the analyte. Freezing of whole blood induces hemolysis and is not recommended for hematological or blood gas specimens. Lamellar body counts are significantly decreased with freezing, thus amniotic fluid specimens should be stored at 4 °C [101]. In unseparated plasma specimens, prolonged storage at 4 °C induces leakage from the cells and falsely elevates potassium [93]. Catecholamines are released from lysed red blood cells and reuptake is slowed, falsely elevating results with prolonged storage at 4 °C [102]. Cryo-activation of pro-renin with long-term storage in the cold (refrigerated or on ice) falsely elevates plasma renin activity assays [103]. Specimens collected for LDH isoenzyme testing should be stored at room temperature prior to analysis because freezing and long-term storage at 4 °C results in loss of lactate dehydrogenase isoenzyme five activities [83]. Prothrombin time is significantly increased with prolonged frozen storage and these specimens should only be stored refrigerated or at room temperature [104]. Because of the number of exceptions to the general in-lab specimen handling procedures, laboratories should consult manufacturers' package inserts for appropriate specimen storage conditions prior to analysis. Laboratories should conduct in-house stability studies prior to changing approved in-lab storage conditions [99].

Some studies suggest that utilization of gel separator tubes eliminates the need to physically separate plasma/serum from cells for short term in lab storage [39]. Prolonged contact with gel does interfere with some analytes especially drugs and estradiol; decreased concentrations maybe observed due to absorption into the gel polymer [39,47]. Gel separator tubes should not be used for these analytes. Further, plasma separator tubes may not be appropriate for storage of common chemistry analytes at 4 °C for greater than 48 h [105]. After long-term storage, barrier seals should be inspected on all separator tubes prior to analysis. Laboratories should consult manufacturers' instructions for a list of analytes that are stable in these tubes for long-term storage. Becton Dickinson released a gel-tube alternative in 2016, the BD Vacutainer Barricor plasma collection tube. This device employs a chemically inert mechanical stopper to separate plasma from cells. These tubes require a shorter centrifugation time than traditional gel separator tubes, yield a sample with less platelet contamination of plasma, and provide improved sample stability [106,107]. At the time of this writing, there were no extensive studies showing the impact of the Barricor tubes on drug concentrations, however one study demonstrated no impact of the Barricor separator on posaconazole concentrations with storage, which is affected by gel separators [108]. This suggests the Barricor separator tubes may be appropriate for rapid processing of therapeutic drug samples, however more work is still needed to verify this conclusively.

Some analytes require preservatives in order to maintain in-laboratory stability. Catecholamines in urine degrade significantly at 4 °C after 48 h at pH 6.0, but are stable frozen or at 4 °C when preserved with acid to achiever pH 2.0–3.0 [109]. Recent studies suggest that glucose is more stable in citrated blood compared to fluoride [22]. Addition of protease inhibitors like aprotinin stabilizes peptide hormones such as parathyroid related protein [90].

Exposure to fluorescent light rapidly degrades bilirubin and other heme products [83]. Vitamins such as carotene and Vitamin A, and red blood cell folate are also degraded by light. In plasma, some drugs, like nifedipine and chloramphenicol [110] are light sensitive. These and other analytes should be protected from light by collecting in brown containers or wrapping the specimen in aluminum foil while stored in the laboratory.

EFFECT OF CROSS-CONTAMINATION ON LABORATORY RESULTS

Cross contamination of specimens occurs when a foreign pathogen, chemical, or other material is introduced into a patient specimen. Cross contamination can lead to inaccurate results in several areas of the laboratory, but is most often a concern in the microbiology and molecular diagnostics laboratories. Specimens processed in laboratories may be contaminated with bacteria and/or DNA [111]. Prevention measures include specimen preparation in dedicated hoods, dedicated pipettes and the use of barrier pipette tips, and frequent decontamination procedures [112]. Cross-contamination of specimens can occur at the time of collection, processing or analysis. Collection errors may involve IV fluid or syringe contamination with incorrect specimen draw order (see Chapter 2) [84,85,113].

Cross-contamination of specimens during aliquoting may also cause errors in laboratory tests. These types of contaminants can be reduced by changing disposable pipette tips with each aliquot and by aliquoting patient specimens one at a time. Cross contamination may also occur on automated analyzers just prior to analysis if instrument sample probes do not use disposable pipette tips. Carryover is most common for analytes with large measuring ranges like human chorionic gonadotropin and some urinary excreted drugs such as opiates, and ultrasensitive assays like hepatitis antigens [114]. Documented carryover checks are required by the College of American Pathologists (CAP) when validating a new method and at intervals outlined by individual laboratories. Instrument manufacturers have reduced carryover cross-contaminations by introduction of wash steps and or disposable pipette tips for sample probes [114].

Case Report: A urine sample was sent to the laboratory for a calcium/creatinine ratio. A 1 mL aliquot of the urine was taken to acidify prior to testing. The technologist adjusted the urine pH to approximately 4.0 with acid, and the sample was submitted for testing. The chemistry analyzer produced undetectable results for both calcium and creatinine, preventing calculation of the ratio. This was significantly different from the previous result so the technologist re-traced her sample preparation steps, and discovered that she had used the wrong bottle of acid to pH the specimen. Rather than using 0.1 N HCl, she had used a less concentrated acetic acid solution that required a much higher volume to adjust the pH, and thus diluted the sample. Another aliquot was taken from the original sample cup, and the tests were repeated following proper preparation procedures. The repeat analysis produced results similar to the previous determination.

Specimen cross-contamination may occur with incorrect preservation of urine specimens. Prior to analysis, urine may be preserved with a variety of additives. The type of additive depends on the laboratory and assay [115]. For many analytes, the preservatives can be added upon arrival to the clinical laboratory. Addition of the incorrect additive may contaminate the urine specimen and interfere with results. In the case described above, acetic acid was added to urine to adjust the pH to the optimal point for analysis, when in fact hydrochloric acid is recommended. Because a higher volume of acetic acid was required the specimen was significantly diluted. It is equally likely that a sample might be alkalinized when it should be acidified. Laboratories can identify incorrect preservatives by measuring pH of all specimens prior to analysis. This type of urine contamination can be prevented by making up 24 h urine jugs containing preservatives for the patients. Laboratories should clearly outline procedures for processing and storage of 24 h urine specimens including adding appropriate preservatives.

SPECIMEN MISIDENTIFICATION

Accurate patient and specimen identification is required for quality patient care. Regulatory agencies like The Joint Commission (TJC) have made it a top priority in order to ensure patient safety. Patient and specimen misidentification occurs in numerous phases of the testing process. During the preanalytical phase, specimen identification begins when the patient presents to the hospital, doctor's office, or phlebotomist. Accurate identification requires collection of at least two unique identifiers from the patient and ensuring that those match the patient's prior records. If a patient is unable to provide identifiers (i.e. neonate or patient in a coma) a family member or nurse should verify the identity of the patient. Information on laboratory requisitions or electronic orders must also match patient information in their chart or electronic medical record. Specimens should not be collected unless all identification discrepancies have been resolved [116]. At specimen collection, phlebotomists should ensure that the area is cleared of identification information from other patients. The sample(s) should be collected and labeled in front of the patient. The specimen should be sent to the laboratory with the test request. Upon acceptance into the laboratory, the identifiers on the specimen should match the requisition and/or electronic order. For non-bar coded specimens, specimens should be accessioned, labeled with a barcode (or re-labeled, if necessary), processed (either manually or on an automated line), and sent for analysis. Identification of the specimen

should be carefully maintained during centrifugation, aliquoting and analysis. Most laboratories use bar coded labeling systems to preserve sample identification.

Patient and specimen ID errors can also occur during the analytical phase. Automated analyzers use bar codes to identify specimens during analysis and results reporting. For manual assays, laboratories should carefully match identifiers on specimens with work lists. Laboratories should carefully monitor repeats, dilutions, add-ons and reflex testing, particularly if these are manual processes. Additionally, procedures should be in place to ensure that barcodes are accurately printed, as poorly or misprinted barcodes may be read incorrectly by laboratory instruments [117]. Such procedures may include regular cleaning and services of label printers.

Misidentification also occurs in the post analytical phase of testing. Results from automated analyzers are electronically transferred to middleware or the laboratory information system (LIS), where rules may dictate whether results are auto verified or require attention from a technologist or pathologist. With manual assays, results are manually entered and technologists must match patient identifiers on specimens, work lists, or result print-outs with information in the LIS. Most LISs are interfaced with hospital information systems to report results in individual patient's charts. In the absence of electronic medical record, laboratory representatives must print laboratory results and fax or mail them to treating physicians.

Misidentification errors can occur at any point in this complex process leading to outcomes ranging from harmless to severe. Outcomes are far less likely to be severe if the error is identified and fixed prior to reporting the results. For this reason groups such as TJC, College of American Pathologists (CAP), and the Institute of Medicine have made misidentification errors a priority [118,119]. Since the implementation of many related initiatives error rates have been reduced, but problems persist [119].

Case Report: An endocrinologist contacted the clinical laboratory regarding discrepant laboratory results. Her patient was a 30 year old female with past medical history of acromegaly and resected pituitary tumor. Although stable after surgery, the patient had occasional headaches, but no visual field disturbances. Pertinent laboratory values included: insulin-like growth factor (IGF1): 1265 ng/mL (normal range: 114−492), growth hormone (GH) 0.1 ng/mL (normal range: <8.0). Upon investigation, the IGF1 testing was performed from plasma while the GH assay was performed on serum. Both specimens were manual aliquots made by specimen processing staff in the laboratory. Testing of the primary tubes retrieved from refrigerated storage, revealed a GH result of 40 ng/mL and IGF1 of 1195 ng/mL. This case is an example of specimen misidentification that occurred during manual aliquoting in the laboratory. Whenever possible, laboratories should use automated specimen processing equipment including aliquoting device. If unavailable or not appropriate for a particular analyte, laboratories should employ a strict procedure for manual aliquoting that ensures careful attention to patient identification. This case prompted the clinical laboratory to implement a system where specimens were aliquot one at a time and not in batch. In addition, the processing technologist is asked to initial all aliquots.

In this case the mislabeled specimen was identified because of a discrepancy between laboratory values and the clinical picture. No treatment decision was made based upon these results. However, it is best to identify mislabels prior to reporting the results. Specimens that arrive with multiple labels should be carefully checked to ensure that all identifiers match. Further, paper requisitions should be matched to specimen tubes. Suspicious specimens should be rejected (see below) or investigated by blood typing, DNA testing or delta checking [116]. In many cases, there are no obvious signs of misidentification on the tube or requisition; therefore laboratories must utilize other tools to identify these specimens.

Case Report: A patient reported to a busy clinic for pre-operative laboratory work. Specimens were collected for complete blood count/differential, electrolytes, and a coagulation panel. The samples were received by and processed in the laboratory. Prior to release of results an instrument flag revealed that a delta check rule had failed. The patient's hemoglobin value changed from 12.3 g/dL to 8.7 g/dL within 48 h. This prompted an investigation by the technologist, who first confirmed that the patient had no recent transfusion history. She sent an aliquot of the sample to the blood bank, where it was confirmed that the blood type in the aliquot did not match that in the patient's record. The error was a result of misidentifica1tion of specimens. All tests ordered for the patient were canceled and specimens were re-drawn and tests repeated. Repeat results revealed a hemoglobin if 12.2 g/dL. In this case, the combination of delta checks and blood typing confirmed a misidentification error and potentially averted an unnecessary transfusion prior to surgery. Further, this case emphasizes the need for a clear hospital wide policy on correction of misidentified specimens outlining times when it is appropriate to change results, recollect, or confirm original results.

Delta checks are a simple way to detect mislabels. A delta check is a process of comparing a patient's result to his or her previous result for any one analyte over a specified period of time [120,121]. The difference or "delta", if outside pre-established rules, may indicate a specimen mislabel or other preanalytical error.

Laboratories should determine the difference in concentration (or relative change) as well as the time interval that is most appropriate for each analyte's delta check. With the increasing use of automation and middleware, delta checks have become a common practice for core laboratories [121,122]. Delta checks are most often set up for assays with little intra-individual variation that are tightly regulated within patients including: men corpuscular volume (MCV), creatinine, BUN, bilirubin, and total protein [120]. Simulation studies demonstrated that delta checks for MCV, HCT, BUN and creatinine are the most sensitive for detecting mislabeled specimens [122]. Further, medical centers should establish their own delta checks based on their individual patient population. For example, delta checks for creatinine and BUN may not be appropriate for a large dialysis clinic, while hematocrit (HCT) delta checks may be ineffective in a large hematology/oncology practice. Further, with the previously identified mislabel rate of ~1/1000 in 2006 [123], only MCV has a high enough positive predictive value to detect all mislabels [122]. Advancements in middleware may allow us to design multi-analyte delta checks with better predictive ability.

Delta checks are not appropriate for all analytes because of high intra-individual variabilities. For example, growth hormone from the case above shows diurnal variation; concentrations at night are significantly higher than in the morning. Thus, laboratories should employ measures to prevent mislabels at their source. Laboratories should adopt a strict specimen rejection policy to reduce entry of questionable specimens into the analytical process [116]. For example, laboratories may decide to reject all specimens that arrive unlabeled or that show disagreement between the requisition and label on the specimen unless they are irreplaceable (i.e., CSF specimens collected from infants or neonates, surgical specimens, etc). Further, laboratories are required by CAP to conduct periodic audits of patient records from requisition to result into the patient chart to look for errors. Intermittent and continual audits of patient ID bands are also helpful in reducing misidentification [116]. Data shows that when laboratories monitor their identification errors on a regular basis, fewer errors occur [123]. Finally, utilization of new technology significantly reduces error rates. Reduction of manual steps removes opportunity for error. Bar coding linked with electronic order reduces manual steps at specimen collection and accessioning [119]. Errors do still exist with barcoding, prompting some laboratories and automation platforms to adopt radio frequency identification (RFID) [116,117,124]. Laboratory automation reduces manual steps, like aliquoting, manual order and results entry; thereby reducing misidentification errors [119].

Case Report: A 68 year old male presented to the hospital with sharp abdominal pain. The patient underwent an appendectomy and received 1 unit of type A blood. The patient developed disseminated intravascular coagulation and died 24 h after receiving the transfusion. Post mortem analysis of the patient's blood revealed that he was actually type O. The patient had been sharing a room with another patient whose blood was type A. The specimen sent to the blood bank had been inappropriately labeled [125].

This is a rare case in which the wrong patient's blood was in the tube sent to the blood bank. Deaths related to ABO incompatible acute transfusion reactions occurred ~1/600,000 patients [126]. The most common error leading to a transfusion reaction is misidentification of a specimen at collection [127], and is responsible for up to 22% of ABO incompatible transfusions [126]. The International Society of Blood Transfusion Biomedical Excellence for Safer Transfusion Committee performed a study looking at the rate of ABO and RhD (rhesus blood group D antigen) testing that did not match previous result(s), this phenomenon was termed "wrong blood in tube" (WBIT) and occurred around 1/1786 specimens [128,129]. A recent analysis of 122 clinical laboratories by CAP found that WBIT occurred ~1/2500 samples [130]. These and other studies prompted TJC to place correct patient identification and prevention of misidentification related transfusion errors as the first goal among their recent National Patient Safety goals [118].

To reduce the rate of WBIT and misidentification errors in the blood bank TJC recommends the use of two unique identifiers for each patient when collecting and administering blood components [118]. The American Association for Blood Banks requires 2 separate determinations of blood type for patients receiving blood in facilities using computer cross-match. CAP checklists require phlebotomists to positively identify patients by verifying two unique identifiers before specimen collection. Further, they require that all specimens be labeled at time of collection [131]. Since international focus on misidentification prevention began in the early 2000's, numerous institutions have published methods to successfully reduce WBIT including: (1) Match requisitions to patient information on the accompanying specimen and in the electronic order system [123], (2) Implementation of an electronic error monitoring system [132], (3) Introduction of patient identification technologies such as barcoding and RFID [119,131,133,134], (4) ABO/Rh testing analysis of two separate specimens for all patients with no blood type on file [135]. For more details on misidentification errors in Transfusion Medicine see Chapters 28.

Despite great successes in reduction of WBIT and other specimen misidentification errors, they still persist [119]. Laboratorians and hospital personnel should implement and continue vigilant error review and process improvement programs to prevent morbidity and mortality associated with specimen misidentification.

CONCLUSIONS

Because most errors occur in the pre-analytical phase of laboratory testing, it is important to have robust procedures in place in the laboratory to eliminate various errors that may occur in the pre-analytical phase. Proper blood collection procedure, transport of specimens, and timely centrifugation ensures not only good specimen integrity but also accurate test results. Sample misidentification may cause serious errors in laboratory test results, and may cause significant morbidity or even mortality, especially if a blood typing specimen is mislabeled. Good laboratory practice not only involves careful attention in the analytic phase, but also in the pre- and post-analytical phases as well.

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CHAPTER

4

Effect of patient-related factors on clinical laboratory test results

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INTRODUCTION

In the United States, more than 7 billion clinical laboratory tests are performed annually [1]. Clinical laboratory test results are an important part of the decision-making process by clinicians for effective diagnosis, prognosis, treatment, and management. It is imperative that the *in vitro* diagnostic testing results accurately reflect the *in vivo* physiological processes of the patient. Inaccurate test results may lead to unnecessary invasive testing, postponement of critical therapies, increased patient anxiety, and expensive healthcare costs (both for the patient and the healthcare system).

Clinical laboratories are required to develop procedures to minimize the effect of pre-analytical, analytical, and post-analytical factors on clinical laboratory analysis. Pre-analytical (steps prior to analysis), analytical (sample analysis), and post-analytical (steps after analysis) factors can affect the accuracy of serum/plasma analytes measured in the laboratory. The pre-analytical phase refers to the processes that occur prior to blood/body fluid testing. These processes include the phlebotomy collection techniques (sample labeling, tourniquet, posture), blood/body fluid tube types (anti-coagulants, gel-separators, clot activators, preservatives), and sample handling (mixing/clotting protocol, temperature, storage, transport).

However, in the pre-analytical process, biological factors, such as age (related variables with age e.g., pregnancy, menopause) are not modifiable. Patient-related factors such as gender (to an extent), dietary intake, and exercise regimens can be modified. Standardized patient preparation prior to blood collection can minimize the effects of pre-analytical factors. Age, and gender specific reference ranges, in some cases, can account for the influence of pre-analytical factors. Understanding how pre-analytical factors influence the accurate measurement of test results will aid in the interpretation of individual patient results within the context of the clinical setting.

This chapter discusses patient-related factors: age, gender, dietary intake, and exercise, and their influence on analytes measured in the clinical laboratory. Furthermore, the contribution of ethnicity/race, a social construct that is used as a surrogate marker for environmental, socio-economic/demographic, and/or genetic factors, on test results is reviewed. Only a few tests have widely used ethnic/race-specific reference intervals, with the exception of the estimated glomerular filtration rate calculation used in the United States. Lastly, other less known influences of fasting, special diets, nutraceuticals, genetic factors in the response to food and nutraceuticals, and cross-sex hormone regimens for transgender patients on laboratory tests are discussed.

EFFECT OF AGE RELATED CHANGES ON CLINICAL LABORATORY TEST RESULTS

Aging is a complex metabolic process that is not fully understood [2]. A myriad of complex physiological changes occur that also significantly affect clinical laboratory test results as an individual transitions through different phases: prenatal, infancy, childhood/puberty, adulthood and elder hood [3]. Understanding the effects of age on laboratory findings can increase diagnostic accuracy. The recognition of age-related changes allows clinicians to distinguish clinically significant changes in

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laboratory test results that are attributed to disease from changes that are associated with healthy aging. Reference ranges for a majority of serum/plasma analytes measured in the laboratory have been established for the healthy adult population [4]. Yet, standardized age-specific reference ranges that distinguish pathologic from non-pathologic processes in the newborn, childhood to puberty, and elderly adult populations are not complete. Understanding the effects of age on laboratory results is crucial.

In 2000, following the passage of the National Children's Act, the U.S. Congress authorized the National Children's Health Study (NCS). NCS is a longitudinal study of 100,000 healthy individuals aged 0–21 years led by the Eunice Kennedy Shriver National Institute of Child Health and Human Development. As a collaborator of the NCS, the American Association of Clinical Chemistry (AACC) has funded several pilot studies to determine age-specific reference ranges [5]. Although the NCS was canceled in 2014, several collaborative studies have emerged under a new project known as Environmental Influences on Child Health Outcomes (ECHOs) program that encompasses multiple longitudinal studies of mothers and their children. ECHO has four key focus areas: upper and lower airway, neurodevelopment, obesity, pre-, peri-, and postnatal outcomes [6].

Prenatal/newborn population

Newborn babies demonstrate different normal values than adults for various analytes. Following birth, arterial blood PO_2 rises to about 80–90 mmHg. Oxygen consumption is significantly higher in the neonates compared to adults. A significant reduction in uric acid concentrations occurs between birth and six days of age. Healthy newborns rapidly metabolize glucose as a result of their high red blood cell count which is not evident in healthy adults [7]. Newborns have increased circulating bilirubin concentrations owing to their immature liver that is unable to convert bilirubin to bilirubin di-glucuronide. Hyperbilirubinemia due to physiologic jaundice is a common condition in newborns and usually resolves within in 5–7 days following birth but after birth it may be difficult to distinguish this normal physiological phenomenon from hemolytic disease of the newborn [8,9]. In newborns, immature kidney function is evidenced by high vascular resistance, low blood flow with preferential blood flow away from the outer cortex, and a low glomerular filtration rate (GFR). Several important physiological functions of the kidney are compromised in newborns: (i) concentrating and diluting functions, (ii) acid base regulation, (iii) sodium reabsorption, excretion and retention, (iv) natriuretic response to sodium, and (v) hydrogen ion secretion [10]. Newborns experience an expanded extracellular fluid volume state. Hypocalcaemia in this population usually resolves within the first two days of life if not associated with a disease processes [11].

Childhood to puberty stages

Growth and development markedly influence laboratory test results. The Pituitary-Gonadal Axis also undergoes changes that contribute to the luteinizing hormone (LH) and follicle stimulating hormone (FSH) fluctuations. In boys and girls, two weeks following birth LH concentrations increase but decline to pre-pubertal concentrations by their first birthday. Similarly, FSH concentrations follow the same trend as LH concentrations after birth but decline to pre-pubertal concentrations in boys by the first year of life and in girls by the second year of life. Reduced LH and FSH concentrations in the early to mid-teen years are not sensitive enough to distinguish between pubertal delay and hypogonadotropic hypogonadism. Gonadal failure indicated by an upward trajectory of LH and FSH concentrations cannot be expected until 10 years of age. Estradiol concentrations are markedly elevated at birth but rapidly decline during the first week to pre-pubertal concentrations (0.5–5.0 ng/dL for girls, 1.0–3.2 ng/dL for boys), and decline to pre-pubertal concentrations again by the sixth month in boys and by the first year of life in girls [12,13]. Skeletal growth and muscle mass development accounts, in part, for the increased alkaline phosphatase (ALP), gamma-glutamyl transferase (γ-GGT), creatinine, and human growth hormone concentrations seen in the childhood to puberty developmental period. ALP concentrations in girls decline after the age of 12 whereas the decline is after the age of 14 in boys [14]. Notably, appreciable alkaline phosphatase concentrations are present during growth spurts but can also be associated with bone diseases (osteoblastic bone cancers, osteomalacia, Paget's disease and rickets). Alkaline phosphatase concentrations are approximately 3-fold higher in adolescents compared to adults [15]. Creatinine increases with age from 12 to 19 years whereas cystatin C concentrations decrease during the same age range, particularly in females. Uric acid concentrations continue to decline up to 10 years of age [16].

Adulthood

In both sexes, total cholesterol increases with advancing age (men age 60 and women age 55). In the second decade of life men have peak uric acid concentrations which are not evident in women until the fifth decade of life [8].

Menopausal pre- and post- period

Post-menopausal women have increased total cholesterol concentrations, which are thought to be due to decreased concentrations of the estrogen. High density lipoprotein (HDL) cholesterol also declines up to 30% [8]. The transition from the peri-to the post-menopausal stage is associated with dramatic endocrine changes. A strong correlation between age and human chorionic gonadotropin (hCG) is observed [17]. Clinical confusion surrounds how to appropriately interpret hCG laboratory results since hCG synthesis is appreciable during healthy pregnancy, cancer or trophoblastic disease. Notably, the pituitary gland produces hCG in addition to LH, FSH and thyroid stimulating hormones, which are all structurally similar glycoprotein [18]. Routine measurements of serum hCG concentrations (reference limit hCG <0.5 mIU/mL) in women are performed to either identify pregnancy or to rule out pregnancy prior to performing invasive medical procedures or administering medications that may be harmful to a developing fetus [19]. Slight increases in serum hCG concentrations (>/ = 0.5 mIU/mL) occur in women between the ages of 41 and 55 years. Thus, it is critical to distinguish the origin of the hCG (placental origin vs. pituitary origin). Misinterpretation of the slightly elevated hCG concentrations in peri- and postmenopausal women may postpone critical clinical treatments. In peri- and postmenopausal women (41−55 years of age), it has been suggested to use serum FSH concentrations to rule out hCG of placental origin and pregnancy. In peri- and postmenopausal women (41−55 years of age) with serum hCG concentrations ranging between 5.0 and 14.0 IU/L, a FSH cutoff of 45.0 IU/L was associated with hCG of placental origin with 100% sensitivity and 75% specificity. Importantly, FSH concentrations >45 IU/L are not present in females with hCG of placental origin. FSH reflex testing should only be used in the setting of pregnancy evaluation in the peri- and postmenopausal population when serum hCG concentrations range between 5.0 and 14.0 IU/L [20]. However, hCG >14.0 IU/L in this age group are indicative of pregnancy unless the clinical setting dictates otherwise. However, hCG concentration related to pituitary origin rarely exceeds 14 IU/L in postmenopausal women [17].

Elderly

The aging population is rapidly increasing in the United States. Between the year 2000 (35 million persons) and the year 2010 (40 million persons), the U.S. experienced a 15% increase in the aged population (65 years and older) [21]. In 2014, there were 46 million Americans over the age of 65 years and this populations growth is expected to be >98 million by 2060 [22]. Aging-related diseases are diagnosed, treated and monitored using various *in* vitro diagnostic testing. However, interpretation of laboratory findings in the elderly adult population is challenging due to multiple confounding factors: (i) physiologic changes that naturally occur with healthy aging, (ii) acute and chronic conditions (kidney disease, diabetes and cardiovascular disease), (iii) diets, (iv) lifestyles, and (v) medication regimens [23]. Age-related changes in serum proteins are evident in elderly adults including albumin, an indicator of malnutrition and disease, which is more significantly affected. After the age of 60, albumin concentrations decline each decade, with significant decreases noted in individuals >90 years old [24]. The majority of circulating calcium is bound to protein. Low serum calcium concentration in the elderly adult population is most commonly caused by low serum albumin concentrations [25]. Protein concentration changes may be entirely due to compromised liver function or poor dietary regimens.

Iron-related changes occur during aging and are noted as decreased iron storage, serum iron, and total iron-binding capacity. Depletion of iron stores may be followed by increases in serum ferritin and decreases in serum transferrin. Dysregulated liver synthesis during aging may account for the reduced transferrin concentrations [4]. In general, decreased iron storage and iron deficiency anemia in the elderly adult population reflect poor dietary iron intake or substantial loss of iron through occult bleeding from the intestinal tract, both of which should be fully investigated prior to interpretation the changes as age-related. It is hypothesized that the anemia in this age group, may, in part, be explained by the age-related decreases in stomach hydrochloric acid (HCl), a key acid responsible for iron absorption in the intestines. Vitamin B12 deficiency is also prevalent in elderly adults due to age-related decreases in serum vitamin B12 concentrations. The underlying cause for vitamin B12 deficiency may be decreased HCI concentrations or chronic atrophic gastritis, which subsequently accounts for insufficient intrinsic factor and vitamin B12 absorption [23].

Age-associated organ function decline is correlated with changes in laboratory findings (e.g., reduced creatinine clearance, glucose tolerance, and hypothalamic-pituitary adrenal axis regulation) that may represent disease or non-disease processes. In 10% or more of the healthy elderly population, physiologic changes in partial pressure of oxygen in arterial blood (between the 3rd and 8th decade decreases by 25%), serum alkaline phosphatase (increases by 20% between the 3rd and 8th decade), 2-h postprandial glucose (after age 40 increases 30−40 mg/dL per decade), cholesterol (by age 60 increases by 30−40 mg/dL), erythrocyte sedimentation

rate (values as high as 40 can be non-pathogenic), and magnesium (decreases by 15%), are apparent in the elderly but may not be associated with disease processes [8,23].

A 30%–40% decline in functioning kidney and the glomerular filtration rate (GFR) is associated with reduced creatinine clearance. Creatinine and blood urea nitrogen (BUN) concentrations can overestimate the kidney functioning capacity, as measured by GFR or creatinine clearance, due to reduced muscle mass [26]. The decline in muscle mass results in reduced creatinine production; thus serum creatinine concentrations remain within normal limits despite the underlying diminished renal clearance capacity [23]. Mean creatinine clearance decreases by 10 mL/min/1.73 m^2 per decade and is significantly different between the adult and geriatric populations. The mean creatinine clearance for a 30-year old is 140 mL/min (2.33 mL/s) per 1.73 m^2 of body surface area, whereas in an 80-year old the creatinine clearance concentration is 97 mL/min (1.62 mL/s) per 1.73 m^2 of body surface area [27]. Small increases in serum aspartate aminotransferase (AST) (18 U/L to 30 U/L) are noted between 60 and 90 years of age whereas serum alanine aminotransferase (ALT) peaks in the fifth decade of life and by the sixth decade gradually declines to concentrations well below those noted in young adults [23]. Gamma-glutamyl transferase (GGT) concentrations rise during aging. A steady increase in serum glucose concentrations and a decrease in glucose tolerance are prevalent in elderly adults. Lower glucose concentrations in elderly adults may be due to poor diet and reduced body mass. Higher serum insulin concentrations are prevalent in elderly adults and may be associated with insulin resistance [23]. In persons older than 75 years, insulin resistance is reportedly responsible for impaired glucose tolerance. These changes may be explained by the fact that the response capacity of insulin receptors is significantly lower in elderly adults compared to young adults. Regarding serum immunoglobulin concentrations, IgA concentration increases slightly in elderly men but overall IgG and IgM concentrations gradually decline. The hypothalamic-pituitary-adrenal axis is compromised during aging and changes include decreases in free thyroxine (FT4), triiodothyronine (T3), corticotropin, and corticosteroid concentrations [28]. Specific to men, free testosterone decreases with age, without significant changes in total testosterone [29,30]. Prostate specific antigen (PSA) concentrations increase up to 6.5 ng/mL in men over the age of 70 without clinical evidence of prostate cancer [23]. Serum electrolytes, such as potassium and calcium, increase during aging. Calcium concentration increase in individuals aged 60 to 90 in the presence of normal albumin concentrations. However, after the age of 90, calcium concentrations gradually decline. Hypercalcemia may be due to a simultaneous drop in serum pH and an increase in parathyroid hormone concentrations. Age significantly impacts lung elastic architecture, alveoli function and diaphragm strength and significantly alters respiratory function. Consequently, the partial pressure of arterial oxygen is decreased while carbon dioxide pressure and bicarbonate-ion concentration are increased [23].

Although age can significantly account for altered clinical laboratory test results, one must consider the overlapping effects caused by disease, such as obesity and hypertension, and/or inadequate dietary intake when interpreting laboratory results that are outside of the reference limits [31]. The abnormal results may highlight age associated disease processes that require clinical intervention. Experimental investigations focused on systematic effects of aging on laboratory analytes are warranted and may provide data useful for the development of effective age-specific diagnostic cutoffs.

GENDER RELATED CHANGES ON CLINICAL LABORATORY VALUES

Gender encompasses a myriad of complex endocrine and metabolic responses. Gender differences in laboratory analytes can be explained by differential endocrine organ related functions and skeletal muscle mass [32]. On average, albumin, calcium, magnesium, hemoglobin, ferritin and iron concentrations are lower in females [8]. Reduced iron concentrations are attributed to blood loss during monthly menses. Mean serum creatinine and cystatin C concentrations are commonly lower in adolescent females compared to adolescent males [16]. Aldolase concentrations are higher in males following the start of puberty. ALP concentrations are higher in girls ages 10–11 and in boys ages 12–13, 14–15, and 16–17 had higher ALP concentrations. A decline in ALP concentrations begins after age 12 for girls and 14 for boys [14]. Menopausal women have higher ALP concentrations than males. Serum bilirubin concentrations are lower in women owing to the decreased hemoglobin concentrations. Gender differences in mean creatinine and creatine kinase concentrations are attenuated when comparing athletic females and males. Females have higher albumin concentrations compared to males of the same age [33]. Lipid profiles are heavily influence by gender. Total cholesterol concentrations vary not only with age but with gender. Females, under the age of 20 years, have higher total cholesterol concentrations compared to males in the corresponding age span. However, between the ages of 20 and 45 males commonly have higher total cholesterol concentrations than females. Males experience a peak in lipid concentrations generally between the ages of 40 and 60 whereas

females peak lipid concentrations occur between the ages of 60 and 80 [34]. Individuals over 90 years of age may have significantly decreased total cholesterol concentrations. Between the ages of 30 and 80, mean high density lipoprotein cholesterol (HDL) decreases by approximately 30% in females but increases by 30% in males [23,35]. These lipid increases may be due to the stimulatory effect of estrogen in the women. In contrast, low-density lipoprotein cholesterol (LDL-C) is higher in men. Men also have higher 24-h urinary excretions of epinephrine, norepinephrine, cortisol, and creatinine excretion compared to women [36]. Women have higher serum GGT and copper, and reticulocyte count (due to increased erythrocyte turnover) compared to their male counterparts.

DIETARY RELATED CHANGES ON CLINICAL LABORATORY VALUES

Diet may affect test results while starvation also has profound effect on clinical laboratory test results. Various dietary factors that affect clinical laboratory test results are discussed in this section.

Food ingestion-related changes on clinical laboratory values

Food ingestion activates *in vivo* metabolic signaling pathways that significantly influence laboratory test results [37]. First, the stomach secretes HCl in response to food consumption which causes a decrease in plasma chloride concentrations. This mild metabolic alkalotic state (alkaline tide phenomenon) results from exaggerated circulating bicarbonate concentrations in the stomach's venous blood and is accompanied by decreased ionized calcium by 0.05 mmol/L (0.2 mg/dL) [38]. Secondly, postprandial-associated impairment in the liver leads to increased bilirubin, and enzyme activities. Depending upon the content of the meal ingested the effects on commonly measured analytes may be short or long lasting. Thus, an overnight fasting for at least 12 h is necessary to obtain an accurate representation of *in vivo* glucose, lipids, iron, phosphorus, urate, urea, and ALP concentrations. Interestingly, ALP activity rises in response to the ingestion of a high-fat meal in Lewis *a* secretors of the blood groups B and O. Lipemia can also interfere with a variety of analytical methods, such as indirect potentiometry. Prior to analysis, lipids can be removed from lipemic samples via ultracentrifugation or by the use of lipid clearing reagents [39]. Carbohydrate (increases glucose and insulin, decreases phosphorus) and protein meals (increases cholesterol and growth hormone within 1 h of food consumption, increases glucagon and insulin) have differential effects on serum analytes. High protein diet can significantly affect various analytes measured in 24-h urine. A standard 700 calories meal markedly increases triglycerides (~50%), AST (~20%), bilirubin and glucose (~15%) and AST concentrations (~10%) [3]. Rapid changes in lipid concentrations are consistent with dietary changes, medications, or disease.

Caffeine intake has significant effects on the human body. It is contained in a variety of foods (coffee, tea, chocolate, soft drinks, and energy drinks) in varying concentrations. The short half-life of caffeine (3–7 h) also varies among individuals. Caffeine induces catecholamine excretion from the adrenal medulla and increases gluconeogenesis, resulting in subsequently increases in glucose concentrations and impaired glucose tolerance following caffeine intake. The adrenal cortex is also vulnerable to caffeine's stimulatory effects as evidenced by increased cortisol, free cortisol, 11-hydroxycorticoids and 5-hydroxindoleaceatic acid concentrations. Caffeine is responsible for a 3-fold increase in non-esterified fatty acids, which interfere with the accurate quantification of albumin-bound drugs and hormones. Spuriously high ionized calcium concentrations can occur following caffeine ingestion since caffeine-induced elevations in free fatty acids can cause a rapid decrease in pH that facilitates the removal of calcium from protein.

Noni juice contains significant amounts of potassium (approximately 56 mEq/L). Ingestion of noni juice can led to hyperkalemia in vulnerable populations such as individuals with renal dysfunction and/or populations receiving potassium increasing regimens such as spironolactone or angiotensin converting enzyme inhibitors. Bran stimulates bile acid synthesis within 8 h of ingestion [38]. However, bran inhibits gastrointestinal absorption of important nutrients including calcium (decreased by 0.3 mg/dL/0.08 mmol/L), cholesterol, and triglycerides (decreased by 20 mg/dL/0.23 mmol/L) [3]. Serotonin (5-hydroxytryptamine) is contained in a myriad of fruits and vegetables such as bananas, black walnuts, kiwis, pineapples, and plantains. Bananas markedly increase 24-h urinary excretion of 5-hydroxyindoleacetic acid in the absence of disease. Avocados suppress insulin secretion causing impaired glucose tolerance.

Special diet-related changes on clinical laboratory values

The ketogenic diet is a low carbohydrate (<40 g/d), moderate-protein, high-fat diet. In the absence of sufficient carbohydrates, the liver is forced to convert fat into fatty acids and ketones. Adherence to a ketogenic diet results in elevated blood and urine ketones within several days and diuresis within 2 weeks. Reportedly, a decline in serum triglycerides and an increase in HDL-C occur over several weeks [40]. The non-vegetarian diet

is associated with higher plasma ammonia, uric acid and urea concentrations compared to a vegetarian diet. The ketogenic diet commonly includes saturated fatty acids, which in the case of palmitic acid can significantly increase plasma cholesterol concentrations. The substitution of saturated fatty acids with polyunsaturated fats and complex carbohydrates can lower LDL-C (low density lipoprotein cholesterol) concentrations. Intake of omega-3 oils may lower triglycerides and very low-density lipoprotein (VLDL) concentrations. Vegetarians have lower LDL-C (approximately 37% lower) and HDL-C (approximately 12%) concentrations compared to non-vegetarians. In contrast, lactovegetarians (vegetarians that consume dairy products) have higher LDL-C (approximately 24% higher) and HDL-C (approximately 7% higher) concentrations compared to vegetarians. Within 20 weeks, a lactovegetarian diet regimen accompanied by low protein and high dietary fiber intake can reduce adrenocortical activity. Lactovegetarians have higher plasma concentrations of dehydroepiandrosterone sulfate (DHEAS) compared to non-vegetarians (individuals who adhere to a moderately protein-rich with diet). Moreover, lactovegetarians have reduced urinary 24-h excretion rates for C-peptide, free cortisol, DHEAS, and total 17-ketosteroid [41]. In middle-aged North American black individuals, urinary 24-h excretion rates of adrenal and gonadal androgen metabolites were reduced after changing from a meat-containing Western diet to a vegetarian diet. The fecal fat test, which measures the amount of fat content in stool to diagnose absorption or digestion abnormalities, is susceptible to dietary influences. It is critical that individuals refrain from significant dietary changes during before and during sample collection.

The hCG diet consists of hCG sublingual drops or injections paired with a low 500-calorie diet. As previously discussed, hCG can be of placental or non-placental origin. hCG is evident in placental trophoblastic (hydatidiform mole, choriocarcinoma), gonadal (ovarian, testicular or extragonadal teratoma), ectopic or non-trophoblastic tumors. Although, exogenous hCG is normally excreted from the body within 10 days, hCG may linger in the body. Individuals on the hCG diet that received injections of hCG have markedly elevated serum hCG concentrations in the absence of pregnancy or malignancy [42]. It is evident that individuals on the hCG diet may have unreliable hCG test results. However, the effects of hCG sublingual drops on other laboratory tests are currently unknown. In healthy males, hCG injections (purified urinary and recombinant hCG) cause a consistent dose-dependent and sustained rise in serum testosterone concentrations through stimulation of Leydig cell testosterone secretion [43].

Fasting/starvation-related changes on clinical laboratory values

Fasting (decreased caloric intake) and starvation (no caloric intake) initiate complex metabolic derangements. Many individuals fast in accordance with culture and religious traditions so understanding the effects of fasting on laboratory results is important. Within 3 days of fasting, glucose concentrations decrease by as much as 18 mg/dL despite the body's coordinated efforts to conserve proteins. Subsequently, insulin rapidly declines while glucagon secretion increases to restore blood glucose to pre-fasting concentrations. The fasting individual undergoes both lipolysis and hepatic ketogenesis, and the metabolic acidosis state is noted by elevated serum acetoacetic acid, beta-hydroxybutyrate, and fatty acids as well as reduced pH, pCO_2 and bicarbonate concentrations. The resultant focal necrosis of the liver is characterized by reduced hepatic blood flow, impaired glomerular filtration and creatinine clearance, and elevated serum ALT, AST, bilirubin, creatinine and lactate concentrations are observed [3].

The body's reduced energy stores mainly account for significant declines up to 50% in both total and free triiodothyronine concentrations. Fasting differentially affects lipid concentrations: within 6 days, cholesterol and triglycerides increase and HDL decreases. Sharp increases up to 15 times the pre-fast plasma in growth hormone concentrations occur early in fasting. Within 3 days of completing a fast, the plasma growth hormone concentration returns to pre-fast concentrations. Albumin, prealbumin and complement 3 (C3) decline during an extended fast but the analytes are rapidly restored to pre-fasting concentrations following protein supplementation.

Starvation triggers the release of aldosterone and excessive urinary ammonia, calcium, magnesium and potassium excretion. In contrast, urinary excretion of phosphorus over time declines. Following a short term, 14-h fast, acetoacetate, beta-hydroxybutyrate, lactate, and pyruvate blood concentrations begin to rise. Long term starvation lasting for 40–48 h causes up to a 30-fold increase in beta-hydroxybutyrate. Reportedly, starvation for four weeks significantly increased AST, creatinine, and uric acid (20–40%) and decreased GGT, triglycerides, and urea (20–50%).

Upon adequate caloric intake, the body begins to restore blood constituents to pre-fasting concentrations and retains sodium as a result of decreased urinary excretion of both sodium and chloride. Subsequently, aldosterone exceeds fasting concentrations and urinary excretion potassium slowly returns to normal.

Nutraceutical-related changes on clinical laboratory values

In 1989, Dr. Stephen DeFelice coined the term "Nutraceutical" from "Nutrition" and "Pharmaceutical". Nutraceuticals, according to the American Nutraceutical Association, includes functional foods with health-promoting and disease-preventing benefits. Rigorous safety and efficacy studies are lacking and little is known about the pharmacokinetic properties of the commercially available nutraceuticals. An estimated 170 million Americans regularly take dietary supplements to enhance their overall wellbeing. Although, nutraceuticals exhibit pharmacological effects, patients do not consider them 'drugs' and often do not disclose usage to their physicians [44]. It is not fully clear how different types of nutraceuticals and conventional drugs interact within the body or the effects of the interaction. Few studies have documented the pharmacokinetic effects of nutraceuticals on laboratory findings [45].

Although it has been known for several decades that biotin is an interfering substance in assays that employ the biotinylated-streptavidin binding, the recent pervasive non-medical ingestion of supraphysiologic doses of biotin, prompted the FDA in 2017 to issue a warning. The warning was directed at consumers, healthcare providers, laboratory personnel, and lab test manufacturers and developers and stated that 'ingesting high levels of biotin in dietary supplements can cause clinically significant incorrect lab test results' [46]. Biotin is a cofactor in enzymatic carboxylation reactions. Significant biotin interference has been reported by several studies in which biotin dosages was 10,000 μg and higher. The typical Western diet can fulfill the adult recommended daily intake of biotin, 30 mcg.

Medically necessary high dose biotin therapy is common for multiple sclerosis, malabsorption syndrome, genetic biotin deficiency, biotin-thiamin responsive basal ganglia disease, and hemodialysis patients. Awareness of potential high biotin blood levels in the fore-mentioned patient populations is important to ensure appropriate alternative non-biotinylated assays are used for testing. Excessive biotin supplementation can produce falsely low results when using a 2-site "sandwich" assay and falsely elevated test results when using a competitive immunoassay (Table 4.1) [47–49].

High protein supplements are not only associated with intermittent abdominal pain but with laboratory manifestations of hyperalbuminemia and transient increases in AST and ALT. Albumin and liver enzyme activities returned to normal once the patients discontinued using the high protein supplements [50]. Widely used as an anti-depressant, St. John's Wort (*Hypericum perforatum*) markedly interferes with the metabolism of prescribed drugs as it is a potent inducer of P-glycoprotein and cytochrome P450 3A4 (CYP3A4) and to a lesser extent CYP1A2 and CYP2C9 [44]. Co-administration of St. John's Wort significantly alters concentrations of cyclosporine (transplant rejection) [51], indinavir (HIV inhibitor) [52], and Digoxin (P-glycoprotein transporter) [53]. Royal jelly is a milky secretion produced by glands in the heads of nurse honey bees for the development and nurturing of queen bees. Ingestion of Royal jelly by an 87-year-old man, who was previously stabilized on warfarin, developed hematuria and was found to have a highly elevated international normalized ratio, (INR) of 7.29 after taking a royal jelly supplement for a week. It is not known how Royal jelly might increase the effects of warfarin. Valerian, also used for its anti-depressant properties, has been acute hepatotoxicity (elevated ALT, AST, GGT) however long-term effects on liver function are unclear. See Chapter 7 for more in-depth discussion on the effects of herbal supplements on clinical laboratory test results.

TABLE 4.1 Effects of excessive biotin supplementation on common endocrine tests measured by immunoassays.

Cortisol (competitive)	Falsely high
DHEAS (competitive)	Falsely high
Estradiol (competitive)	Falsely high
Free thyroxine (competitive)	Falsely high
Free triiodothyronine (competitive)	Falsely high
Insulin (2-site "sandwich")	Falsely low
FSH (2-site "sandwich")	Falsely low
LH (2-site "sandwich")	Falsely low
NT-proBNP (2-site "sandwich")	Falsely low
Parathyroid hormone (2-site "sandwich")	Falsely low
PSA (2-site "sandwich")	Falsely low
Prolactin (2-site "sandwich")	No effect observed
Testosterone (competitive)	Falsely high
Thyroglobulin (2-site "sandwich")	Falsely low
TSH (2-site "sandwich")	Falsely low
TSH receptor antibodies (2-site "sandwich")	Falsely low
Troponin T (2-site "sandwich")	Falsely low
Vitamin B12 (competitive)	Falsely high
25-hydroxyvitamin D (competitive)	Falsely high
25-hydroxyvitamin D (2-site "sandwich")	Falsely low

Cross-sex hormone therapy effects on laboratory test results

In many clinical laboratories gender-specific reference intervals are appended to test results. These reference intervals are not appropriate for patients on cross-sex hormone regimens and thus may be challenging for the clinician to interpret. Although limited, there are studies that have examined analyte changes in transgender individuals and may serve as a reference for laboratories establishing reference intervals (Table 4.2) [54]. Interestingly, in male-to-female patients on cross-sex hormone therapy, hematocrit, low-density lipoprotein, and hemoglobin reference ranges were within the cis female reference intervals. Yet, creatinine, alkaline phosphatase, and potassium reference ranges were within the cis male reference ranges. Overall, triglycerides were higher than both the cis female or cis male reference intervals (Fig. 4.2) [55]. To eliminate health disparities in the delivery of care provided to transgender patients, additional studies that examine analyte changes in patients on cross-sex hormone therapy are needed to identify: (i). analytes that should be monitored during the transitioning period; (ii) analytes that have diagnostic and prognostic strength for acute and chronic diseases in this population [56–58].

TABLE 4.2 Differences in routinely measured analytes in male to female trans patients on hormone therapy.

Analyte	Male	Male-to-female	Female
ALP	34.3–83.8[a]	24.4–90.7[a]	31.0–98.0
ALT	8.2–37.5	6.3–35.3	9.7–34.1
AST	9.7–37.6	11.7–36.9	11.1–32.6
BUN	7.2–19.9	5.9–18.8	4.9–21.9
Creatinine	0.73–1.3[a]	0.55–1.3[a]	0.65–1.0
Hgb	13.3–16.2	12.6–14.7[a]	11.9–14.6[a]
HCT	38.4–45.7	34.6–43.7[a]	34.4–41.9[a]
Na	134.9–141.7	134.2–141.2	135.7–140.8
K	3.6–4.6[a]	3.4–4.9[a]	3.6–5.2
Cholesterol	120.5–246.3	110.2–244.5	104.5–254.3
Triglycerides	5.7–183.3	34.2–242.6[b]	31.2–110.6
HDL	24.9–63.9	19.5–85.1	32.0–76.2
LDL	73.8–151.2	4.3–161.4[a]	45.6–166.0[a]

ALP, alkaline phosphatase; *ALT*, alanine aminotransferase; *AST*, aspartate aminotransferase; *BUN*, blood urea nitrogen; *HCT*, hematocrit; *HDL*, high-density lipoprotein; *Hgb*, hemoglobin; *K*, potassium; *LDL*, low-density lipoprotein; *Na*, sodium.
[a]*Indicates consistent changes aligned with either natal or identified gender.*
[b]*Indicates consistent changes intermediate to natal and identified gender.*
Source: Roberts TK, Kraft CS, French D, et al. Interpreting laboratory results in transgender patients on hormone therapy. Am J Med 2014;127:159–62, Table 1. ©Elsevier reprinted with permission.

EXERCISE RELATED CHANGES ON CLINICAL LABORATORY VALUES

The effects of exercise on laboratory findings varies and is highly correlated with the health status of the person [59], temperature, and dietary intake (food or liquid) that occurs during or following exercise [60]. Fig. 4.1 shows that the frequency distribution of serum creatinine concentrations in athletes and controls (sedentary people) [59]. Thyroid function is markedly affected by the exercise intensity. Anaerobic exercise (characterized by 70% of maximal heart rate and lactate values $4.59 + 1.75$ mmol/L) caused a rise in T4, free T4 and thyroid stimulating hormone (TSH) but a fall in T3 and free T3 [61]. Physical exercise significantly alters plasma volume as a resultant of fluid volume loss due to sweating and fluid shifts between both the intravascular and interstitial bodily compartments [62]. Exercise reduces urinary erythrocyte and leukocyte content and the volume of urine while increasing the urinary protein excretion. Elevated urinary protein will clear within 24–48 h. Following exercising, a transient increase in the white blood cells, hematocrit and platelets occurs in parallel with electrolyte abnormalities (serum potassium decreases by 8%), which are present due to the altered hydration state and usually normalize with rehydration. Dehydration is also associated elevated creatinine and BUN concentrations. In the setting of severe dehydration, a sharp rise in BUN occurs but creatinine is only mildly elevated. Again, rehydration will gradually decrease these concentrations to normal.

Regular vigorous exercise raises HDL-C and lowers triglycerides, very low density lipoprotein cholesterol (VLDL-C), and LDL-cholesterol. On the other hand, weightlifting exercise in healthy men (aged 18–45) induced pathological liver function test results [63]. AST, ALT, LD, CK and myoglobin were significantly increased following weightlifting and remained increased for approximately 7 days post exercise. Yet, bilirubin, GGT and ALP were within normal limits. These findings highlight the importance of refraining from weightlifting prior to clinical laboratory testing. Among healthy males, exercise comprised of 30-min cycling at 70–75% of maximal heart rate with a 30-min recovery significantly increased concentrations of hematocrit, red blood cell count, plasma albumin and fibrinogen concentrations, plasma viscosity, and whole blood viscosity from baseline in all three age groups (20–30 years, 40–50 years, and 60–70 years) in male subjects [64]. However, the changes were temporary and returned to baseline after the 30-min recovery period. In endurance runners exercise-associated iron deficiency is common. In moderately trained female long-distance runners, long-term endurance exercise

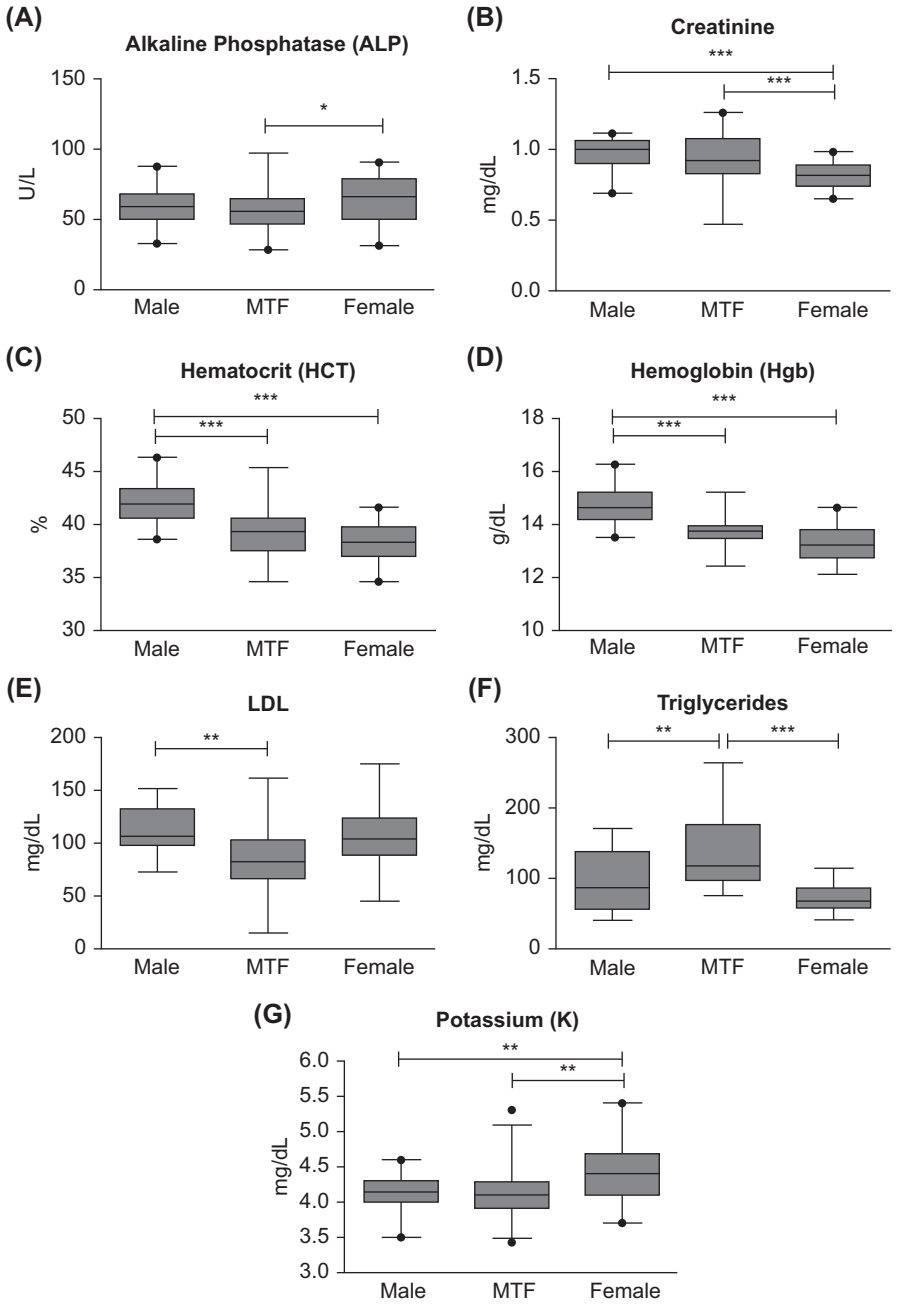


FIG. 4.1 Consistent differences in laboratory measured and routinely monitored in male-to-females on hormone therapy. Values for male-to-female trans patients on hormone therapy for ≥6 months (n = 55) compared with healthy male (n = 20) and female (n = 20) controls. Data are expressed as the 25th, 50th (median), and 75th percentiles (boxes) and the 2.5th and 97.5th percentiles (whiskers). *P < .05; **P < .01; ***P < .005. *LDL*, low-density lipoprotein. *Source: Roberts TK, Kraft CS, French D, et al. Interpreting laboratory results in transgender patients on hormone therapy. Am J Med 2014; 127:159–62, Fig. 1 ©Elsevier, reprinted with permission.*

(8 weeks) was evaluated and no differences were apparent in high sensitivity C-reactive protein (CRP) concentrations, suggesting that inflammation is not a normal process of endurance exercise in females. On the other hand, both serum hepcidin and soluble transferrin receptor (sTfR) were affected and may explain the higher prevalence of iron deficiency in this population. Analytes that are affected by exercise are summarized in Table 4.3.

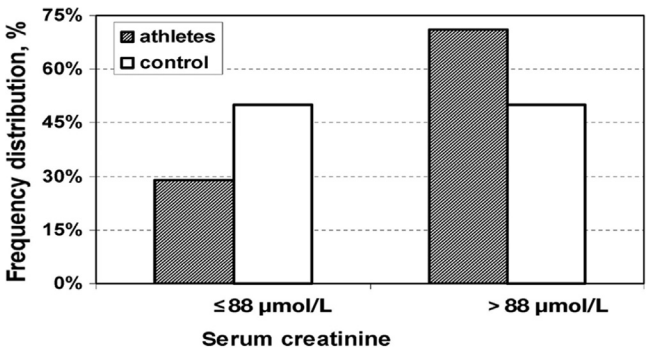


FIG. 4.2 Frequency distribution of serum creatinine concentrations in the 2 groups, athletes and controls. Data are divided considering as threshold the median value of the control group [88 µmol/L (1.0 mg/dL)]. *Source: Banfi G, Del Fabbro M. Serum creatinine values in elite athletes competing in 8 different sports: comparison with sedentary people. Clin Chem Feb 2006;52(2):330–1. https://doi.org/10.1373/clinchem.2005.061390, Fig. 1. Reproduced with permission from the American Association for Clinical Chemistry.*

TABLE 4.3 Analytes that are affected by exercise.

Analyte	Effect of exercise
Urea	Value may increase after exercise
Creatinine	Value may increase after exercise
Aspartate Aminotransferase (AST)	Value may increase after exercise
Lactate Dehydrogenase (LDH)	Value may increase after exercise
Total Creatinine Kinase (CK)	Value may increase after exercise
CK-MB	Value may increase after exercise
Myoglobin	Value may increase after exercise
WBC Count	Value may increase after exercise
Platelet Count	Value may increase after exercise
Prothrombin Time	Value may increase after exercise
D-Dimer	Value may increase after exercise
Packed Cell Volume	Value may decrease after exercise
Activated Partial Thromboplastin Time	Value may decrease after exercise
Fibrinogen	Value may decrease after exercise

DIFFERENCE IN LABORATORY TEST RESULTS AMONG POPULATIONS

Several analytes have been reported to exhibit small to large differences among different ethnic populations [65]. The clinical significant of these differences are not always evident and may 'racialize' the delivery of patient care [66]. It is imperative that laboratory professionals and clinicians refrain from using ethnicity or race as the ultimate indicator for diagnosis, management, and prognosis as such use can increase the risk of serious medical errors. Self-identification and social categorization of populations continues to evolve as many individuals identify with more than one race.

Population differences in total serum protein concentration was reported to be higher in black individuals compared to white individuals and was attributed to gamma-globulin concentrations although serum albumin concentration was lower in black individuals. Along similar lines, creatinine kinase was reportedly higher in a study comparing black and white subjects noting higher muscle mass in the black subjects. However, each patient is different and a white individual's muscle mass can be equivalent or exceed a black individual's muscle mass. Healthcare professionals must incorporate 'personalized medicine' when evaluating clinical results. One study reported that black children exhibited higher ALP compared to white children.

Another study showed that serum cystatin C concentrations were different among non-Hispanic white, non-Hispanic black, and Mexican American adolescents (aged 12 to 19) [16]. Cystatin C concentrations were higher in non-Hispanic white compared to non-Hispanic black and Mexican Americans. In contrast, creatinine was lower in non-Hispanic white and Mexican Americans compared with non-Hispanic black Americans. In an adult population-based sample (aged 50–67), black men had higher 24-h urinary excretion of creatinine and higher concentrations of the stress hormones epinephrine and norepinephrine compared to white men [36]. Hematological differences were reported between African Americans and white subjects in which the hematocrit, hemoglobin, mean corpuscular volume, serum transferring saturation, and white blood cell counts were lower in the African American group. Ferritin was higher in the African American group [67].

Currently, in the United States the Modified Diet of Renal Disease (MDRD) calculation the ethnicity factor of 1.2 is used to estimate the glomerular filtration rate (GFR) in Black/African Americans. However, two large studies conducted in sub-Saharan Africa (Ghana $n = 944$ and South Africa $n = 100$) showed the MDRD equation performed better in the absence of the ethnicity factor of 1.2 [68]. In a Saudi population ($n = 32$), GFR estimated by MDRD strongly correlated with the

measured inulin clearance [69]. Yet, in a Japanese population (n = 248), GFR estimated by the 0.881xMDRD equation correlated better with measured inulin clearance than with the 1.0xMDRD equation [70]. Clearly, estimation of GFR by MDRD varies by global region and most likely this variability correlates with genetic/environmental factors that are associated with body muscle mass. Interpretation of laboratory findings based solely on the presumed effect of ethnicity/race should not occur.

CONCLUSIONS

It is important for laboratory professionals to implement age and gender specific reference ranges for certain analytes and such reference range information should be a part of routine reporting of laboratory test results. Nevertheless diet, exercise and other factors may alter expected laboratory test results and proper investigation must be made to interpret such laboratory test results for proper patient management. Especially for pharmacogenetics testing.

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CHAPTER

5

Interferences of hemolysis, lipemia and high bilirubin on laboratory tests

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INTRODUCTION

The preanalytical phase of the laboratory testing process includes all of the procedures prior to the start of sample analysis, and typically involves a variety of healthcare professionals. There are a wide variety of preanalytical factors that can adversely impact the integrity of specimens that are submitted for analysis. These factors include the improper or incorrect use of collection containers, improper tourniquet application, contamination from infusion routes, excessive time delay from specimen collection to analysis, failure to store specimens at an appropriate temperature prior to analysis, failure to shield the specimen from direct light, and collection of the specimen at the wrong time of day or at an inappropriate time following the administration of certain medications [1]. Prevention of medical errors in healthcare has received a great deal of attention since the publication in 2000 of the report from the Institute of Medicine which estimated that medical errors result in approximately 44,000 to 98,000 preventable deaths and 1,000,000 excess injuries each year in U.S. hospitals [2]. Therefore, accurate laboratory test results are important in order to prevent medical errors because diagnoses of many diseases today are based on laboratory test results. While medication errors are most often cited as the number one cause of medical errors, inappropriate treatment of patients due to incorrect test results caused by interfering substances has been noted to be a factor contributing to medical errors [3–5]. One study found a significant decrease in the number of errors observed in the clinical laboratory between 1996 and 2006. However, the proportion of pre-analytical errors observed during this time span remained constant [6]. Interference in clinical assays is often underestimated and too often undetected in clinical laboratories [7]. Preanalytical errors due to endogenous interfering substances are perhaps one of the most common causes of errors that occur in laboratory testing.

In principle, interferences that affect the spectrophotometric measurement of a sample can be reduced by use of an adequately blanked analytical method. However, this is often not practical or easy to implement. In addition, endogenous interfering substances can cause not only spectral interference, but can also cause chemical interferences in some assays. Also, interference due to hemolysis can increase the concentrations of those analytes that are present in the erythrocytes themselves. While common endogenous interferences such as hemolysis, lipemia and icterus are known to interfere with photometric assays, interference with turbidimetric methods and immunoassays have also been reported.

The prevalence of endogenous interfering substances seen in patient samples submitted for analysis can be significant, but the actual frequency at which interferences occur can be difficult to estimate. One study that investigated the prevalence of endogenous interferences seen in outpatients found that 9.7% of samples submitted for analysis contained at least one endogenous interfering substance [8]. Of the samples considered to have some type of endogenous interfering substances, 76% were considered to be lipemic, 16.5% were hemolyzed and 5.5% were icteric. However, significant differences in the incidence of endogenous interferences have been noted with respect to where patients are located within a hospital setting.

Observations by both physicians and clinical laboratory staff suggest that the rate of hemolysis in samples collected in emergency departments significantly exceeds those collected in other hospital locations. One study that evaluated a total of 4,021 samples found that hemolyzed samples were more frequently seen

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in samples collected in the emergency department compared to samples obtained from the medical unit [9]. Of the 2,992 specimens collected in the emergency department, 372 (12.4%) were hemolyzed while of the 1,029 samples from the medical unit, 16 (1.6%) were hemolyzed. The use of trained phlebotomists to collect blood from patients in the medical unit, versus the use of nurses not formally trained in phlebotomy practices to collect blood in the emergency department, was suggested to play a significant role in the differences seen in the rates of hemolysis. Another factor cited as a cause for higher rates of hemolysis in the ED is the common use of intravenous catheters for blood collection in this particular setting [10].

The incidence of endogenous interfering substances seen in specimens submitted to the clinical laboratory is dependent upon a number of factors including the patient population being served (i.e., neonates, diabetics, elderly, patients on total parenteral nutrition, inpatients vs. outpatients, etc.), use of skilled phlebotomists versus minimally trained healthcare providers, and elapsed time from collection of sample to processing and analysis. Patient characteristics such as gender and age have also been cited as factors that affect the rates of hemolysis. One study found a significantly higher rate of hemolysis in samples collected from males (13.1%) versus females (10.1%). This trend for higher rates of hemolysis in males was seen regardless of whether samples were collected in a primary healthcare setting, nursing home or the emergency department [11]. In addition to gender, age has been cited as a contributing factor to the rate of hemolysis. Higher rates have been noted in patients greater than 63 years of age compared to younger individuals [11]. However, samples collected from infants and neonates typically show higher levels *in vitro* hemolysis.

Another important factor in the assessment of endogenous interferents is the mechanism that is used to identify the presence of interfering substances. The use of visual inspection of samples for identification of interfering substances is still in use by some laboratories, although this practice is rapidly being supplanted by the use of instruments with the capability to detect and quantify the amount of interference present. Manual visual detection of endogenous interferences is noted to suffer from significant lack of agreement between individuals and also vastly underestimates the actual number of samples that have levels of endogenous interfering substances that can cause assay interference. Also, increased concentrations of bilirubin can result in underestimation by visual means of the amount of plasma hemoglobin that is present in hemolyzed samples. This situation is frequently seen in samples collected from newborns who often show increased bilirubin concentrations and whose samples are often hemolyzed. Thus, the use of automated systems for detection of endogenous interfering substances is imperative if proper evaluation of these types of samples is to be accomplished. One study that utilized an algorithm for the detection and processing of clinically or analytically relevant amounts of hemolysis found that automated detection of relevant hemolysis was approximately 70 fold higher compared to when sample hemolysis was assessed by manual detection [9].

Despite the vast number of publications that have addressed the problems of endogenous interfering substances, there is still a significant lack of understanding concerning sources of endogenous interferences. For example, artificial substitutes such as Intralipid used to mimic lipemia, often do not behave the same as samples with native lipemia [12]. Despite this, virtually all studies designed to assess the effect of lipemia utilize Intralipid to evaluate this interference. Other aspects of interference testing often ignored or overlooked include whether the interfering substance produces similar interference effects at different analyte concentrations. Evaluation of endogenous interfering substances should be performed at several different concentrations of analyte. For example, a 10% bias in the measured concentration of 100 mg/dL of glucose due to 300 mg/dL of plasma hemoglobin may be insignificant when the glucose concentration in the sample is 200 mg/dL.

EFFECT OF HEMOLYSIS ON LABORATORY TESTS

Hemolysis is the disruption of the erythrocyte membrane with release of hemoglobin and other intracellular components into the surrounding serum or plasma. Intracellular components released in high concentrations from erythrocytes include enzymes such as lactate dehydrogenase (LD) and aspartate aminotransferase (AST), as well as electrolytes such as potassium and magnesium. In cases of massive hemolysis, the release of intracellular fluid from erythrocytes can even result in the dilution of serum or plasma analytes, such as sodium, that are usually present in low concentrations within erythrocytes.

Normally, serum and plasma contains very low concentrations of free hemoglobin, with plasma containing less than 2 mg/dL and serum less than 5 mg/dL of free hemoglobin. Visual detection of hemolysis does not occur until the free hemoglobin concentration is greater than 20–70 mg/dL. The ability to visually detect hemoglobin in serum or plasma is affected by the concentration of other compounds present in the sample. For example, serum or plasma from patients who are jaundiced can mask hemolysis when hemolysis is estimated by visual means. Thus, samples with moderate

TABLE 5.1 Potential causes of *in vivo* and *in vitro* hemolysis.[a]

IN VIVO *HEMOLYSIS*
Extravascular hemolysis
Enzyme deficiencies (e.g., glucose-6-phosphate dehydrogenase deficiency)
Hemoglobinopathies (e.g, sickle cell, thalassemia)
Erythrocyte membrane defects (e.g., hereditary spherocytosis)
Infection (e.g., Bartonella, Babesia, malaria)
Autoimmune hemolytic anemia
Other (e.g., hypersplenism, liver disease)
Intravascular hemolysis
Microangiopathy (e.g., prosthetic heart valve, thrombotic thrombocytopenic purpura)
Transfusion reaction
Infection (e.g., sepsis, severe malaria)
Paroxysmal cold hemoglobinuria
Paroxysmal nocturnal hemoglobinuria
IN VITRO *HEMOLYSIS*
Excessive aspiration force (blood drawn too vigorously, especially through small or superficial veins)
Catheter partially obstructed
Blood forced into the tube from syringe
Specimen frozen
Mechanical damage (e.g., shaking, excessive force in pneumatic tubes)
Gender
Age
Equipment used to collect sample (higher rates noted with use of IV catheters)
Tube size (higher rates noted in larger tubes compared with smaller tube sizes; 10 mL vs. 5 mL)
Tube fullness (higher rates when tubes are underfilled compared with properly filled tube)
Delay in analysis

[a]*Source of data, Refs. [11] and [50].*

hemolysis and free hemoglobin concentrations of 100–150 mg/dL may go undetected when visual assessment of samples is performed [13].

Hemolysis can be caused by a variety of mechanisms including physical disruption of cells, and immunological and chemical means. Hemolysis can be divided into *in vivo* and *in vitro* hemolysis, with *in vivo* hemolysis accounting for less than 2% of cases [14]. *In vivo* hemolysis occurs when red cell lysis within the body with subsequent release of intracellular components into the plasma. In contrast, when lysis of cells following the collection of blood occurs, it is termed as *in vitro* hemolysis. Common causes of *in vivo* and *in vitro* hemolysis are shown in Table 5.1.

In vivo hemolysis

In vivo hemolysis can be divided on the basis of where red cell destruction occurs. Intravascular hemolysis occurs when erythrocytes are destroyed while the cells are still within the vascular system, while extravascular hemolysis is due to destruction of red cells by the phagocytic system in the liver, spleen or bone marrow. It is important to recognize and differentiate between *in vivo* and *in vitro* hemolysis since analytes such as potassium or LD that might not be reported in samples showing *in vitro* hemolysis, should be reported in samples with *in vivo* hemolysis. For example, increased potassium in a sample with *in vitro* hemolysis can be assumed to be artifactual and not representative of the patient's true potassium concentration. However, increased potassium measured in a patient with *in vivo* hemolysis represents the true intravascular potassium in the patient.

In vivo intravascular hemolysis is typically characterized by an increased LD, decreased haptoglobin and increased urine hemoglobin concentration. The increased LD is the result of release of the enzyme from erythrocytes, while the decreased haptoglobin is the result of binding of haptoglobin to free hemoglobin. Hemoglobin, which is a tetramer, is rapidly broken down in plasma to dimers, with the resultant hemoglobin-haptoglobin complex being rapidly cleared. The half-life of the hemoglobin-haptoglobin complex is approximately 10–30 min due to rapid elimination by the monocyte-macrophage system, while the half-life of free haptoglobin is 5 days [15]. Haptoglobin becomes saturated when free plasma hemoglobin concentrations exceed approximately 150 mg/dL. Plasma hemoglobin not bound to haptoglobin is readily filtered through the glomerulus and will be excreted in the urine resulting in hemoglobinuria. It should be noted that low haptoglobin concentrations without *in vivo* hemolysis may be seen in newborns and young children and in those individuals with haptoglobin deficiency [16].

The free hemoglobin will produce a positive reaction for heme protein when measured using a urine dipstick. The free hemoglobin dimers that remain in circulation are oxidized to form methemoglobin, which dissociates to produce free heme and globin chains. The oxidized free heme binds to hemopexin and is removed from circulation by the liver, spleen and bone marrow. Heme is further metabolized to eventually form unconjugated bilirubin. Measurement of hemopexin, methemoglobin and unconjugated bilirubin can be helpful in the differentiation of *in vivo* intravascular or extravascular

hemolysis from *in vitro* hemolysis. *In vivo* hemolysis due to immunohemolytic causes such as ABO transfusion reactions, paroxysmal cold hemoglobinuria, idiopathic autoimmune hemolytic anemia, or due to use of certain drugs can result in further hemolysis of sensitized erythrocytes during the process of blood collection, clot formation and centrifugation. Thus, the collection of a sample anticoagulated with heparin can help eliminate further hemolysis that might occur *in vitro* during clot formation. Recommended criteria have been published for helping differentiate *in vivo* from *in vitro* hemolysis [17]:

- Collect both serum and plasma sample.
- Because anticoagulation of blood helps minimize *in vitro* hemolysis, perform all tests on plasma whenever *in vivo* hemolysis is suspected.
- Measure hemoglobin and potassium concentrations and LD activity in the serum and plasma specimens.
- Specimens with increased LD activity and hemoglobin concentrations, but normal potassium concentrations suggest *in vivo* hemolysis
- Measure haptoglobin, hemopexin and the reticulocyte count to confirm *in vivo* hemolysis

Case report

A 58-year-old female was admitted with a diagnosis of paroxysmal nocturnal hemoglobinuria (PNH). Results of her physical examination were normal. Laboratory results obtained on Days 1 through 7 of her admission are shown below [17]:

Test (reference interval)	Specimen[a]	Day 1	Day 4	Day 5	Day 7
Potassium (3.2–4.6 mmol/L)	P			4.6	3.9
	S	4.9	5.5	5.7	4.6
Lactate Dehydrogenase	P			2830	3020
(133–248 U/L)	S	2630	3170	3000	3305
Hemoglobin	P	52		162	132
(0–50 mg/L)	S	297	301	221	210
Haptoglobin (410–2100 mg/L)	S	<150	<150	<150	<150
Reticulocytes (0.5–1.5%)		10.6	9.5		

[a]*P, plasma; S, serum.*

Blank DW, Kroll MH, Ruddel ME, Elin RJ. Hemoglobin interference from in vivo hemolysis. Clin Chem 1995;31:1566–9. Reproduced with permission from the American Association for Clinical Chemistry.

Results for the plasma samples collected from this patient demonstrated increased plasma LD and hemoglobin and a normal potassium concentration. These findings, along with low serum haptoglobin and increased reticulocyte count, strongly suggest *in vivo* hemolysis. Of interest was the finding of higher serum potassium and hemoglobin concentrations and LD activity when compared with plasma. These findings suggest that further lysis of erythrocytes occurred during the process of clotting of serum samples, with subsequent release of potassium, LD and hemoglobin into the serum. Thus, this patient had *in vivo* hemolysis due to PNH. In addition, there was concomitant *in vitro* hemolysis following the collection of blood into tubes not containing anticoagulant, with further lysis of erythrocytes occurring during the process of clot formation. It may be advantageous to collect blood from patients with immunohemolytic anemia into tubes containing an anticoagulant such as heparin in order to prevent further hemolysis of cells that might occur during clotting.

In vitro hemolysis

Poor specimen quality is recognized as the most frequent source of errors in the preanalytical phase of testing. Among samples that are submitted to the clinical laboratory for testing that are deemed unsuitable for analysis, *in vitro* hemolysis accounts for approximately 40%–70% of the cases [1,14,18]. *In vitro* hemolysis can occur at a variety of stages including during phlebotomy, sample handling and processing, and during storage. In addition, red cell fragility may be more pronounced in some patients resulting in higher likelihood of *in vitro* hemolysis [19]. Table 5.2 lists the various causes of *in vitro* hemolysis.

In vitro hemolysis can cause a positive or negative bias in an analyte. The mechanisms causing bias with *in vitro* hemolysis include proteolysis of analytes due to release of intracellular compounds, release of thromboplastic substances, dilution of the analyte due to release of cytoplasmic contents, release of the analyte itself from erythrocytes, and analytical interference due to hemoglobin and other intracellular substances [14]. Analytes such as potassium, LD and AST that are present within erythrocytes at concentrations greater than approximately 10 times that seen in extracellular fluids will cause an increase following hemolysis. Also, the greater concentration for some analytes such as potassium, neuron-specific enolase and acid phosphatase observed in serum compared with plasma is due to release of these compounds from platelets during fibrin clot formation [20]. Interference from hemoglobin itself may be due to the spectral properties of this compound, which has an absorbance peak at 420 nm and shows significant absorbance between 340 and 440 nm and between 540 and 580 nm. In addition to the spectral interference effects of hemoglobin, this compound may also interfere due to the reactivity of its iron atoms, which can participate in oxidation-reduction reactions

TABLE 5.2 Common causes of *in vitro* hemolysis and the various stages in which they occur.

Step	Specific causes
Phlebotomy (and specimen collection process)	• Collection from catheter • Collection of capillary blood (fingerstick or heel stick) • Needle gauge • Length of time that tourniquet is used • Fist clenching by patient • Tube under filled • Vigorous mixing of sample following collection • Lack of mixing following collection • Transfer from syringe into Vacutainer tube
Specimen transport	• Transport via pneumatic tube • Significant time delay in specimen transport to laboratory • Transport of specimen at inappropriate temperature
Specimen processing	• Significant time delay between receipt of specimen and centrifugation • Centrifuge temperature extremes • Speed of centrifugation • Re-centrifugation of previous centrifuged specimen • Poor barrier separation if gel barrier tubes are used
Specimen Storage	• Improper storage temperature following analysis • Length of time that specimen is stored following analysis

or in reactions that involve hydrogen peroxide (see Chapter 9 for examples of tests using hydrogen peroxide and peroxidase assays). While many analytes are subject to interference effects from *in vitro* hemolysis, the influence of *in vitro* hemolysis on measured potassium concentrations is probably the most widely recognized. The frequency that potassium is measured in the clinical laboratory, along with the serious consequences of misdiagnosis of hypokalemia or hyperkalemia, has resulted in this analyte being widely recognized as being affected by *in vitro* hemolysis.

The prevalence of *in vitro* hemolysis can vary widely depending on the patient population being tested, whether trained phlebotomists or inexperienced individuals are collecting the sample, and whether the sample is processed on site or is sent to a remote site for processing, with significant time delays between collections and processing [21]. With respect to the type of patient population that is being tested, the prevalence of *in vitro* hemolysis in outpatients has been found to be approximately 90 times less when compared to samples collected from patients in the Emergency Department, where studies often show *in vitro* hemolysis to be present in approximately 10% of samples from this location [10]. Other hospital locations associated with a high prevalence of *in vitro* hemolysis include patients in the pediatric and neonatal wards, where fingerstick and heelstick samples are often collected, and which are associated with high rates of *in vitro* hemolysis.

Poor blood collection practices contribute to *in vitro* hemolysis. One study which evaluated the root causes of *in vitro* hemolysis found that approximately 80% of the samples with *in vitro* hemolysis was attributed to aspirating blood too vigorously through a needle into a syringe (31%), collecting blood from a butterfly needle into a syringe (20%), collecting from an intravenous catheter into a syringe (17%), or collecting from an infusion port into a syringe (12%) [18]. Of interest, errors in handling, including freezing of specimens, accounted for 1% of the reasons for *in vitro* hemolysis. A recent study found that a significant improvement in hemolyzed samples could be achieved by use of small-volume/vacuum sample collection tubes [22]. With lower vacuum pressures, the pressure gradient and velocity of blood flow into the collection tube is decreased.

The ability of automated analyzers to measure plasma hemoglobin in patient blood samples as a means for identifying *in vitro* hemolysis has proven to be a reliable means for identifying samples containing this interfering substance. Some instruments report *in vitro* hemolysis using a semiquantitative scale while other reports the actual plasma hemoglobin concentration. However, once identified, laboratories utilize a variety of different mechanisms for dealing with specimens that are hemolyzed. These options include outright rejection of hemolyzed samples, analysis of hemolyzed samples and reporting of results with a disclaimer stating that those analytes affected by *in vitro* hemolysis may be incorrect, or correction or adjustment of the measured analyte concentration using a correction factor based on the magnitude of hemolysis. The vast majority of laboratories either rejects samples with *in vitro* hemolysis or analyzes the sample and reports the result with a comment stating that the result may not be accurate.

Reporting a result that has been corrected or adjusted based on the magnitude of *in vitro* hemolysis is not a recommended approach [23]. The wide range of correction factors that have been proposed for correcting measured potassium for *in vitro* hemolysis demonstrates how problematic the use of correction factors can be. For example, correction factors that have been proposed for adjusting potassium in samples with *in vitro* hemolysis range from an increase of 0.20 mmol/L of potassium per 100 mg/dL of plasma hemoglobin, to an increase of 0.51 mmol/L per 100 mg/dL of plasma

hemoglobin [23,24]. The wide range of correction factors that have been proposed highlights the fact that factors influencing hemolysis are not as simplistic as they seem. Factors such as interindividual variability in erythrocyte hemoglobin concentrations or in the concentration of erythrocytes themselves, the effect of erythrocyte age on intracellular concentrations of various analytes, and differences in red cell membrane fragility likely contributes to the differences that are seen in recommended correction factors [24–26]. For example, decreased erythrocyte concentrations may lead to faster flow of cells through the needle during phlebotomy resulting in increased shear forces and cell membrane rupture [27]. Also, variability in erythrocyte membrane permeability as a result of disease can impact the effects of *in vitro* hemolysis. Older erythrocytes have greater permeability to cations compared with younger cells, and contain approximately only half the potassium content of younger cells [28,29]. Hemolysis that occurs as result of mechanical trauma to cells during blood collection is likely to cause lysis of older cells containing different concentrations of certain analytes, while hemolysis induced by lysis of all cells, due for example to freezing and thawing of blood, will induce lysis of all cells. In addition, certain conditions such as chronic lymphocytic leukemia have been associated with increased fragility of leukocyte membranes, with release of intracellular contents from these cells during the collection of blood [30].

The mechanisms causing *in vitro* hemolysis during collection of blood from a patient may be very different from the mechanisms that have been employed in studies designed to investigate the effect of *in vitro* hemolysis. Mechanisms used to simulate *in vitro* hemolysis include osmotic lysis of cells, freeze-thaw cycles, and physical disruption of cells by forcing through a small-bore needle, physical disruption of clotted blood with a wooden applicator stick or homogenization of whole blood in a blender [24,27,29,31–34]. In addition, some studies removed platelets and leukocytes prior to inducing hemolysis while other studies do not. The use of these different methods used to simulate *in vitro* hemolysis probably account for the wide variability in results that are sometimes reported in the effects of *in vitro* hemolysis on measured analyte concentrations.

Evaluation of the effects of hemolysis on laboratory test results using paired blood samples collected during the same phlebotomy draw, where one sample is hemolyzed and the other sample free of hemolysis, shows that the effects of *in vitro* hemolysis can be much different when compared to studies where hemolysis is introduced by artificial means [23]. Methods used to mimic *in vitro* hemolysis such as the addition of a whole blood lysate prepared by the freezing and thawing of whole blood, or osmotic lysis of cells following the addition of distilled water to packed cells typically results in the lysis of all cells, including reticulocytes, mature erythrocytes, platelets and leukocytes. However, *in vitro* hemolysis due to mechanical disruption of cells during blood collection typically does not result in lysis of all cells. Older erythrocytes are more prone to shear stresses that occur during sample collection compared with younger cells [35]. Shear stress that occurs during phlebotomy can result in the formation of erythrocyte membrane pores that allow the leak of small ions such as potassium, but block passage of larger molecules such as LD, AST and hemoglobin [36]. Thus, the use of plasma hemoglobin as a marker of *in vitro* hemolysis may not show a direct relationship with the loss of small ions, such as potassium, from cells due to shear stress forces.

Case report

A 74-year-old male was seen by his physician for a routine physical. A general chemistry screen consisting of electrolytes and liver and renal function tests were ordered and a blood sample is collected into a plain evacuated tube. The phlebotomist mentioned to the physician that she had a difficult time collecting blood from the patient due to the lack of “good veins”, and she needed to use a 23 gauge needle to collect the sample since she had no more 21 gauge needles left. After collecting the sample, the phlebotomist placed the tube of blood in a rack and took her lunch break. She returned an hour later, and she centrifuged the sample and removed the serum from the cells. The technologist analyzing the sample noted that the serum had a pink to red color, but failed to note that on the report that was sent to the physician. Laboratory results obtained on serum are shown below:

Test (reference interval)	Result
Glucose (≤99 mg/dL)	55
Potassium (3.2–4.6 mmol/L)	5.8
Lactate Dehydrogenase (133–248 U/L)	322
Aspartate transaminase (AST) (≤48 U/L)	62
Alanine aminotransferase (ALT) (≤55 U/L)	18
Creatinine Kinase (CK) (≤200 U/L)	93

The results obtained on this patient were consistent for a sample that had been delayed in processing to remove the serum from cells, and in addition was also hemolyzed. The relative low glucose suggested some delay in processing, with metabolism of glucose by cells. The rate of disappearance of glucose in the presence of blood cells has been reported to be approximately

10 mg/dL per hour, but the rate increases with glucose concentration, temperature, and white blood cell count. The increased AST, LD could be indicative of liver or skeletal muscle injury, but the normal ALT and normal CK values suggested that liver or skeletal muscle injury was not present in this patient and were likely increased due to *in vitro* hemolysis. The increased potassium was also consistent with *in vitro* hemolysis. In addition, the lengthy delay in processing of the sample could have led to leakage of potassium out of cells into the serum. The use of small bore needles like the one used for collection of blood from this patient is discouraged as needles over 21 gauges have been found to be associated with increased rates of *in vitro* hemolysis. Small bore needles can cause a larger vacuum force being applied to the blood, causing increased shear stresses on cells causing them to rupture.

LIPEMIA

Unlike *in vitro* hemolysis, lipemia is something not easy to avoid. Diseases frequently associated with lipemia include ethanol use, diabetes mellitus, hypothyroidism, chronic renal failure, pancreatitis, primary biliary cirrhosis, total parenteral nutrition, and use of certain medications such as steroids, estrogen and protease inhibitors. Interference from lipemia is due to the ability of lipoprotein particles to scatter light as well as absorption of light, affecting tests that use spectrophotometric methods. Lipemia can also cause interference by volume displacement, affecting the measurement of electrolytes that utilize indirect ion selective electrode methods. Other possible effects of lipemic specimens include sample non-homogeneity which can affect the ability of analyzers to sense and pipette specimens [37].

The scattering of light due to the turbidity caused by triglycerides can increase the absorbance values of solutions, thereby decreasing the operating scale for colorimetric methods. The interference effects due to lipemia can cause either increased or decreased values depending on whether a sample blank is used. If the turbidity is too great, no measurement may be possible due to limits on the linear range of the assay [38]. Methods based on nephelometry can be adversely affected as a result of light scatter.

The displacement of plasma water by high triglyceride concentrations can interfere with those methods that are based on measurement in the aqueous portion of the sample and assume that all samples contain approximately 93% plasma water. Samples containing very high concentrations of triglycerides can contain much less plasma water per unit volume of sample due to the volume displacement effects of triglycerides. Pseudo-hyponatremia, caused by increased triglyceride concentrations in samples where the sodium is measured using an indirect method involving dilution of the sample prior to analysis, is one common example of interference encountered with lipemic samples (see Chapter 8 for more details on the water exclusion effect). Following centrifugation of a lipemic sample, or if the sample is allowed to stand for a period of time prior to analysis, the lipids in the sample are not evenly distributed but tend to concentrate within the upper portion of the sample. Thus, analytes dissolved in the aqueous phase of the sample will be at much lower concentrations in the upper layer, which is predominantly occupied by lipid. The converse is true for lipids and lipid soluble components such as drugs that are present in higher concentrations in the upper layer consisting primarily of lipid.

In addition to the interference effects of lipemia caused by interference in spectrophotometric analysis and by displacement of plasma water, lipids can also cause interference by physical-chemical mechanisms. An analyte that is located primarily in the lipid layer may not be as accessible to reagents used in measurement of the analyte, or may not be accessible to an antibody used in immunoassay methods. Also, lipids may affect electrophoretic and chromatographic separation of analytes [39].

Lipemia causing test interference can be the result of recent food intake, altered or deranged lipid metabolism, or infusion of lipid-containing solutions. The most common pre-analytical cause of lipemia is the failure to fast prior to the collection of blood. Plasma triglyceride concentrations, primarily in the form of chylomicrons, increase substantially between one and 4 h following eating and the chylomicrons and chylomicron remnants remain elevated for six to 12 h afterward. Patients should be fasted for at least 12 h before blood samples are collected. For patients that are receiving parenteral infusion of lipids, the treatment should be stopped for at least 8 h prior to collection of blood.

Common secondary causes of lipemia include diabetes mellitus, alcoholism, nonalcoholic fatty liver disease and renal disease. In addition, intravenous infusion of lipid emulsions for parenteral nutrition or as a diluent for poorly water-soluble medications can result in lipemia [37].

In whole blood it is difficult to visually detect lipemia unless the triglyceride concentration is greater than 1000 mg/dL. In serum or plasma, visual detection is usually observed when triglyceride concentrations are greater than 300 mg/dL, although the ability of triglycerides to cause turbidity is dependent upon whether the triglycerides are present primarily in the form of chylomicrons, very low density lipoprotein (VLDL), or low density lipoprotein (LDL) particles. Thus, while measurement of triglycerides can provide a rough

assessment of the degree of lipemia, the degree of turbidity does not show good correlation with triglyceride concentrations [40]. Most analyzers are able to evaluate samples for lipemia by measuring at wavelengths above 600 nm.

A variety of methods have been proposed for removing lipids from serum or plasma. High-speed centrifugation is often performed as this procedure produces a clear infranatant. Other methods used include the extraction of lipids with organic solvents and the precipitation of lipids using various compounds. Some manufacturers add detergents or enzymes to their reagent in order to remove turbidity. Use of centrifugation to separate lipids depends on the type of lipids contributing to the interference. If lipemia is due to chylomicrons, centrifugation at 12,000 *g* can help separate chylomicrons. However, if the lipids present consist primarily of VLDL or LDL particles, much higher centrifugation is required. Lipemia can be removed from EDTA samples used for hematology by centrifugation. However, the cell free supernatant that is removed must be replaced by an equal volume of saline.

The investigation of interference due to lipemia is difficult due to the fact that the light scattering properties of chylomicrons and VLDL particles is dependent upon the size of the particles present. VLDL is broken into three size categories referred to as small VLDL (27–35 nm), intermediate VLDL (35–60 nm), and large VLDL (60–200 nm). Only the intermediate and large VLDL particles are effective at scattering light. Chylomicrons are a heterogenous mixture of particles that range in size from 70 to 1000 nm. Since VLDL particles and chylomicrons vary greatly in size and triglyceride content, measured triglyceride concentrations correlates poorly with sample turbidity [41].

The use of artificial fat emulsions such as Intralipid to mimic lipemia is often used to establish the effect of lipemia on assay interference. Particles in Intralipid range in size from 200 to 600 nm. Thus, Intralipid misses the range of values for large VLDL particles and misses the lower and upper ranges seen with chylomicrons [42]. Thus, extreme care should be exercised when interpreting the results of interference studies that use samples with Intralipid or other synthetic emulsions added to mimic lipemia [12]. Such artificially derived samples may not behave in the same manner as true lipemic samples obtained from patients.

Case report

A 20 year-old female with a history of diabetes presented to the emergency department 7 months following discontinuation of her insulin therapy. She was diagnosed with diabetic ketoacidosis, acute pancreatitis and severe hypertriglyceridemia. Values obtained upon admission and 24 h later are shown below.

	Result	Result
Test (reference interval)	**Admission**	**24 h**
Glucose (≤99 mg/dL)	312	158
Triglycerides (<150 mg/dL)	15,300	13,500
Sodium (134–143 mmol/L)	125	122
Lipase (<85 U/L)	458	616
Amylase (<200)	154	955

The sodium in this patient was measured using indirect potentiometry where the sample is diluted prior to analysis by the ion-selective electrode. This method for analysis of sodium is known to be adversely affected by the presence of compounds such as lipids, which displace the aqueous portion of the sample. Use of an alternate method for measuring sodium would be appropriate in this case. This patient presented with severe abdominal pain and was diagnosed with pancreatitis. The elevated lipase measured upon admission is consistent with pancreatitis. However, the normal amylase measured in this same sample was an unexpected finding. Centrifugation of the sample to remove the lipemia and reanalysis of the sample showed the amylase in the sample collected 24 h following admission to be 955 U/L. The laboratory director confirmed that the method used for analysis of amylase is affected by lipemia, which causes falsely decreased values [43].

ICTERUS

Increased concentrations of bilirubin are another source of endogenous interference. Bilirubin has high absorbance between wavelengths of 340 nm and 500 nm. Thus, methods that rely on measurement at these wavelengths are prone to interference effects from bilirubin. In addition to the spectral properties of bilirubin, this compound can also react chemically with reagents. For example, bilirubin can react in oxidase/peroxidase-based assays such as those used for measurement of glucose, cholesterol, triglycerides, creatinine, and uric acid [44]. Bilirubin reacts with hydrogen peroxide formed in the test system resulting in lower than expected test results. Also, bilirubin can interfere with dyes that bind to albumin. The reduction in absorption of bilirubin due to oxidation in an alkaline environment is the main cause for bilirubin interference in some versions of the Jaffe method for creatinine. In a strongly acidic environment, the absorption of conjugated bilirubin shifts to the UV wavelengths that

can result in interference with phosphate when the phospho-molybdate method is used [45].

Visual inspection of samples for the detection of hyperbilirubinemia is not very sensitive. Samples containing hemolysis make detection even more difficult. Automated detection of bilirubin by analyzers that assess the presence of common interfering substances is the recommended method for identifying samples that are icteric. Spectral interference from bilirubin can be eliminated by the use of blanking procedures. However, chemical interference caused by bilirubin cannot be eliminated by blanking. The addition of potassium ferrocyanide or bilirubin oxidase to the reagent can eliminate interference from bilirubin in methods based on formation of hydrogen peroxide (see Chapter 8).

METHODS FOR EVALUATING THE EFFECT OF ENDOGENOUS INTERFERING SUBSTANCES

Guidelines have been established for the assessment of interference effects and various experimental designs have been advocated by others [4,46–48]. One common method used to evaluate the effect of an interfering substance is to measure the analyte of interest by use of an alternate method know to not be affected by the interfering substance. The most common approached used to assess interference effects is to add serially higher concentrations of the interfering substance to aliquots of the same matrix and then measuring the substance of interest in each aliquot. The effect of the interfering substances can be assessed by use of regression analysis by plotting measured analyte concentration versus the concentration of interfering substances. If the slope obtained from the regression analysis differs by some predetermined value (i.e., ±10%), then interference is determined to be present. Unfortunately, most of the experimental models used to assess the effect of interfering substances make the assumption that interference is not related to the concentration of the analyte being measured [46–48]. For example, a 0.5 mg/dL positive bias caused by 150 mg/dL of plasma hemoglobin, observed in a sample with a baseline total bilirubin concentration of 0.5 mg/dL would represent a 100% increase in total bilirubin. This finding would be extrapolated to infer that a sample with a baseline total bilirubin concentration of 10.0 mg/dL would show a measured concentration of 20 mg/dL if *in vitro* hemolysis of 150 mg/dL of plasma hemoglobin was present. Unfortunately, this type of analysis may be inappropriate and misleading when applied to analytes where the effect of interfering substances is dependent on analyte concentration.

It has been demonstrated that there are at least three types of interference that can contribute to the effect of interfering substances [49]. These three types of interference have been classified as analyte-dependent, where the magnitude of the interference effect is dependent on the concentration of the analyte of interest; analyte-independent, where the magnitude of the interference effect is constant regardless of the analyte concentration; and a combination of the first two, where the effect of an interfering substance is dependent on both the concentration of the analyte and the concentration of the interfering substance. A model for assessing the relative contribution of each of these three types of interference effects has been described [49]. Briefly, this model involves the creation of a series of aliquots containing the analyte of interest at various concentrations. Each aliquot containing the analyte of interest at a particular concentration is then subdivided and various concentrations of suspected interfering substance added. This results in a matrix containing the analyte of interest at several different concentrations, with each concentration containing the suspected interfering substance at various concentrations.

CONCLUSIONS

The effects of endogenous interfering substances on laboratory test results are a topic that has been widely studied, with numerous publications, and oftentimes conflicting or inconclusive results. Endogenous interfering substances can cause erroneous test results and lead to inappropriate patient care if acted upon. Collection of another sample not containing the interfering substance, or providing an alert that the test result may be inaccurate due to the presence of an interfering substance is recommended. Many instruments now evaluate and report the level of endogenous interfering substances that may be present. Utilization of these automated methods for assessing endogenous interfering substances should help identify samples that may be inappropriate for analysis. Manufacturer's information on the effects of endogenous interfering substances on specific test methods is often a useful guide as to which analytes are subject to the effects of interfering substances. In addition to the information provided by manufacturer's a number of guidelines are available that can help laboratorians evaluate the effects of interfering substances.

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CHAPTER

6

Immunoassay design

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INTRODUCTION

Immunoassay is an important component in modern clinical laboratory. Various kinds of analytes, hormones, serum proteins, antibodies to infectious or allergic agents, therapeutic drug monitoring (TDM), and drugs of abuse (DAU) are measured using immunoassays. The analytes may require high sensitivity and wide analytical range; they may need qualitative or quantitative results. Immunoassay technology today can meet all such analytical needs.

In the early days of immunoassays, the methods were laborious, requiring skilled labor. The manual immunoassays either used antibody coated micro-titer plates or tubes to which samples and label reagents were added, and after separation and wash, the signal was read. Further developments in this field made the assays easy to use and exploit their inherent promise of specificity and sensitivity. However, revolutionary developments in immunoassay automation over the last 20 years made immunoassays today as fast and precise as chemistry assays. Currently hundreds of immunoassays are commercially available and routinely used in clinical laboratory analysis. They help in the diagnosis and monitoring of various diseases, including allergy, anemia, autoimmune, cardiovascular, neurologic, diabetes, metabolic, endocrine, and cancer. Most immunoassay methods use specimens without any pretreatment, are easy to use, and are run on fully automated, continuous, random-access systems. The assays use very small amounts of sample volumes (most $<100\ \mu L$), reagents are stored in the analyzer, most have stored calibration curves on the system, often stable for 1–2 months, and report results in 10–30 min. Immunoassays offer fast throughput, automated rerun, relatively inexpensive tests of high sensitivity and specificity, and results are reported in a format that the laboratory wants.

Immunoassays measure the analyte concentration in a specimen by forming a complex with a specific binding molecule, which in most cases is an analyte-specific antibody (or a pair of specific antibodies). The complex generates some kind of signal which is then converted into the analyte concentration via a 'calibration curve'. The immunoreaction is further utilized in various formats and labels, giving a whole series of immunoassay technologies, systems, and options. However, like every analytical method, immunoassays also suffer from some interferences and pitfalls.

IMMUNOASSAY METHODS AND ASSAY PRINCIPLE

The main principle in immunoassay technology is that binding of the analyte with a specific binding molecule must generate a difference in signal between free versus bound analyte. There are many different designs of the immunoassay to achieve this objective: homogeneous versus heterogeneous, different assay formats, and different types of signal generated. There are two types of immunoassays by assay format: competition and immunometric (commonly referred as 'sandwich'). Competition immunoassays work best mostly for small molecular weight analytes, requiring limited amounts of a single analyte-specific antibody, and a labeled analyte. In the classical method, the antibody may be conjugated to a solid phase, allowing the separation of bound versus free antigen (and label). In such type of immunoassay, the analyte in the specimen competes with the labeled analyte for antibody binding sites, and thus the generated assay signal is *inversely* proportional to the analyte concentration in the specimen.

Sandwich (also called immunometric) immunoassays, on the other hand, are mostly used for large molecular weight analytes, predominantly proteins or peptides, and use two different specific antibodies. In this assay format, the analyte in the specimen binds to both antibodies simultaneously at different epitopes.

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Again, in the classical assay, one antibody may be conjugated to a solid phase, and the other to a label. The bound complex is separated from other assay components, and the relevant amount of label expressed as signal. The signal in sandwich assays is directly proportional to analyte concentration; with a low background signal, this type of immunoassay can be highly sensitive, detecting very low concentrations of the analyte. The signals generated are mostly optical − absorbance, fluorescence, or chemiluminescence. Since two antibodies (mostly recognizing different recognizing sites or 'epitopes' of the analyte) are used in sandwich assays, in contrast with a single antibody in competition assays, the sandwich assays have higher specificity than the competition assays.

Depending on the need of separation between the bound versus. free labels, the immunoassays may be homogeneous or heterogeneous. In the former, the bound label has different properties than the free label, and no physical separation between the two is needed. For example, in fluorescent polarization immunoassay (FPIA), the free label has different Brownian motion than when the relatively small molecular weight (a few hundred to thousand Daltons) label is complexed to a large antibody (140,000 Da). This results in a difference in the fluorescence polarization properties of the label. This difference is utilized to quantify the bound label [1]. Although FPIA was one of earliest immunoassays marketed by Abbott Laboratories, this assay is no longer available. In another type of homogeneous immunoassay, an enzyme is the label, whose activity is modulated differently in the free versus the complexed conditions of the label. This forms the basis of the EMIT (enzyme multiplied immunoassay technique) or CEDIA (cloned enzyme donor immunoassay) technologies [2,3]. In the EMIT method the label enzyme, Glucose 6-phosphodehydrogenase is inactive unless in the antigen-antibody complex. The active enzyme reduces NAD to NADH, and the absorbance is monitored at 340 nm. Similarly, in the CEDIA method, two genetically engineered inactive fragments of the enzyme beta-galactosidase are coupled to the antigen and the antibody reagents. When they combine, the active enzyme is produced and the substrate, a chromogenic galactoside derivative, produces the assay signal.

In a third commonly used format of homogeneous immunoassay (turbidimetric immunoassay, or TIA), analytes (antigen) or their analogs are coupled to colloidal particles, e.g., of latex [4]. Since antibodies are bivalent, the latex particles agglutinate in presence of the antibody. However, in the presence of free analytes in the specimen, there is less agglutination. In a spectrophotometer, the resulting turbidity can be monitored as an end-point or as a rate. The TIA can also simply be done just by reacting antigen and antibody in presence of a precipitating agent (for example, polyethylene glycol or PEG of different molecular weight). Use of microparticles in the turbidimetric reaction increases the assay sensitivity and speed.

Nephelometric immunoassays use the same precipitation reaction, but instead of absorbance, scattered light at 90-degrees to the original light path is measured. Nephelometric immunoassays have several advantages over the turbidimetric assays: they are more sensitive and suffer from less endogenous interference.

Another example of homogeneous chemiluminescent immunoassay technology is the LOCI (luminescent oxygen channeling immunoassay) in which the immunoassay reaction is irradiated with light generating singlet oxygen molecules in microbeads ('Sensibead') coupled to the analyte. When bound to the respective antibody molecule, also coupled to another type of bead ('Chemibead') that reacts with singlet oxygen, chemiluminescence signals are generated, proportional to the concentration of the analyte-antibody complex. This technology is used in the Siemens Dimension Vista automated assay system [5,6].

Kinetic interaction of microparticle in solution (KIMS) is another assay format where in the absence of drug molecules, free antibodies bind to drug microparticle conjugates forming particle aggregates and a resulting increase in absorption is observed. When drug molecules are present in a urine specimen, drug molecules bind with free antibody molecules and thus prevent formation of particle aggregates, therefore diminishing the absorbance in proportion to drug concentration.

In heterogeneous immunoassays, on the other hand, the bound labels are physically separated from the unbound labels, and their signal is measured. In these types of assays, there are two separate reagents reacting with the sample containing the analyte: a solid phase and a label. After the immunoreaction, typically lasting 5−10 min, the solid phase, containing the ternary complex of antibody 1-antigen-antibody 2, is separated. The separation is often done magnetically, where the reagent analyte (or its analog) is provided as coupled to paramagnetic particles (PMP), and the antibody is labeled. Conversely, the antibody may be also provided as conjugated to the PMP, and the reagent analyte may carry the label. After separation and wash, the bound label is reacted with other reagents to generate the signal. This is the mechanism in many chemiluminescent immunoassays (CLIA), where the label may be a small molecule that generates the chemiluminescent signal. Examples of immunoassay systems where the chemiluminescent labels generate a signal by chemical reaction are the ADVIA Centaur from Siemens and the Architect from Abbott [7]. An example where the small

label is activated electrochemically is the ELECSYS automated immunoassay system from Roche [8]. The label also may be an enzyme (enzyme-linked immunosorbent assay, or ELISA) that generates chemiluminescent, fluorometric, or colorimetric signals depending on the enzyme substrates used. Examples of commercial automated assay systems using the ELISA technology and chemiluminescent labels are the Immulite (Siemens) and the ACCESS from Beckman-Coulter [9,10]. Another type of heterogeneous immunoassay uses polystyrene particles. If these particles are of micro-sizes, that type of assay is called micro-particle enhanced immunoassay (MEIA) [11]. In older immunoassay formats, the labels used to be radioactive: radio-immunoassay or RIA. But because of safety and waste disposal issues, RIA is rarely used today. Various commercially available assay formats commonly used in clinical laboratories are listed in Table 6.1.

TABLE 6.1 Various types of common immunoassay kits.

Immunoassay type	Example	Signal
Competition (analytes of MW $\leq$ 1000 Da)	FPIA (TDx from Abbott) assays for therapeutic drugs and drugs of abuse	Fluorescence polarization, but Abbott Laboratories discontinued this format.
Competition	EMIT (Siemens) on: ADVIA Chemistry, Dimension, or Viva SYNCHRON (Beckman- Coulter) Assays for small molecules	Colorimetry (Enzyme Modulation) All these assays measure signal at 340 nm due to conversion of NAD to NADH (but Dimension and SYNCHRON uses particle enhanced turbidimetric inhibition immunoassay method).
Competition	CEDIA (Microgenics) Assays for small molecules	Colorimetry (Enzyme Modulation); at system-dependent visible wavelengths, commonly using beta-galactosidase enzyme (fragments).
Competition	LOCI (Siemens)	chemiluminescence
Sandwich (analytes MW > 1000 Da)	ADVIA Chemistry assays (Siemens), proteins, hormones	Turbidimetry latex
Sandwich	Centaur assays (Siemens), Proteins, hormones	Chemiluminescence
Sandwich	Elecsys assays (Roche) proteins, hormones	Electro-Chemiluminescence
Sandwich	Architect assays (Abbott), proteins, hormones	Chemiluminescence

IMMUNOASSAY REAGENTS

The main reagent in immunoassays is the binding molecule, which is most commonly an analyte-specific antibody or its fragment. Several types of antibodies or their fragments are now used in immunoassays. There are polyclonal antibodies, which are raised in an animal when the analyte (as antigen) along with an adjuvant is injected into the animal. Small molecular weight analytes are most commonly injected as conjugates of a large protein. Appearance of analyte-specific antibodies in the animal's sera is monitored, and when sufficient concentration of the antibody is reached, the animal is bled. The serum can directly be used as the analyte-specific binder in the immunoassay. Most commonly, however, the antibodies are purified from serum and used in the assay. Since there are many clones of the antibodies specific for the analyte, these antibodies are called polyclonal. In newer technologies, however, plasma cells of the animal can be selected as producing the optimum antibody, and then can be fused to immortal (usually myeloma) cells to form a hybridoma. The resulting tumor cell grows uncontrollably producing only the single clone of desired antibody. Such antibodies, called monoclonal antibodies, now may be grown in live animals or cell-culture. There are several benefits of the monoclonal antibodies over polyclonal ones: (a) the characteristics of polyclonal antibodies are dependent on the animal producing the antibodies; if the source individual animal must be changed, the resultant antibody may be quite different; (b) since polyclonal antibodies constitute many antibody clones, they have less specificity than monoclonal antibodies. Sometimes, instead of using the whole antibody, fragments of the antibody, generated by digestion of the antibody by peptidases, e.g., Fab, Fab′, or their dimeric complexes, are also used as reagents.

The other main reagent component of immunoassays is the label. There are many different kinds of labels, generating different kinds of signals. For example, use of acridinium ester labels, when treated with peroxide, produces a chemiluminescent signal. As described earlier, an enzyme may be used as the label, which in its turn can generate different types of signals depending on the substrate used for the enzyme.

For ease of manufacturing, some manufactures use a solid phase with a common binding protein like streptavidin immobilized, and a common signal reagent. In such immunoassays the specific antibodies are conjugated with biotin. This assay architecture can be used with both competition and sandwich assays. While such assays render assay sensitivity and ease of manufacturing, they may become vulnerable to interference from exogenous biotin interference; this is discussed in Chapter 8.

The versatility of immunoassays is demonstrated by its use even in Point of Care (POC) assays. In these assays, sample fluid moves along microchannels of a disposable assay cartridge by capillary actions, reacting with immobilized antibodies and reagents. Generated signals are mostly colorimetric or electrochemical. POC assays demand fast reactions, ease of use, and less complicated instrumentation for easy, patient bed-side portability. POC immunoassays are discussed in greater details later in Chapter 30.

A newer development in immunoassay technology is multiplexing, where a small amount of sample reacts simultaneously with multiple immunoassay reagents specific for a class of analytes. The Luminex xMAP technology is based on polystyrene or paramagnetic microspheres, or beads, that are internally dyed with red and infrared fluorophores of differing intensities. Each dyed bead is given a unique number, or bead region, allowing the differentiation of one bead from another. Individual bead sets are then coated with a capture antibody qualified for one specific analyte. Multiple analyte-specific beads can then be combined in a single well of a 96-well microplate-format assay to detect and quantify multiple targets simultaneously, using one of the Luminex instruments for analysis. The multiplex assays can be run using either polystyrene or paramagnetic beads.

Another version of multiplex immunoassays is disease specific arrays, for example, Randox Laboratories' Biochip arrays.

Gordon and Michel in a review discussed several versions for lateral flow parallel micro-channel immunoassays on chips with various kinds of separation and detection technologies: chromatographic, surface plasmon reflectance spectroscopy, Raman resonance spectroscopy, refractive index, electrochemical, and Field-Effect Transistors [12].

LIMITATIONS OF IMMUNOASSAYS

Even though the immunoassay methods are now widely used, there are a few limitations and drawbacks of the method. One of them is the limitation of the binding molecule, i.e., the antibody, in terms of its specificity. Many of the endogenous metabolites of the analyte may have very similar structural recognition motifs as compared to the analyte itself. There are also various molecules very different from the analyte, but producing recognition motifs similar to the analyte. These molecules are generally called cross-reactants. When present in the sample, obviously they will produce false results in the relevant immunoassay [13–15]. One of the major problems of immunoassays used for measuring immunosuppressants is significant positive bias observed in immunoassays compared to more specific liquid chromatography-tandem mass spectrometry (LC-MS/MS) based assay due to significant cross-reactivities of metabolites. For example, the Chemiluminescent Microparticle Immunoassay (CMIA, Abbott) for tacrolimus showed an average of 24% positive bias while the Roche tacrolimus assay on the Cobas e411 analyzer showed an average 22% positive bias when compared with values obtained by using LC-MS/MS [16].

Other components in a specimen, e.g., bilirubin, hemoglobin, protein, or lipid, may interfere in the immunoassay by interfering with the assay signal, and thus produce incorrect assay results (please see Chapter 6). A third type of immunoassay interference involves endogenous human antibodies in the specimen, which may interfere with the assay reagent components – the assay antibodies, or the antigen-labels. Such interference includes that from heterophilic antibodies or various human anti-animal antibodies. All these sources of pitfalls in immunoassay have been described later in Chapter 7.

SPECIMEN TYPES FOR IMMUNOASSAYS

Serum and plasma are the most common type of specimens used in immunoassays. Whole Blood specimens must be used for some analytes, e.g., most of the immune-suppressant drugs. Urine is the most commonly used specimen in DAU testing. Urine samples are less frequently interfered by heterophilic antibodies, hemoglobin or icterus. Turbidity interference is possible in urine, but the cause is most likely bacterial growth. Preservatives in urine, like acetic acid, boric acid, or alkali, may interfere in some urine assays. Cerebrospinal fluid (CSF) specimens are used in monitoring the integrity of the blood brain barrier (by analyzing plasma proteins) or infection in CSF. The most common CSF interferent is hemolysis. Interference from turbidity is also possible in such specimens. Oral fluid is sometimes used for drugs testing where initial screening may be conducted by immunoassays, but further confirmation using mass spectrometry may be necessary [17]. Interestingly, testing of HIV in oral fluid using immunoassay is commercially available [18]. Hair, nails and sweat can also be used for drug testing but immunoassays have limited application [19]. Amniotic fluid, cord blood, meconium, and breast milk have been used to determine fetal and perinatal exposure to drugs [20–23]. However, these drugs testings are mostly conducted in the legal setting. Therefore, drug confirmation is essential using a specific chromatographic method.

Immunoassays are commercially available for a variety of analytes (Table 6.2).

TABLE 6.2 Examples of common analytes where immunoassays are commercially available.

Type of analyte	Specific example
Anemia markers	Ferritin, folate, vitamin B12 etc
Auto-antibodies: to diagnose and monitor auto-immune diseases	Antinuclear antibody (ANA), Rheumatoid factor etc
Cardiac markers	Troponin I, Troponin T, B-natriuretic peptide, Myoglobin, CK-MB etc
Diabetes markers	Insulin, C-peptide, HbA1c
Drugs of Abuse	Amphetamine, Benzoylecgonine (cocaine metabolite), Opiates, Oxycodone, Methadone, Marijuana metabolite, Phencyclidine, Benzodiazepines, Barbiturates etc
Hormones	ACTH, LH, FSH, Prolactin, hCG, Cortisol, Parathyroid hormone (PTH) etc.
Infectious diseases	Antibodies to and antigens from infectious microorganisms such as hepatitis and HIV
Therapeutic drug monitoring	Digoxin, Phenytoin, carbamazepine, Valproic acid, Phenobarbital, Primidone, Theophylline, Various aminoglycosides, Vancomycin, Immunosuppressants (Cyclosporine, Tacrolimus, Sirolimus, Everolimus, Mycophenolic acid) etc
Thyroid diseases	T4, T3, TSH, Thyroglobulin etc
Tumor markers	Alpha-fetoprotein, Beta-2 microglobulin, CA 15-3, CA 19-9, CA-125, calcitonin, Carcinoembryonic antigen (CEA), Gastrin, Human chorionic gonadotropin (hCG), Prostate specific antigen (PSA) etc

CONCLUSIONS

Immunoassays on automated systems are one of the main stays of analysis in today's clinical laboratories. Various types of immunoassays have been developed to analyze all types and sizes of antigens. The assays use photometric, luminometric, or fluorimetric signals, and homogeneous or heterogeneous reaction types. Serum and plasma are the main specimen types used for immunoassays. Other alternate types of specimens also have been used. Immunoassays are used not only on the central laboratory analyzers, but on the patient bed-side point-of-care systems as well.

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CHAPTER

7

Overview of other sources of interferences in immunoassays: prozone effect and interferences from heterophilic antibodies and autoantibodies

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INTRODUCTION

Since antibody-antigen immunoreactions depend on the recognition and binding of the antigen with the antibody, anything that interferes in the binding reaction will cause false negative or false positive results (depending on assay architecture). The various kinds of immunoassay architectures have been described in Chapter 5 (Immunoassays). The immunometric (or 'sandwich' assays) using two separate antibodies (Stationary Phase and Label antibodies), as expected, have less interference than the competition assays (which uses a single antibody). This chapter explores the sources of various types of antibody interference observed in immunoassays.

LIMITATIONS OF IMMUNOASSAYS

Even though immunoassays are widely used and a main workhorse in the clinical laboratory, they suffer from four major types of interferences, rendering false negative or positive results:

1. Interference from a specimen's endogenous interferant [1,2], which interferes non-specifically via 'matrix effect' or in signal generation; most common examples of such interferants for serum samples are: bilirubin, hemoglobin, lipids, and paraproteins.
2. Interference from a specimen's endogenous and exogenous interferant, which mimics the analyte (antigen) − antibody binding and thus causes interference. This is termed as cross-reactivity of the assay [3].
3. System or method related errors. These are more common among today's oft-used automated assay-systems (e.g., system probe carry-over).
4. Heterophilic interference, which is caused by endogenous human antibodies in the sample. These antibodies bind to assay components, generating false results. They include interference from auto-antibodies, heterophilic antibodies, macro-analytes (endogenous conjugates of analyte and antibody), macro-enzymes, and Rheumatoid Factors.
5. Prozone (or, 'hook') effect. If the sample has high excess of antigens, they saturate the binding sites of all antibodies, and the 'sandwich' complex does not form, generating a lower than expected assay signal, and false negative results.
6. Another kind of interference in homogeneous colorimetric assays is observed when certain clones of paraproteins in high concentration may precipitate during the assay reaction. Depending on the assay architecture and calculations, such precipitation may increase the photometric absorbance, leading to false positive or negative results, or system error flags (for saturated photometer) [4].

It is important to recognize the sources of such discordant results and conduct follow-up studies to provide clinically useful and important results.

HETEROPHILIC ANTIBODY INTERFERENCES

Heterophilic antibodies are human antibodies in a specimen that interact with assay antibodies (raised in

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various animals) to give false positive or negative results. The heterophilic antibodies are polyclonal heterogeneous in nature, consisting of the following types: (a) heterophilic antibodies that interact poorly and nonspecifically with the assay antibodies, (b) anti-animal antibodies, which interact strongly and specifically (with respect to the animal species in which the assay antibodies have been raised), (c) auto-antibodies, endogenous human antibodies interacting to an analyte, (d) therapeutic antibodies, where the antibody given therapeutically interferes with an assay, (e) macro-analytes or macro-enzymes, which are oligomeric or polymeric conjugates of an analyte, and/or conjugated with the endogenous antibody, and (f) rheumatoid factors. Heterophilic antibodies may arise in a patient in response to exposure to certain animals or animal products, or to infection by bacterial or viral agents, or nonspecifically. Although many of the immunoglobulin (Ig) clones in normal human serum may display anti-animal antibody properties, only those antibodies with sufficient titer and affinity towards the reagent antibody used in an assay will cause interference. Rheumatoid Factors also have been reported to interfere in immunoassays. Among the anti-animal antibodies, the most common occurrence is of human anti-mouse antibodies (HAMA), because of wide use of murine monoclonal antibody products in therapy or imaging. Heterophilic antibody and anti-animal antibody interference are often lumped together as heterophilic antibody interference. Such interference has been mostly found with immunometric sandwich assays, less often with competition assays. Sample dilution and depletion or removal of interfering antibodies has been recommended to remove heterophilic antibody interference. A patient history with respect to exposure to animals or animal products, or auto-immune diseases alerts the laboratarian towards the possibility of encountering heterophilic antibody interference.

Since heterophilic antibodies are found mainly in serum, plasma or whole blood, but not in urine, such interference is not found in urine specimens. This gives an excellent way to detect the interference for analytes that may be present in both matrices. For example, many case studies with false positive human chorionic gonadotropin (hCG) in serum/plasma have been described in the literature. In every such case, if hCG was measured in parallel urine samples, the false results and the resulting dire consequences could have been easily avoided [5].

Heterophilic antibody interference may cause critical impact and clinical misjudgment, resulting in unnecessary follow-up testing and unneeded but potentially dangerous therapy, leading to significant patient morbidity and even to mortality. The fact that such interference may not be suspected always from the patient history, or that such effect may be transient in nature, complicates the responsibility of the clinical laboratory to report accurate patient results.

Mechanism of heterophilic antibody interference

The interfering antibodies exert their effect by interacting with the assay antibody/antibodies or the analyte (in the specimen, or that used in the assay). In many cases, especially with heterophilic antibodies, the interaction among interfering and assay antibodies are nonspecific and of lower avidity. In such cases, if the assay employs equilibrium conditions, no interference is found. However, most immunoassays today are performed on automated analyzers. Since the attending physicians demand laboratory results ever faster, most auto-analyzers use immunoassays under non-equilibrium conditions, which are more prone to assay interferences.

In the sandwich type immunoassays heterophilic antibodies can form the 'sandwich complex' even in absence of the target antigen, generating mostly *false positive* results. However, if the interfering antibody binds only one of the assay reagent antibodies (capture or label), reducing reagent concentration, *false negative* results (though less frequent than false positive results) are observed.

In the competition type immunoassays, such interferences follow the following mechanisms:

1. If the assay uses a labeled analyte (or its analog), the interfering antibody (e.g., auto-antibody) may interact with the label, reducing the assay signal, and generating a *false positive* result. The same thing happens even if the assay antibody is labeled; the competing analyte (or its analogs) on the 'capture reagent' will bind to the interfering antibody, again generating a reduced signal, and false positive results.
2. However, if the analyte only (but not the competing analyte-reagent; this happens when the assay uses not the analyte in the reagent to compete, but an analog) interacts with the interfering endogenous antibody, then the analyte concentration in the assay reaction decreases, and *false negative* results are seen.
3. Heterophilic antibody may interact with the assay antibody, reducing its effective concentration (via steric-hindrance), which then decreases the assay signal, and generates a *false positive* result.

While the competition immunoassays are in general less affected by heterophilic antibody interference, the frequency of auto- and anti-animal antibody interference, however, is the same in these types of assays. Two-site, immunometric 'Sandwich' assays (e.g., cardiac troponin I, human chorionic gonadotropin, etc.), are more affected by the antibody interference.

How problematic is heterophilic antibody interference?

Heterophilic antibodies are poorly defined polyreactive human antibodies, recognizing IgG from different species. They have no clear immunogen and are of more non-specific type. They bind mostly to the Fc region of assay antibodies to cause interference; however, incidences of heterophilic antibody binding to other parts of the assay antibody (e.g., idiotope or the 'hinge' region) have been reported. Such interference is normally more pronounced if the assay antibody-pair is from the same (animal) source. If monoclonal antibodies (mostly of murine type) are used in the assay, the frequency of heterophilic interference is less, but the magnitude of interference is higher (however, see the anti-animal antibody section below). Heterophilic antibodies are found more in sick & hospitalized patients with reported prevalence of 0.2%–15%. However, during the last decade most commercial assays have started incorporating blocking reagents against the heterophilic antibodies in their assay reagent formulation, reducing this percentage. Thus a 2005 report lists 0–4% heterophilic antibody interference (measured by assay responses with and without an interference-blocking reagent) in eight automated tumor marker immunoassays [6]. However, since the heterophilic antibodies are very heterogeneous in nature, and their concentrations may differ among individuals, as described later, no blocking-reagent can guarantee 100% protection against such interference. Thus, it is important to note here that while frequency of heterophilic antibody interference may have gone down, the cases that are reported now, have a large magnitude of the interference, affecting the clinical interpretation of assay results much more.

D-Dimer immunoassays seem more susceptible to heterophilic interference; Wu et al. reports that 66.7% of incorrect D-Dimer results were caused by heterophilic antibodies [7]. Use of heterophilic antibody blocker reagents minimized the interference. Similarly, thyroid function immunoassays are also frequently impacted by heterophilic antibody interference; one has to be extra-vigilant in diagnosing thyroid disorders because the heterophilic antibody interference falsely raises the TSH (commonly, a sandwich assay) while suppressing free thyroid results (a competition assay) [8,9]. Kiyo reported a falsely elevated prostate-specific antigen (PSA) result in a patient after radical prostatectomy; they resolved the clinical versus PSA immunoassay result discrepancy by repeating the result in several PSA immunoassays [10].

In competitive immunoassays only a single analyte-specific antibody is used in the reagent, and so the reported cases of heterophilic antibody interference in such types of immunoassays are infrequent. However, just like free hormone immunoassays can be affected by heterophilic antibody interference, the immunoassay for the immunosuppressive drug cyclosporine A [11] has been described to suffer from a similar kind of interference. Liendo, Ghali, and Graves [12] reported a case where high serum digoxin levels (4.2 ng/mL) were observed in a patient 24 h after his last digoxin dose. However, there were no clinical symptoms of digitalis toxicity. The affected immunoassay, a competition type, used a monoclonal assay antibody. However, when the sample was assayed in another digoxin immunoassay that required protein precipitation before the assay, they found no digoxin in the sample. Similar results were found in two other digoxin assays, using different assay antibodies, as well. One of these two assays was an RIA, known for its interference from digoxin-like immunoreactive factors (DLIF), which gave essentially negative digoxin results. The researchers tried but found no evidence of any cross-reacting analogs or metabolites of digoxin causing such discordance. The specimen showed marked non-linearity in the original assay. Ultrafiltration of the sample (30kD exclusion membrane) followed by assaying the filtrate in the original assay detected no digoxin. By using protein A pretreatment of the sample to remove interfering endogenous antibodies, they found a reduction in observed digoxin results proportional to the amount of Protein A used. These results demonstrated that the original false positive results were caused by an heterophilic antibody interference in the sample.

Rheumatoid Factors (RF) are IgM type antibodies that interact with assay antibodies at the Fc area. RF is present in serum from >70% patients with rheumatoid arthritis. RF is also found in patients with other autoimmune diseases. RF concentration increases in infection or inflammation. RF interference follows the same mechanism as interference from other types of antibodies. Thus, in two-antibody immunometric assays, RF bridges the capture and label antibodies without involving the antigen and generates false positive signal and results. In single antibody competition type immunoassays, RF binds to assay antibody, preventing its reaction to the label reagent through steric-hindrance, thus reducing signal and generating false positive results. If RF is suspected to cause interference, the patient history needs to be examined for diseases that may increase serum RF concentration. RF concentration in the sample may be measured by many of the commercial RF assays available. RF can be removed from the sample by the many separation steps described in Section 6 of this chapter. In an interesting study [13] where RF interference gave false positive cardiac troponin I results in an immunometric assay, the authors inactivated RF by incubating the sample with anti-RF antibody and the interference was removed.

Astarita et al. described a case study of false positive thyroglobulin in a woman with history of rheumatoid arthritis [14]. They confirmed RF interference by (a) non-linear dilution, (b) alternate thyroglobulin immunoassay employing different antibodies, and (c) precipitating out interfering RF and retesting. Mongolu et al. described a case of 60-year old male where RF was suspected to interfere in multiple immunometric assays (LH, FSH, SHBG, PRL, hCG, TSH) resulting in false positive results; polyethylene glycol precipitation of the interferant brought all results to normal [15]. Lavoie et al. described false positive HIV Ag/Ab results, which on alternative HIV assays or with Heteroblocker treatment yielded negative results [16]. RF interference in multiplex cytokine assays could be neutralized by commercial Heteroblocker [17].

Another source of heterophilic antibody related interference is the conjugation of analytes to antibodies in certain patients' sera, leading to macromolecules of altered antigenicity (e.g. macroamylasemia and macroprolactinemia). Additionally antigens can polymerize in patient sera, resulting in altered antigenicity as well. This is the case in so named 'big prolactin' or 'big big prolactin'; these macroprolactin molecules often produce reduced prolactin immunoassay results compared to the physiologically active hormone concentrations.

INTERFERENCE FROM AUTO-ANTIBODIES AND THERAPEUTIC ANTIBODIES

Autoantibodies are endogenous patient's antibodies that bind to either the analyte itself or to one of the reagents used in the assay. Such antibodies are more frequently observed in auto-immune disease patients.

Autoantibodies to the analyte

The autoantibody may bind to the analyte-label conjugate in a competition type immunoassay, reducing the signal, and giving a false positive result. Alternatively, it may bind to the analyte in a sandwich assay or competition assay that uses a label containing an analog of the analyte, giving false negative results. An example has been described in a cardiac Troponin I assay (marker for cardiovascular diseases) where the autoantibody to troponin caused negative interference [18].

Autoantibodies to a component in the reagent

In one example, an endogenous anti-avidin antibody interfered in a theophylline assay that used the avidin-biotin system [19]. In this competition type immunoassay, the autoantibody interacted to avidin in the reagent, interfering in complex formation, thus lowering signal, and giving false positive results. Of course, if the assay was of sandwich type, the reduced signal would have caused false negative results. There are many examples of anti-streptavidin antibodies mimicking heterophilic antibodies in assays using streptavidin-biotin reaction. Also increased serum concentrations of biotin either prescribed therapeutically or ingested as over-the-counter supplement interfere in the streptavidin-biotin reaction employed in the immunoassay. As in the case of heterophilic interference, the problem is more acute in thyroid tests; because the interference causes lower signal in both immunometric TSH assay and competition T3 or T4 assay generating false negative TSH result and false positive T3 or T4 [20,21]. See Chapter 8 for a more detailed discussion of biotin interference in immunoassays.

Auto-antibodies to various label enzymes (e.g., alkaline phosphatase, peroxidase) have been reported; these may interact with the label enzyme in an ELISA, decreasing the signal, and falsely elevating the results in a competition type immunoassay.

Therapeutic antibodies are antibodies used to bind and inactivate toxic components from circulation and treat toxicity of various substances. Thus, Digibind (Glaxo/Burroughs Wellcome), Fab-fragments from ovine anti-digoxin antibodies and used to treat toxicity from the cardioactive drug, digoxin, interferes in most digoxin assays that do not use sample pretreatment. This interference is removable by ultrafiltration or protein precipitation [22].

INTERFERENCE FROM HUMAN ANTI-ANIMAL ANTIBODIES (HAAA)

HAAA are different from heterophilic antibodies by their specificity to the antibodies from certain species, and by their stronger avidity. HAAA arise mostly when the patient is exposed to a defined animal antigen. In the majority of the cases, the exposure is from diagnostic (i.e., tumor targeted imaging agent) or therapeutic applications of tumor-specific monoclonal antibodies. Since these antibodies are mostly murine in source, the most prevalent examples of HAAA interference are from human anti-mouse antibodies (HAMA) [23,24]. Most antibody-targeted imaging agents and antibody-targeted drugs use murine or rat antibodies. Digibind, used to treat digitalis toxicity, are Fab fragment of sheep anti-digoxin antibodies. Therapeutic insulin used to be from pigs, thus generating anti-pig antibodies in the patients. Now most insulin used as drug are humanized and bioengineered, eliminating this problem. Factor VIII used as drug is also from pigs. Many vaccines are generated in rabbits or chickens (eggs).

Anti-animal antibodies may also arise from contact with the animals (e.g., animal husbandry or keeping of animals as pets) [25] and the transfer of dietary antigens across the gut wall in conditions such as celiac disease [26].

The human anti-animal antibodies can belong to IgG, IgA, IgM, and rarely, the IgE class and can be directed to any part of the monoclonal antibody used in the assay (Fc, Fab, idiotope, etc.). When the HAAA are elicitated by animal immunoglobulins, the HAAA can have anti-idiotype or anti-isotype specificity. Anti-idiotype antibodies are directed against the hypervariable region of the immunoglobulin molecule, and anti-isotype antibodies are directed against the constant regions. The anti-idiotype antibodies may again generate endogenous anti-anti-idiotype antibodies. Assuming the 'mirror image' principle of idiotypic antibodies, the anti-anti-idiotype antibody will recognize the original analyte and bind to it. However, in general, most HAAA are isotype antibodies.

The magnitude and duration of HAAA vary wildly. Serum concentrations of HAAA range from micrograms to grams per liter. The HAAA may be transient lasting a few days to months and years. The possibility of interfering antibodies being transient [27] may make detection and solution of discordant results from such types of interference difficult. The prevalence estimates of HAAA, especially HAMA, have been studied by many authors; they vary from <1% up to 80% among different hospitalized or outpatient hospitals. Several commercial assay kits for HAMA estimation are available [28]. However, because HAMA are so heterogeneous, a negative HAMA assay result does not confirm absence of all types of HAMA in a sample. In fact, the difference in the prevalence estimates not only arises from population differences but from assay specificities as well.

HAMA interfere in immunoassays that use murine antibodies to the analyte. As more and more monoclonal (most common source is mouse) antibodies are used in commercial and other immunoassays (because of the specificity of the antibody and the reliability of the antibody supply), the impact of HAMA interference in terms of incorrect result and resulting inaccuracy in diagnosis and therapy has become more serious. As expected, the HAMA can be of varied prevalence, specificity, titer, and binding capacity. Most common HAMA concentration is <10 μg/mL, however HAMA concentrations as high as 1000 μg/mL have been reported. Since HAMA arise from exposure of patients to mouse antibodies, cancer patients who may have used such antibodies as part of imaging or therapeutic agents, have higher prevalence of HAMA occurrences (40–70%).

Antibodies to other species

After mouse, rabbit and goat are the most common animals used to generate assay antibodies in; so, like HAMA, immunoassay interference caused by human anti-rabbit (HARA) and anti-goat antibodies have been described. While HARA interference was shown in transthyretin, haptoglobin, and C-Reactive Protein (CRP) assays, no example has been described in literature yet about these antibody interferences in TDM/DAU assays [29,30]. There has been no report of Digibind (anti-digoxin sheep antibody fab fragments) giving rise to human anti-sheep antibodies; this may be because the small molecular weight Fab fragments are rapidly removed from circulation and the treatment is rare.

DETECTION AND CORRECTION OF HETEROPHILIC ANTIBODY INTERFERENCES

Assay developers take steps like sample blanking to minimize interferences. Auto-analyzers may include measurements of icterus, hemolysis, and turbidity to flag results that may be affected by such interferences. Sample blanking and robust assay design can be used to minimize these interferences, including matrix effect arising from protein and other non-specific constituent in the specimen. When suspected, the interferant may be removed from the specimen by specific agents, ultrafiltration, or centrifugation, before reanalysis. Alternatively the specimen may be analyzed by a different method known to be free from such interferences.

If a discordant result is suspected to be caused by interference from some endogenous antibody, the best ways to confirm such interference are: (a) a dilution linearity study with the specimen, (b) examination of the patient history (exposure to immunogenic animals or animal products; history of hyperactive immune system), and (c) treatment of the sample to block the interference or remove the interfering antibody, and repeating the assay.

Fig. 7.1 shows the effect of successive dilutions of a HAMA containing sample (spiked with 32 μg/mL of theophylline) versus those of a serum-based calibrator for the assay (=60 μg/mL) in a theophylline immunoassay using mouse anti-theophylline antibody (author's unpublished data). The HAMA sample interferes in the assay, and reads 59 μg/mL when assayed undiluted. After successive dilutions (by 1.3, 2, 4, 6, and 12-fold with the assay diluent), the interfering antibody is diluted enough as to not cause any interference in the assay (which had some heterophilic antibody blocker

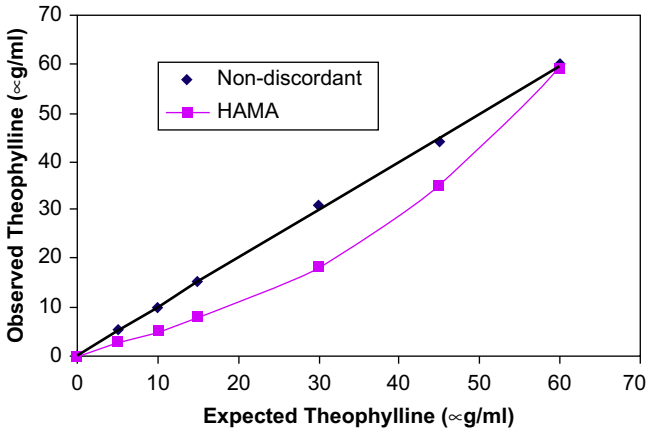


FIG. 7.1 HAMA interference detected by sample serial dilution.

in the reagents). The slope of a line fitted through the lowest three dilutions indicates a theophylline concentration of 31.1 μg/mL, close to the original spiked value. However, dilutions do not always give the correct analyte value in the sample because of increased imprecision in the low end of the assay, and the 'matrix effect' between the calibrator matrix and a patient sample.

As described previously, a patient history in terms of any exposure to animal antibody, illness, or exposure to animals should also alert for heterophilic antibodies or HAAA as possible sources for inaccurate results. At that time, the assay insert should be examined for the types of antibodies and heterophilic antibody blockers used in the assay.

There are various types of commercial or home-brew blockers for heterophilic antibody or HAAA [31,32]. The blocker can be non-immune animal serum, polyclonal antibody, polymerized IgG, nonimmune (irrelevant) mouse monoclonal, and a mixture of monoclonal antibodies or fragments of IgG [Fc, Fab, F(antibody')$_2$] preferably from the same species used to raise the reagent antibodies. As expected, although the nonspecific heterophilic interference can be mitigated by addition of non-immune serum to the reagents, purified IgG, preferably of the same subtype as used in assay, is better than serum in reducing the more specific and stronger binding HAAA.

Several blocking agents are commercially available: Immunoglobulin Inhibiting Reagent (IIR; Biorecalamation), Heterophilic Blocking Reagent (HBR; Scantibodies), Heteroblock (Omega Biologicals), and MAB 33 (monoclonal mouse IgG1) and Poly MAB 33 (polymeric monoclonal IgG1/Fab; Boehringer Mannheim). IIR is a proprietary formulation of high-affinity anti-animal antibody (10^9 L/mole), and HBR is monoclonal mouse anti-human IgM. A suspected discordant sample, e.g., sample giving false positive hCG results may be separately incubated with the blocker and then re-assayed [31]. Reinsberg studied the efficacy of various blocking reagents to eliminate HAMA interference [32]. Most commercial assay reagents include such blockers. However, because of the heterogeneous nature of the interfering antibodies, no blocker can guarantee success in all samples.

Non-specific and weaker heterophilic antibody interferences can even be mitigated by incubating the sample with any non-immune animal serum (even one different from the source of the assay antibody). Use a 'cocktail of animal sera' to reduce heterophilic interference has been suggested [28]. If the interference is complement mediated involving Fc parts of the interfering antibodies, heat inactivation of the sample (56 °C for 30 min) sometimes removes the interference [28]. A limitation of this method is that not many analytes can survive such an antibody denaturing process.

As previously mentioned, a general concept of prudent assay design is to use Fab or F(ab')$_2$ fragments of the analyte specific antibody, thereby reducing the interference from human anti-isotype antibodies [33]. Kuroki et al. used human/mouse chimeric antibody in a CEA assay to reduce HAMA interferences [34]. Another interesting concept is to use chicken antibodies (IgY) in the assay, because so far no heterophilic antibody interference against chicken assay antibodies has been reported in the literature [35]. By changing the reaction temperature and allowing longer time to achieve equilibrium, one may be able to reduce heterophilic antibody interference, but not HAAA interference [36]. However, with modern auto analyzers, changing reaction temperature or reaction time is mostly not possible.

Removal of interfering substances

If the methods described above to correct the interference do not work or cannot be applied, then the interference can be resolved by removing the interferant and reassaying the clean specimen. Some commercial assays routinely use sulfo-salicylic acid precipitation of proteins to make protein-free specimens, which are then assayed in the analyzer.

Of course, a simple solution to remove antibody interference is the selective removal of the antibodies from a sample. This can be achieved by selective adsorption of human IgG by a solid phase containing protein A or protein G [12]. This, however, does not work if the majority of the interfering antibodies are of IgM type. Alternately, the antibody fraction in the sample may be precipitated out with a polyethylene glycol (preferable PEG 6000) reagent [37]. Alternately, small molecular weight analytes, if they are not highly protein bound, could be extracted away from the interfering immunoglobulins by preparation of a protein-free filtrate via ultrafiltration or protein precipitation (using TCA, sulfosalicylic acid, or ammonium sulfate). The centrifugal ultrafiltration is a fast

and relatively easy method, using 10- or 30 kDa molecular weight cut-off filter membrane ultra-centrifugal cartridges (example: Amicon's Microcon or Centricon). Centrifugation can be done in table-top instruments (preferably at 4 °C). As little as 300 µL of sample is required. For digoxin assays, this step could not only remove antibody interference, but also removes interference from protein-bound interferants or Digibind [38]. The harsh conditions of acid precipitation, on the other hand, may damage many analyte molecules. Furthermore, this method will only work for small molecular weight analytes that can be easily filtered from large molecular weight antibodies.

PROZONE EFFECT

Prozone (or 'Hook Effect') is a phenomenon only observed with immunometric assays for analytes with a large analytical range; for example, alfa-fetoprotein, calcitonin, cardiac troponins, hCG, PSA, TSH, etc. In such assays, erroneous results are obtained if a correct antibody-antigen 'sandwich' is not formed. In the presence of a large excess of analyte (antigen), each and all of the antibody binding sites (on both the 'solid phase' and the label antibodies) may be occupied by single analyte molecules, and no sandwich complex forms, resulting in reduced signal and false *negative* results.

As an example, a turbidimetric immunoassay for α1-Acid-Glycoprotein (AAG) showed a prozone effect with analyte concentrations above 2000 mg/dL (author's unpublished data) (Fig. 7.2):

A similar prozone or 'Hook Effect' has been described with immunoradiometric assays of calcitonin [39,40]. The false negative results, suspected to be caused by prozone, will show a serial dilution curve as above; with increased dilution the antigen concentration will decrease leading to sandwich formation. When a dilution is reached whereby the prozone effect disappears, the observed analyte concentration may be multiplied by the corresponding dilution factor to obtain the true antigen concentration in the sample.

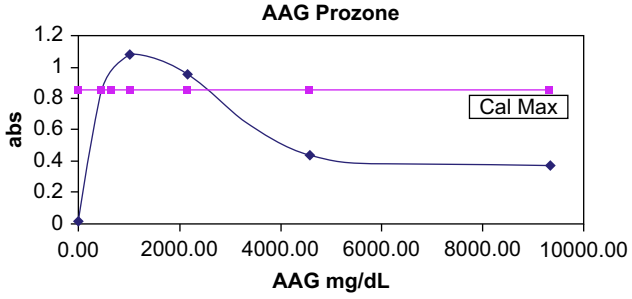


FIG. 7.2 Example of prozone effect in an alpha -1 acid glycoprotein (AAG) assay. abs = Absorbance, Cal Max = absorbance generated from the assay with the highest assay calibrator (650 mg/dL) as sample.

CONCLUSIONS

In spite of the excellent sensitivity and specificity of the immunoassay, they do suffer from interferences: serum constituents, cross-reactants, endogenous antibodies, or prozone effect. There are various ways to detect and mitigate such interferences to obtain accurate results.

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CHAPTER

8

Biotin interference in clinical laboratory tests: sporadic problem or a serious clinical issue?

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INTRODUCTION

The biotin: streptavidin complex is an enduring feature of modern immunoassay design that has been widely incorporated into routine diagnostic assays for several decades [1]. With a dissociation constant approaching the femtomolar range [2], biotin and streptavidin form one of the strongest non-covalent bonds occurring in the natural world. Why evolution has favored such tenacious binding between a water-soluble prosthetic group and a bacterial protein is intriguing. Biotin is a B-group vitamin that functions as a co-factor for carboxylase enzymes, which are ancient and found across all forms of life [3]. Streptavidin is synthesised by *Streptomyces avidinii*, but more than 100 homologues are likely to exist across distantly related organisms [4] with avidin, from chicken egg white, the best characterised. Streptavidin-like proteins sequester biotin, and this is variously exploited to competitive advantage by the organisms that synthesise them [5–9].

UTILIZATION OF BIOTIN IN IMMUNOASSAYS

Unique characteristics as a ligand-binding pair have seen streptavidin and biotin used in diverse applications in biotechnology, from immunoassays and immunohistochemistry, purification of proteins and nucleic acids, cell biology and live cell imaging, to more recent use in drug delivery and radiographic imaging [1,4,10–13]. Naturally, the affinity of the interaction is central to its utility, as is its stability, resistant to extremes of temperature and pH, detergents, denaturants and organic solvents [1]. Biotin (MW 244.31) can be covalently linked to macromolecules without appreciably altering their function, and many effective biotinylation approaches exist for different target molecules, permitting versatile application. Biotinylation renders an antibody "bivalent" and able to bind both its cognate antigen as well as streptavidin. Because biotin: streptavidin binding is essentially irreversible, this provides a highly resilient bridge mechanism in heterogeneous immunoassays. Streptavidin (MW 56 kDa) can be readily coated onto a solid phase with high uniformity, unlike direct adsorption of antibody, which is often heterogeneous in conformation and subsequent antigen binding capacity [1]. The net benefit is enhanced signal capture when streptavidin is used as a bridge, and this is further amplified by the presence of four biotin-binding sites within streptavidin homotetramers. Given that the limit of detection is highly dependent on the number of signal molecules captured, incorporating the biotin: streptavidin pair into immunoassays can greatly increase sensitivity.

BIOTIN INTERFERENCE IN IMMUNOASSAYS

For all the strengths and versatility of biotin: streptavidin assays, their conspicuous vulnerability relates to the potential effect of high free biotin concentrations in the sample. Excess biotin can compete with biotinylated reagents, potentially displacing their binding to the streptavidin coated solid phase during the assay reaction and reducing the amount of signal that is captured. Whether this causes a positive or a negative bias

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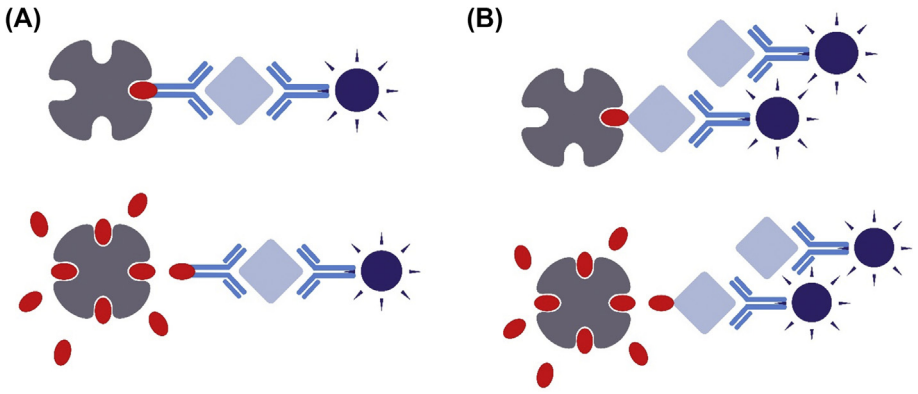


FIG. 8.1 Mechanism of biotin interference in (A) sandwich and (B) competitive immunoassays.

depends upon the assay architecture: in sandwich immunoassays, biotin interference causes falsely low results whereas in competitive immunoassays, falsely high results ensue because of the inverse relationship between signal and analyte concentration (Fig. 8.1). Assays in which biotinylated antibodies are already bound to streptavidin coated microparticles are generally not affected by biotin interference. The biotin: streptavidin interaction is almost irreversible and pre-formed biotin: streptavidin is not readily displaced by excess free biotin in the sample.

Even amongst assays with a biotin-susceptible architecture, there is wide variation in individual assay tolerance. Many manufacturers report thresholds of biotin tolerance to indicate the concentration of biotin expected to cause significant analytical bias, usually defined as 10%. The tolerance of various assays has been extensively cataloged [14–16]. At the time of writing, all Roche Elecsys immunoassays are sensitive to biotin, with tolerances ranging from approximately 10 to 120 ng/mL and more than 90% at 30 ng/mL or above [17]. Immunodiagnostic Systems immunoassays also depend heavily on a dynamic biotin: streptavidin structure; the majority show a biotin tolerance of just over 70 ng/mL, but the renin and aldosterone assays have thresholds under 10 ng/mL [18]. More than 60% of Ortho Clinical Diagnostics Vitros assays have biotin tolerances of 20 ng/mL or less, with several at 5 ng/mL and under, including Troponin ES [19]. Siemens Healthineers have harnessed the versatility of biotin: streptavidin technology widely in varying configurations [20]. Many of the Centaur immunoassays do not employ biotin: streptavidin, but for those that do, the majority are resistant to biotin interference, using streptavidin-magnetic particles pre-complexed with biotinylated reagents. About 15% of Centaur and Immulite assays are biotin-sensitive, including the Troponin I Ultra assay. The remaining Immulite assays incorporate pre-bound streptavidin-biotin and are very robust to biotin interference. The Dimension EXL and Dimension Vista LOCI include numerous biotin-sensitive assays, but the tolerance for the majority is greater than 100 ng/mL. Certain Beckman Coulter immunoassays are sensitive to biotin interference, including the thyroid cancer marker, thyroglobulin [21]. DiaSorin immunoassays are resistant to biotin interference with the exception of a small handful of serology assays that show sensitivities of 10 ng/mL or less [22]. The Abbott Architect and Alinity assays are unaffected by biotin [23].

In vitro spiking studies capture biotin induced bias over a wide range of concentrations, generating a much more detailed picture of biotin interference than that expressed by a 10% analytical bias figure [24–28]. Biotin tolerance across the Roche competitive (Fig. 8.2A) and sandwich immunoassays (Fig. 8.2B) is highly variable, with the sensitive assays showing steep dose-response curves, but more resistant assays exhibiting flatter curves.

The convergence of susceptible methods and supra-physiological biotin intake

Biotin: streptavidin based immunoassays have long been used in clinical diagnostics. By necessity, they were designed to tolerate the concentrations of biotin usually found in human serum, estimates of which range from under 0.1 to approximately 1 ng/mL [29–35]. The history of biotin interference parallels the history of supra-physiological biotin intake, whereby biotin is consumed as a drug, not as a trace micronutrient. Before chronicling this history, it is useful to outline biotin's role in health and disease, and consider the settings in which high dose biotin is used, therapeutically or otherwise.

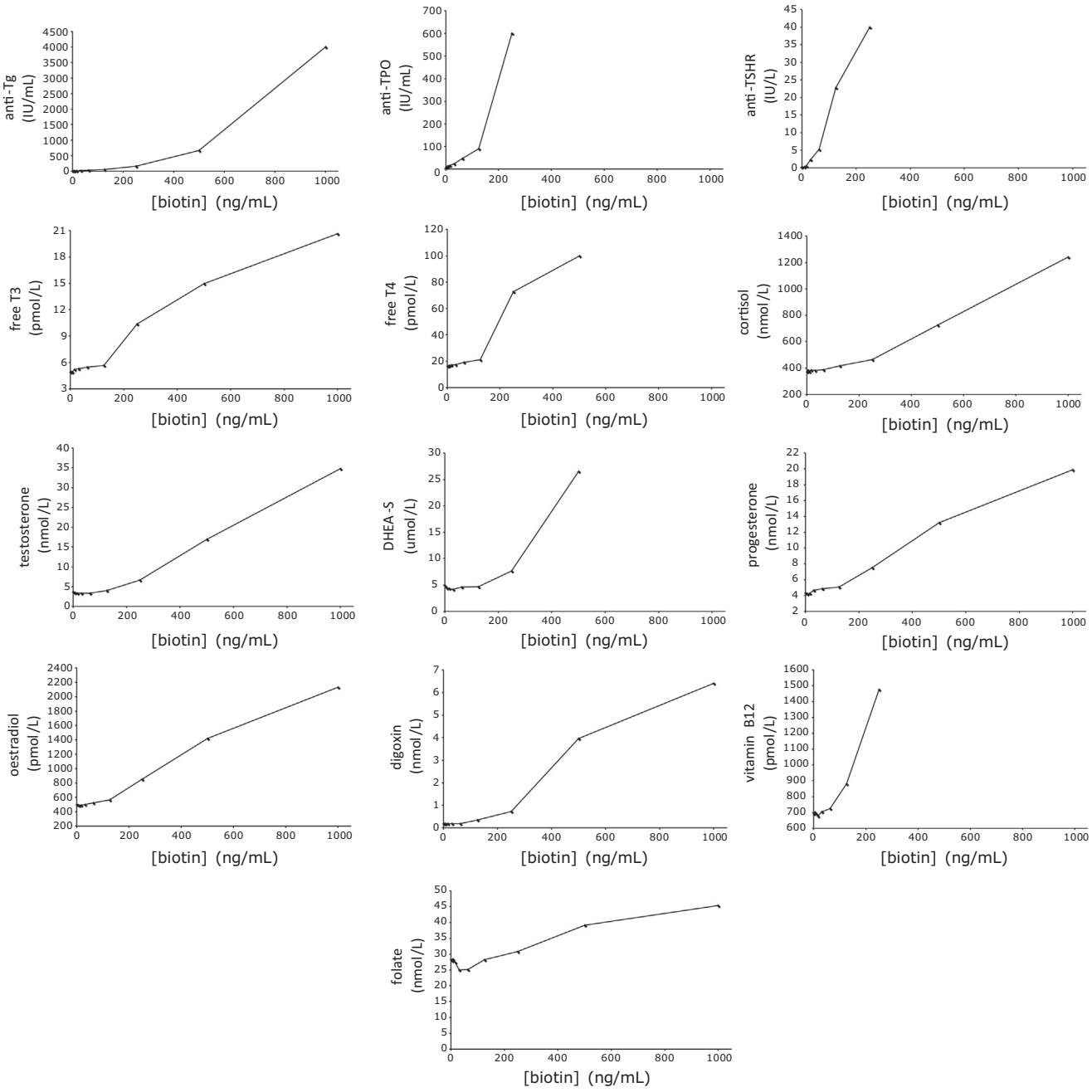


FIG. 8.2A Falsely elevated values of various analytes due to positive interference of biotin in biotin based competitive immunoassays.

BIOTIN REQUIREMENT AND PHYSIOLOGICAL FUNCTIONS

In humans, biotin serves as a co-factor for five carboxylase enzymes that perform critical steps of gluconeogenesis, fatty acid metabolism and branched chain amino acid catabolism. Biotin covalently attaches to apo-carboxylases through the action of holocarboxylase synthetase, generating active carboxylases capable of transferring bicarbonate to organic acids [36]. As an integral component of intermediary metabolism, absolute biotin deficiency is presumably incompatible with life [37]. Humans and other mammals cannot synthesise biotin and it must be derived exogenously. Although the

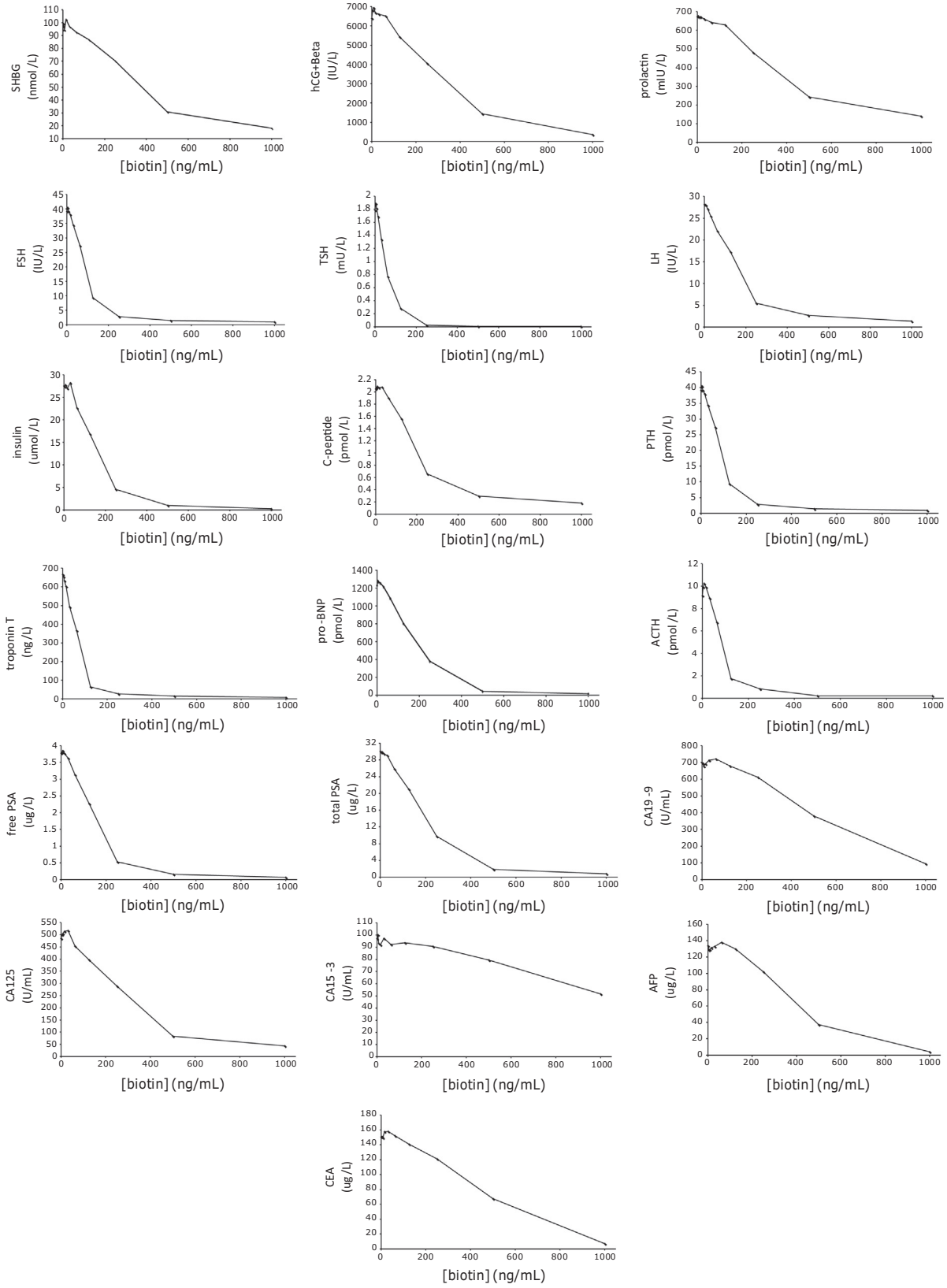


FIG. 8.2B Falsely lower values of various analytes due to negative interference of biotin in biotin based sandwich immunoassays.

absolute biotin requirement is unknown [38], humans need only trace amounts and the WHO recommended adequate intake (30 μg daily in adults) is easily met, as biotin is widespread in foods [39]. Biotinidase is crucial for absorption of dietary biotin from the gastrointestinal tract, liberating free biotin from degraded proteins, which is actively taken up by the Na-dependent SMVT transporter [40].

Inborn errors of biotin metabolism

As expected by their pivotal role in biotin metabolism, mutations that reduce the activity of holocarboxylase synthetase and biotinidase impair biotin utilisation and cause ensuing deficits in all of the biotin-dependent carboxylases. Deficiency of holocarboxylase synthetase causes a "functional" biotin deficiency: though present, biotin cannot be incorporated into the carboxylase enzymes, and their activity is impaired [41]. Biotinidase is crucial for the endogenous recycling of biotin, and mutations affecting its activity lead to excessive urinary loss of biotinylated peptides and depletion of the internal biotin pool [41–43]. Both holocarboxylase synthetase deficiency and biotinidase deficiency are inherited in an autosomal recessive manner [41]. Shared clinical features include metabolic acidosis, neurological abnormalities (hypotonia, ataxia, mental retardation and seizures), and the classical features of biotin deficiency, dermatitis and alopecia. Clinical expression is variable [44], depending in part on the extent of enzyme deficiency. The estimated incidence of holocarboxylase synthetase deficiency is 1 in 87,000 live births [45], and combined partial and severe biotinidase deficiency, 1 in 67,000 [46].

Acquired biotin deficiency

Outside of the hereditary metabolic disorders, frank biotin deficiency is very rare [46], reinforcing the very low biotin requirement for human health, and also hinting at the efficacy of physiological mechanisms that optimise biotin utilisation and prevent its wastage. Frank biotin deficiency has been reported in cases where total intravenous nutrition [47–49] and infant formulae [50–52] were devoid of biotin. Perhaps the most well-known example of acquired biotin deficiency occurs not because of lack of biotin in the diet, but an inability to absorb it. Excessive consumption of raw egg white for a prolonged period can cause biotin deficiency [53] because of sequestration of biotin by native avidin. So called "egg white disease" is extremely uncommon; while sporadic cases are documented [54], most reports are in clinical studies, where it is employed as an experimentally induced form of biotin deficiency [55,56].

High dose biotin therapy in inborn errors of metabolism

Pharmacological concentrations of biotin are used in several settings, with a recently extending niche. Free biotin many hundred times more than average daily intake rescues the respective functional biotin deficiency and true biotin depletion of holocarboxylase synthetase deficiency and biotinidase deficiency. In the former, high dose biotin therapy promotes biotinylation activity of residual holocarboxylase synthetase, somewhat reconstituting functional holocarboxylases. Good clinical response is usually achieved with 10–20 mg biotin daily; very rarely, patients require higher doses [41]. In biotinidase deficiency, supra-physiological concentrations of biotin effectively compensate for the excessive urinary biotin excretion and decreased bioavailability from food [42]. Clinical response is usually achieved with 5–10 mg oral biotin in children, but adolescents and adults may require 10–20 mg [57]. For both diseases, treatment is lifelong [37,41].

Biotin-responsive basal ganglia disease (BRBGD) is a separate entity also successfully treated with high dose biotin. This autosomal recessive disorder usually presents in childhood, with recurrent bouts of encephalopathy that may lead to coma and death, but supra-physiological biotin (5–10 mg/kg/day) effects startlingly prompt resolution of symptoms and prevents neurological disability [58]. In a proportion of subjects, acute crises recur unless high dose thiamine therapy is added [59], which fits conceptually with known causative mutations in the thiamine transporter, hTHTR2 (SLC19A3). Biotin is not a substrate for this transporter, however, and the basis for its striking clinical efficacy remains unclarified.

High dose biotin therapy beyond metabolic disease

Progressive multiple sclerosis (MS) is the most recent disease to be treated with high dose biotin, although efficacy is still under investigation. MS is an autoimmune demyelinating disease of the central nervous system that leads to neurodegeneration, neurological deficit and disability [60]. MS unfolds as two broadly distinct clinical courses; an unpredictable, relapsing remitting form and a progressive form in which continuous clinical deterioration occurs. The major therapeutic advances in MS treatment in the last two decades have targeted

acute episodes with immunosuppressive/anti-inflammatory strategies [61]. Unfortunately, progressive MS has remained stubbornly resistant to treatment.

In a pioneering pilot study, objective improvement in disease activity was reported in a small cohort of patients with progressive MS who were treated with 300 mg biotin daily [62]. Improvement largely localised to tetraparesis/paraparesis in those with spinal cord disease, but some patients also demonstrated visual improvement. A follow-up 12 month randomised, blinded, placebo-controlled trial also demonstrated improvement in MS-related disability in a subset (approximately 15%) of patients with progressive MS [63]. Given the paucity of agents shown to improve progressive MS, these studies were met with receptive enthusiasm by neurologists and patients alike. Because biotin was considered very safe, there was rapid uptake in parts of the world even before the randomised control trial was published. The efficacy of high dose biotin therapy in MS has attracted critical questioning, however [64], and a recent observational study failed to find clinical improvement [65]. Another trial was stopped prematurely due to lack of improvement in visual acuity in patients with fixed visual deficits [66]. More work is required, in larger and longer phase III clinical trials, to substantiate the initial findings. Currently in process, these trials are keenly awaited.

Thus far, there is no clear mechanism for biotin's therapeutic action in MS; two current hypotheses relate to re-myelination and improved energy metabolism [67]. Justification for the extremely high dose of biotin is also lacking, and no clear dose-response data has been presented [62]. Whether responders in the MS trial harbor biotinidase mutations is a pertinent question [68], given that biotinidase deficiency is fraught with misdiagnosis with a cluster of neurological diseases that includes MS [69–76]. The number of patients who improve with biotin exceeds the prevalence of biotinidase deficiency, however, implying misdiagnosed biotinidase deficiency cannot explain biotin's putative efficacy in MS [77].

Biotin supplementation for skin, hair and nails

Beyond inborn errors, frank biotin deficiency is exceedingly rare. There are no major reports of a wider incidence of biotin deficiency causing alopecia and dermatitis in the general community, yet biotin therapy is frequently adopted in various hair and skin disorders [78]. Biotin is also a common treatment for various nail disorders, which may have its origin in livestock practices [79–81].

Select case reports suggest clinical efficacy of biotin therapy in very specific hair and nail disorders, including the rare disorder cheveux incoiffables [82,83], and rare cases of valproate-associated alopecia [84]. Several studies purport to show clinical efficacy of mg strength biotin in treating brittle nails [85–87], however the general absence of appropriate controls and assessment of biotin status preclude firm conclusion without further investigation [81]. In recent years, a trend for biotin supplementation in mg amounts has emerged in the general "healthy" community, to whom biotin is marketed for skin, hair and nail cosmesis [88]. Lack of regulation of the vitamin industry has allowed unsubstantiated claims of efficacy to persist. As yet, there is no compelling evidence that biotin supplementation in an already replete individual can improve growth, quality or appearance of skin, hair or nails [78].

Biotin metabolism and pharmacokinetics

Bioavailability of oral biotin is extremely high relative to typical dietary intake [89], and recent preliminary evidence suggests the SMVT transporter is only saturated at biotin doses approaching 100 mg [90]. More than 80% of biotin circulates in the free form [91,92], with the remaining fraction bound to proteins, including biotinidase [93]. Investigations in animal models indicate the liver is the primary storage site [92,94], and also the predominant site of biotin catabolism, where it undergoes β- and sulfur-oxidation [36,40,95]. Biotin excretion is principally renal, with biliary excretion minimal [96,97].

Recent studies provide much needed data on biotin pharmacokinetics. Peak concentrations are reached approximately 1 h post ingestion. After 5, 10 and 20 mg biotin, respective mean peak concentrations of 40, 91 and 184 ng/mL have been reported, with moderate inter-individual variability evident [98]. Single dose schedules have shown mean concentrations of approximately 500 and 800 ng/mL after 100 and 300 mg biotin [90] and peak levels in excess of 1000 ng/mL have been documented in healthy controls after ingesting 300 mg biotin [99]. Given evidence of biotin bioaccumulation [31,98], higher concentrations are likely to occur in those chronically dosed.

HISTORY OF BIOTIN INTERFERENCE

Serum biotin concentrations exceeding the normal level by more than 1000-fold could not have been conceived of when manufacturers developed the first biotin: streptavidin based immunoassays. Biotin therapy

for inborn errors of metabolism emerged in the 1980s and, not unexpectedly, the earliest report of biotin interference occurred in this setting, when a newborn treated with biotin appeared biochemically hyperthyroid [100]. In the three decades since, there have been sparse but ongoing case reports documenting thyroid function test derangement in biotin-treated patients with metabolic diseases [101–106].

The highest density of biotin interference reports, however, has come from the MS setting, where the introduction of high dose biotin therapy was promptly followed by a succession of case reports. Again, thyroid function test abnormalities have dominated the literature [107–114]. Different patterns of thyroid derangement occur on different platforms, depending on the specific TSH, free T4 and free T3 methods and their biotin sensitivities. At the time of writing, gross biotin interference on the Roche, Siemens Dimension EXL and Vista Loci platforms appears as profound biochemical thyrotoxicosis, with suppressed TSH and elevated free thyroid hormones. The free T4 elevation is prominent on Roche, giving an unusually high free T4:free T3 ratio that occurs only rarely in genuine thyroid disease, and an autoimmune aetiology is suggested by elevated anti-thyroid and TSH-receptor antibodies [104,107,110,111,114–117]. Biotin interference affecting Beckman thyroid function tests can masquerade as thyroid hormone resistance or TSHoma, with unaffected TSH (and anti-thyroid antibodies) but elevated free thyroid hormones [102,118]. On the Vitros platform, a subclinical hyperthyroidism picture occurs, with suppressed TSH but normal free thyroid hormones [116,119,120].

Interference affecting other analytes is not as commonly reported as thyroid function tests. The scope of interference has been highlighted by *in vivo* studies of volunteers and MS patients [99,102,112,121] but case reports of erroneous results found in routine presentation have affected fewer analytes. In the MS setting, these include falsely low PSA [122], falsely elevated vitamin B12 [109], and falsely elevated 25-hydroxy vitamin D. Falsely low PTH [123], falsely normal PTH in a patient with chronic kidney disease [118,124], and deranged LH, FSH, testosterone, oestradiol, progesterone and cortisol [119,120] have been reported in individuals taking over-the-counter biotin.

ADVERSE EFFECTS FROM BIOTIN INTERFERENCE

Given that false diagnoses of thyrotoxicosis have dominated the literature, it is not surprising that inappropriate anti-thyroid therapy [103,110,115,122], and unnecessary thyroid ultrasounds [114,122] and radioactive uptake scans [104,107,113] are the most commonly reported adverse event. When biotin interference is not picked up, the failure to respond to anti-thyroid medications can be misconstrued as treatment refractory thyrotoxicosis, prompting more definitive "management". Near-miss radioactive iodine [24] and near-miss thyroidectomy (unpublished) have been reported. Biotin interference can also mask biochemical hypothyroidism, and both inappropriate reduction in thyroxine dose, and delay in treatment of hypothyroid patients have occurred [100,105]. Unnecessary adrenal, pituitary and pelvic imaging and near-miss hysterectomy/oophorectomy have also been reported [120]. However the most serious potential adverse event concerns a fatality in a biotin-treated MS patient presenting with chest pain and admitted to ICU. The possibility of a falsely negative high sensitivity Troponin T result at presentation has neither been confirmed nor excluded, and any causal role in the ultimate clinical outcome remains unclear [125].

Assessing the risk of adverse events

Changes in clinical practice and uptake of over-the-counter mg strength biotin supplements have increased the likelihood that samples presenting for laboratory testing contain high biotin concentrations. This implies an increased risk of adverse events, which is consistent with increased reports of diagnostic errors. However, in comparison to total number of tests conducted, it must be acknowledged that these reports are rare. What is the likelihood of clinically-relevant biotin interference? It is necessary to estimate risk beyond potential to probability, so that appropriate risk mitigation strategies can be implemented. In this regard, it is important to distinguish the risk of analytical bias from the risk of misclassification of disease and the risk (and severity) of an adverse event. Such risk assessment is necessarily complex, and will be both analyte- and test-specific, as well as patient- and context-dependent. What follows is a brief discussion of some critical elements that require consideration in a formal risk assessment, focusing on the diagnostic rather than disease monitoring setting.

A pivotal determinant of the risk of biotin interference is the likelihood that the patient is taking high dose biotin when they present for testing. Unfortunately, at any general population level, this is difficult to estimate, as published prevalence studies are scarce at the time of writing. From Nielsen sales data, consumption of >1 mg biotin supplements in the US was

estimated at 0.7% in 2017 [126]. The US is believed to consume high levels of supplements, and how these figures compare in other countries is not clear. Additional demographic and regional differences are likely to occur, and dedicated prevalence studies are needed. Beyond the overall prevalence of biotin use in any given population, for certain sub-groups the presumption of higher than general prevalence is imperative. For the biotin-responsive inborn errors of metabolism, there is a high probability of patients presenting with biotin-affected samples, as biotin treatment is lifelong for each disorder. For patients with MS, uptake of high dose biotin therapy has been heterogeneous; nonetheless, the MS population must be treated as a group at high risk of biotin-affected samples at the time of testing.

Assuming a biotin-affected sample does present for testing, the first component of the biotin risk equation is analytical bias, and the intersection of two major elements must be considered: biotin concentration and assay tolerance. Serum biotin concentration will depend on the dose of ingested biotin, the time of blood collection in relation to that dose, and the individual's renal function, which will govern biotin clearance. As discussed earlier, assays vary in their biotin tolerance, thus a given biotin concentration will exert differential analytical bias on different assays.

Whether that analytical bias translates to a change in disease classification depends, to some extent, on the true result, and whether a reference interval or a binary classification applies. For the former, true results approaching the upper and lower percentiles will flag more readily when biotin concentration exceeds the assay tolerance for respective competitive and sandwich immunoassays than results at the median. Interference causing flagged results is more likely to come to clinical attention, although it is does not follow that it will necessarily translate into clinical harm, as will be discussed below. In contrast, for disease flagged by a binary classification where normal appears as "negative" and disease is flagged as positive, false negative results may prove much less visible, as they are absorbed into the mass of normals. Invisibility may exacerbate clinical risk.

Visibility converges with the clinical picture in governing the impact of interference. Whilst a characteristic pattern of thyroid function test results might well mimic hyperthyroidism, severe biochemical hyperthyroidism in a completely asymptomatic individual will hopefully raise the question of interference. There are numerous examples where discordant results from biotin interference were promptly intercepted by clinicians because they did not fit clinically [106,108,111,115–117]. In contrast, the convergence of distorted results with a consistent clinical presentation is much more likely to lead to further investigations and inappropriate therapy [120].

Disease prevalence also intersects the risk of a false result. For disease flagged by binary classification, the clinical risk of a falsely negative analytical result is highly dependent on the prevalence of that particular disease. In Bayesian terms, the probability of a false negative result for a very low prevalence disease is much lower than the probability of a false positive result for that same disease [127]. It follows that for very rare diseases, the probability of biotin interference causing false negatives and associated clinical harm is low because most biotin-affected patients will be unaffected by that low prevalence disease (unless there were an association between biotin therapy and other rare disorders).

For higher prevalence diseases, the probability of false negative results is greater, however the "penetrance" of risk will still be governed by the specific clinical setting. For acute myocardial infarction (AMI), where a false negative troponin result may potentially lead to delayed or missed diagnosis, risk penetrance is modulated by the clinical features of the patient and the specific acute coronary syndrome protocol employed by the physician. Many algorithms are in current use, incorporating clinical features, ECG and troponin results [128]. Early rule-out approaches open the possibility of early discharge in patients with very low risk, and retain patients with high-risk features. Protocols differ in the number and weighting of risk factors, and whether single or serial troponin results are collected. Paradoxically, the highest risk of biotin interference causing missed AMI diagnosis and inappropriate early discharge may well occur in "low-risk" patients. A single troponin protocol is also more vulnerable to biotin interference than serial troponin protocols, with a longer interval between tests more robust than a shorter interval, allowing greater time for biotin clearance. An absolute delta change might also buffer biotin interference somewhat, as clearance of biotin in the interval may unmask a significant change in troponin results. However for grossly elevated biotin concentrations, the negative analytical bias is profound [28] and a patient who happened to present with such high biotin concentrations whilst having a non-ST elevation MI would be at very high risk of a false negative troponin result contributing to either delayed or missed diagnosis if other high risk features were absent.

It is clear that the risk of biotin interference translating into clinical harm is complex and holds many contingencies. Nonetheless, attempts at formal

risk assessment are important as estimated risk guides the mitigation strategies that a laboratory or hospital should explore and employ. Arguably, confirming a higher risk in the general population would warrant general measures of risk mitigation, whereas risk that localises in certain patient groups requires adoption of targeted approaches to protect those rare individuals at high risk.

SOLUTIONS TO THE PROBLEM OF BIOTIN INTEFERENCE

Biotin is a unique interferent in multiple respects, presenting both challenges and opportunities for intervention. An ingested interferent, biotin affects particular groups of patients, is cleared with reasonably predictable kinetics, and exerts defined effects on susceptible immunoassays. All of these properties can be harnessed for risk mitigation approaches, and analytical bias can be prevented if effective strategies are put in place. A multi-pronged approach is warranted, encompassing both general and targeted strategies, the selection of which should be informed by, and tailored to, formal risk assessment for a particular clinical service.

Education and awareness: general and specific approaches

Risk mitigation begins with awareness. Biotin interference needs to be communicated to the wider scientific community and relevant clinicians and health care professionals. At a local level, this can readily be achieved through the existing channels of laboratory communication, including electronic alerts and commenting, as appropriate.

Responsible medication management: notification of biotin interference

Education strategies often address clinicians and health care professionals, but arguably it is the patient and consumer who should be targeted most vigilantly. Harm from biotin interference can be viewed as a particular case of adverse drug event, whereby the risk operates *ex vivo*, when false results are reported by laboratories and acted upon by clinicians. It follows that standard medication alert pathways should be employed for risk mitigation, including mandatory notification of the risk of harm at the time of dispensing. In Europe, MS patients participating in biotin trials were given an alert card to be presented to all health care consultations, including emergencies [129]. Such an approach arms at-risk patients with a notification alert to present to healthcare encounters - their time of greatest risk - and is a most important step in protecting them.

The over-the-counter setting is more challenging as the supplements industry has historically evaded regulatory bodies [130,131]. High dose biotin supplements likely hold little clinical benefit to healthy consumers but expose them to risks, about which they are not informed. In this regard, the problem of biotin interference accords with wider calls for greater regulation of the supplements industry to protect consumers [132]. Importantly, the FDA safety communication on biotin interference was addressed to the public and consumers of biotin, as well as the wider laboratory and medical community, and can therefore be seen as a pivotal first step in alerting consumers to risks associated with biotin [133].

Notification of biotin use at the time of blood collection

The FDA recommends that high dose biotin consumption/therapy is specifically elicited at the time of blood collection [133]. In practice, this may be difficult to administer when individuals may be unaware they are consuming biotin. Clinicians and patients can be educated to indicate high dose biotin therapy on referrals. Laboratories then require a robust system of detecting this and responding appropriately. Education can also be aimed at withholding biotin prior to blood collection (see below).

Surveillance

Electronic surveillance through laboratory middleware or laboratory information systems can be used to target high-risk samples for further integrity checks and/or reflex testing. High-risk patients and results can be identified through clinical notes or drug/script alerts, and suspicious analytical results or patterns thereof. Certain biotin-sensitive tests can be used as markers of interference, to flag samples for further testing [117].

Chemical surveillance may be possible in the near future. Measuring biotin at the time of testing offers a means of identifying affected samples in real time, analogous to the measurement of haemolysis, icterus and lipaemia. At least two major manufacturers are presently developing methods for biotin measurement on main platforms. Liquid chromatography-mass spectrometry (LC-MS) is also an option, though rather than a real-time test for screening a high volume of samples, is generally better suited to confirmation of samples

triaged as suspicious through other means. Biotin measurement using LC-MS can also assist with validation of biotin depletion methods (see below).

Harnessing the pharmacokinetic parameters of biotin

Emerging pharmacokinetic data is valuable in guiding periods of washout that can be recommended before testing using biotin-affected platforms. Recommendations must be platform specific, due to wide variation in biotin tolerance, and manufacturer guidance would be valuable in this regard. An 8 h withdrawal period is required to reduce biotin to below 30 ng/mL in individuals on 10 mg daily dosing, which would suffice for many Roche assays [98], but would still exceed the tolerance of all affected Vitros assays. For MS patients on 100–300 mg biotin, further advice on adequate withdrawal periods is awaited. Whilst many laboratories currently recommend at least 48–72 h, longer periods are necessary for very sensitive tests [103]. Care is required, particularly as pharmacokinetic variability is likely [98], and scrutinising such results is prudent as an additional safety check. Further caveats must be borne in mind: (i) recommendations do not hold in renal impairment, where biotin clearance will be delayed; (ii) for some inborn errors of metabolism, withholding biotin may not be safe and (iii) washout is not an option in the acute setting.

Depletion of biotin

Biotin can be easily removed *in vitro*; two very similar protocols have been independently developed employing streptavidin-coated magnetic particles from affected assays [99,134]. In smaller studies, a similar approach has also been trialled using streptavidin-coated agarose or magnetic beads [24,124]. Such methods provide a useful means of verifying biotin interference. Whether results are reportable post *in vitro* biotin-depletion warrants further consideration, particularly for laboratories without access to alternative methods. This would constitute an *in vitro* diagnostic test and require comprehensive evaluation by laboratories. However once validated, these protocols endow laboratories with a means of analysing samples on their main platforms, with workable delay. For specific analytes and in specific settings, this may prove a pragmatic and acceptable approach; in this regard, the accuracy and precision of thyroid function test measurement has been shown to be unaffected by one depletion protocol [134]. The development of alternative methods that selectively neutralise or remove biotin without otherwise changing the sample matrix is presumably an area of commercial interest, and may be a future option.

Assay re-design to improve biotin tolerance

Close liaison between laboratories and manufacturers has facilitated improvement in immunoassay performance, leading to reduction in heterophile and anti-animal antibody interference [135,136]. It follows that recent reports of biotin interference will provide positive selection pressure for manufacturers to modify their assays and attenuate the biotin risk. It is likely that resistance to biotin interference will be a key criterion of immunoassays of the future.

Alternative methods

The use of biotin-unaffected platforms removes the potential for biotin interference, though of course no platform is resistant to interferences in general. Laboratories with access to multiple platforms may have scope to move certain tests to an unaffected method if deemed sufficiently high-risk. Many laboratories will not have such capability. Deliberate change of platform is not widely considered a necessary response to the threat of biotin interference [137], however in certain high-risk settings, contingency plans are required. For Emergency Departments served by biotin-sensitive troponin assays, access to a biotin-unaffected cardiac biomarker would be prudent in the rare event of a patient on high dose biotin therapy presenting with possible acute coronary syndrome.

CONCLUSION

Once the realm of fine print on package inserts, biotin interference is now occurring in routine practice, and has shifted from the theoretical to the empirical. The recent flurry of case reports has brought legitimate concern over safety, only reinforced by the FDA warning of a possible biotin-related fatality. This comes about through an unusual convergence: an experimental therapy and a cosmetic fad have collided with a trusted technology long in use in diagnostics. The biotin:streptavidin interaction has brought tangible improvement to immunoassay performance over the last three decades. At the time of its original incorporation into template immunoassays, the notion that biotin would one day

be prescribed at 10,000 times the average daily intake would have been inconceivable. So, too, the existence of cosmetic demand for grossly supra-physiological biotin intake in health, despite the absence of evidence for any benefit. Such changes in therapy and in consumer behavior have unmasked the latent risk of an assay principle favored by many manufacturers.

Although still rare in relation to total number of tests, the increased probability of biotin interference implies an increased risk of clinical harm. The obligations of the supplements industry and biotin producers in alerting to potential harm have been relatively overlooked in a discussion that has centered upon laboratories, *in vitro* diagnostic manufacturers and clinicians. Collective responsibility will ultimately ensure greatest protection for patients and consumers.

Laboratories using biotin-affected methods are obliged to evaluate their risk of biotin interference and implement commensurate mitigation strategies. Biotin deviates from many other interferences in that there are systematic steps that can be taken to reduce risk. General and targeted approaches are required, the latter directed to the groups at highest risk of clinical harm. Mitigation approaches that target the high risk individual at the time of highest risk are intuitively more robust. However, the juxtaposition of rare events and common clinical pathways is a recurring challenge in risk mitigation, and the layering of general approaches remains important. Arguably, the patients at highest risk of harm from biotin interference are those with MS on 300 mg daily dosing. Serum biotin concentrations exceeding 1000 ng/mL can occur in such patients, which are high enough to cause gross interference on every susceptible assay. Infants and children with inborn errors of metabolism also attain very high serum biotin concentrations and are at a similarly high risk. Most adults with biotin-responsive metabolic disease are at somewhat lower risk, with daily dosing under 20 mg typical.

The evidence base for efficacy of high dose biotin therapy in MS is preliminary, and it is not clear that it will remain an enduring therapy. However, the literature hints at other experimental applications of high dose biotin [117,138], and we have seen that the possibility of clinical benefit from a supplement considered safe may drive uptake even before a solid evidence base is established. Unfortunately, biotin's *in vivo* safety profile is not currently matched *in vitro*. A prescient response from manufacturers in re-engineering biotin: streptavidin assays to remove the risk of biotin interference is the ultimate long-term solution. This will ensure continued performance benefits of the biotin: streptavidin complex, without future risk of clinical harm from biotin interference.

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CHAPTER

9

Challenges in routine clinical chemistry testing analysis of small molecules

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INTRODUCTION

General or routine chemistry is the area of laboratory medicine dealing with measuring commonly ordered analytes, usually in serum or plasma, and is responsible for the highest volume of testing in clinical laboratories. In the author's medical center (Columbia New York Presbyterian Hospital), clinical chemistry tests account for 75% of the test volume in the clinical laboratory, with the top ten chemistry tests (creatinine, glucose, urea, calcium, potassium, chloride, sodium, carbon dioxide, total protein, and alkaline phosphatase) adding to about 45% of the total test volume. The ability to handle large volumes of testing is due to the availability of highly automated, high-throughput analyzers, often linked to a robotic laboratory automation system that identifies, processes, and distributes specimens to the analyzers with high efficiency.

The analytical methods used in routine chemistry include the following assay types:

1. Chemical methods use chemical reactions to produce a measurable product, most commonly yielding changes in color or optical absorbance proportional to the analyte levels. An example is the Jaffe reaction to measure creatinine, in which creatinine reacts with a chromogen to form a colored product.
2. Electrochemical reactions result in changes in redox potential or in exchange of electrons at electrodes with consequent current generation proportional to the analyte concentration. An example is an oxygen-sensitive electrode used to measure O_2 consumed in a glucose oxidase reaction, which is proportional to the glucose concentration.
3. Enzymatic assays use enzymes to measure analyte concentrations or substrates to measure enzyme levels. The action of the enzyme on the substrate generates a product that can be measured by a chemical method. Sometimes a cascade of enzymes is necessary to generate a measurable product. An example of an enzymatically measured analyte is creatinine, assayed by a cascade of creatininase, creatinase, sarcosine oxidase, and peroxidase enzymes. Examples of clinically useful enzyme analytes are alanine and aspartate aminotransferases, alkaline phosphatase, creatinine kinase, lactate dehydrogenase (LDH), lipase, and amylase.
4. Ligand assays use proteins with high affinity for the analyte to measure its concentration. Most often, these assays are immunoassays, in which antibodies or antibody fragments are used to bind the ligand, although in a few cases, other specific-binding proteins are used. Examples of immunoassays include drug testing and monitoring assays, measurements of cardiac biomarkers such as cardiac troponins and B-type natriuretic peptides, and assays for hormones and tumor biomarkers. Examples of ligand assays using non-antibody high-affinity binding proteins include assays for folates and vitamin B_{12}. Whereas many of the low-volume ligand assays are part of "specialized chemistry" often handled in separate areas of the clinical laboratory, with the advent of multifunctional high-throughput analyzers able to handle a multitude of assay methodologies, many of the high-volume immunoassays have migrated to the highly automated routine chemistry area.

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Some methods use combinations of these categories. For example, a glucose assay may use glucose oxidase to generate O_2, which is then measured by an oxygen-specific electrode in an electrochemical reaction. In this chapter, frequent sources of inaccurate results in commonly ordered small molecule analytes, focusing on common pre-analytical variables (Table 9.1) and analytical interferences (Table 9.2), are addressed. In Chapter 10, common errors in enzymes and other protein assays are reviewed. Chapters 11 and 12 focus on challenges in endocrinology testing and tumor markers testing, respectively. In many institutions, common therapeutic drug levels, as well as initial toxicology screens, are done in the core laboratory section by high-throughput analyzers and immunoassays, and these are reviewed in Chapters 13–17. Comprehensive listings of pre-analytical variables and interferences in clinical chemistry testing are available in reference books [1–3].

The most commonly ordered tests in clinical chemistry are often ordered as panels. The Centers for Medicare and Medicaid Services, formerly called Health Care Financing Administration (HCFA), recognizes six panels of tests delineated by the American Medical Association CPT editorial board (Table 9.3), of which the Basic Metabolic Panel (BMP) comprising five electrolytes (sodium, potassium, chloride, carbon dioxide, and calcium) and three small organic analytes (glucose, urea, and creatinine) is the most commonly ordered panel of tests. Note that all the test components of a panel need to be medically justified for the panel to be reimbursed.

CREATININE ANALYSIS

The small molecule creatinine is a product of the catabolism of creatine phosphate, a major source of rapidly available reserve energy, particularly in striated muscle and brain. Creatine phosphate can donate a phosphate group to adenosine diphosphate (ADP) to form adenosine triphosphate (ATP), a reaction catalyzed by creatine phosphokinase (CPK). Both creatine phosphate and creatine are converted non-enzymatically to creatinine at an almost steady rate of 2% per day [4].

Striated muscle and neural tissues have the highest requirements for quickly available energy and, therefore, the highest concentrations of creatine. Given the much larger volume of skeletal muscle, this tissue is the source of up to 94% of creatinine [4], and in healthy individuals the blood levels of both creatinine and CPK are proportional to skeletal muscle mass.

TABLE 9.1 Common pre-analytical sources of variation in small molecule analytes[a].

	Na	K	Mg	iCa	Ca	Cl	CO_2	Phos	Glu	Bili	Lac	NH_4	Trig	Chol	LDL-C	HDL-C	UA	BUN	CR
Elderly	=	=	=	↓/=	=	=	=	=/↓	=/↑	=			=	=	=/↑	=/↓	=/↑	=/↑	↑
Female/male			=	=/↓	=			=/↑	↓	↑	↓		↓	↓	↓	↑	=/↓	↓	↓
Meals	=	0–5	=	=	=	=	4	15	50	16			78	=	=	=	0–3	=	0–50
Starvation/ malnutrition	=	=/↓				=	↓		↓	240		17	↓35	↓10			5–20	↓20	0–20
Obesity	=	=		=	=		↓	=/↓	=/↑		↑		↑	=/↑	=	↓	↑		
Exercise	0–3	Var	Var	=/↑	Var	=/↑	=/↓	Var	Var	↑	↑	300	Var	Var	Var	↑	↑	Var	=/↑
Caffeine		↓		↑			↓		↑					=	=	=			
Smoking	=								=	↑		↑	=/↑	Var	Var	↓	=	↓	=
Alcoholism	=/↑	↓	Var	↓			↓	↓	Var	↑	↑		20	10	↓40	↑	=/↑		↓
Pregnancy	=	=	↓10	↓	↓10	=	↓	Var	=/↓	=/↓		=	↑	↑	↑	↑	Var	↓	=/↓
Menstruation	↓		5			↑	↓	↓					=	↑		=			
Anxiety									↑		↑		↑	↑		=		↑	=
Supine to upright	=	=	4	=	5–10	=	=	=	=			=	12	10	10	10	=	=	=
Tourniquet	3	↑	↑	=/↑	3		↓4	↓3	↓4	8	↑	↑	7	5–20					
Serum/plasma	=	5	=	↓	=	=	=	2–8	=	=	5–20	30	=	=	=	=	=	=	=
Storage of whole blood at room temperature	=	↑	10	Var	↓3	↓	↓	=/↑	↓	=	↑	↑	=/↓	=	=	=	=	=	=

Var, variable effect, depending on the study. For details, see Refs. [1,2].

[a]*Numbers represent typical percentage increase or decrease due to the pre-analytical variable.*

TABLE 9.2 Common interferants in small molecule chemistry assays[a].

Strong redox agents	**Urea**	**Sodium, potassium**
Acetaminophen	Ammonium	Strong redox agents
Acetoacetate	Fluoride	**Chloride**
Ascorbic acid	Hemolysis	β-Hydroxybutyrate
Azide	Levodopa	Bicarbonate
Bilirubin	Methylbenzethonium	Bromide
Bromide	**Uric acid**	Heparin
Catecholamines	Bilirubin	Iodide
Cysteine	Borate	Lactate
Diatrizoate	Hemolysis	Salicylates
X-ray contrast agents	Strong redox agents	Strong redox agents
Dopamine	Theophylline metabolites	Thiocyanate
Gentisic acid	Xanthine	**Total CO_2**
Glutathione	**Ammonia**	Hemoglobin > 800 mg/dL
Guanidine	Alanine aminotransferase	Bilirubin >30 mg/dL
Hydralazine	Glucose >600 mg/dL	Paraproteins
Hydroxyurea	Hemolysis	**Total calcium**
Iodide	**Bilirubin**	Bilirubin
Isoniazid	Amphotericin B	Calcium contaminants
Lactate	Levodopa	Citrate
Methylene blue	Methotrexate	EDTA
N-acetylcysteine	Nitrofurantoin	Hemolysis
Nitrates	Naproxen	Lipemia
Procainamide	Sulfasalazine	Oxalate
Sulfasalazine	Hemoglobin	**Total magnesium**
Tetracycline	Light	Calcium
Thiocyanate	Strong redox agents	Heparin
Uric acid	Paraproteins	**Phosphate**
Glucose	Lipemia	Alteplase
Bilirubin	**Cholesterol**	Bilirubin
Fructose	Strong redox agents	Fluoride
Galactose	**HDL cholesterol**	Hemolysis
Hemoglobin	Strong redox agents	Heparin
Hemolysis	Paraproteins	Lipemia
Icodextrine	Triglycerides	Liposomal amphotericin B
Lipemia	**Triglycerides**	Mannitol
Maltose	Strong redox agents	Paraproteins
Mannitol	Heparin	Tissue plasminogen activator
Mannose	Glycerol	
Oxygen levels		
pH levels		
Strong redox agents		
Xylose		
L-Lactate		
Citrate		
D-Lactate		
Glycolate		
Glyoxylate		
Oxalate		

[a]*Creatinine interferents are shown in Table 9.4. For details, see text.*

Creatinine is widely used as a marker of renal function—specifically as a surrogate for the glomerular filtration rate (GFR). The GFR is the volume of urine filtered by the glomeruli per unit of time. Clearance of a substance is defined as the amount of plasma cleared of that substance per minute, and it can be calculated by the following formula:

$$\text{clearance} = \frac{U \times V}{P} \tag{9.1}$$

where U is the urinary concentration, P is the plasma concentration, and V is the flow of urine in milliliters per minute. Clearance equals the GFR if the following assumptions are correct for the substance:

1. Production and plasma concentrations are constant.
2. There is no metabolism of the substance.
3. Excretion is exclusively by renal filtration, without secretion or reabsorption.

Whereas these assumptions are mostly correct for creatinine in healthy individuals, they may become less valid in patients with impaired renal function, leading to lower than expected plasma creatinine levels and overestimation of the GFR. It has been estimated that 16–66% of the creatinine formed in patients with chronic renal failure is subject to metabolism, mostly by reconversion to creatine [4]. Tubular secretion accounts for only 7–10% of the creatinine excretion in healthy individuals, but it increases proportionally to the degree of creatinine levels. Likewise, extra-renal excretion, particularly by degradation mediated by fecal bacteria, is increased in patients with chronic renal failure. Conversely, creatinine production decreases with higher blood levels of creatinine, possibly due to feedback inhibition of creatine synthesis. Despite these limitations, creatinine measurement in the plasma is one of the most widely used tests in clinical chemistry and a cost-effective approach to screening and evaluating kidney disease.

Creatinine clearance can be calculated by measuring both plasma and urine creatinine concentrations together with an assessment of urinary flow, usually by measuring the volume of urine excreted per 24 h. Although directly measuring creatinine clearance should be a more accurate estimation of the GFR, in practice it suffers from inaccuracy resulting from compounding the errors of three measurements and from difficulties in properly timing urine collection.

Interestingly, estimation of the GFR based on measurement of plasma creatinine alone has generally shown better reproducibility and more accurate estimation of GFR than creatinine clearance. A widely used formula recommended by the National Kidney Foundation is the Modification of Diet in Kidney Disease (MDRD) equation that takes into consideration, in addition to plasma creatinine, age, sex, and race (Caucasian vs. African American) [5]. The use of age, sex, and race accounts for the influence of these factors in creatinine production, mainly through varying average muscle mass. The estimated GFR (eGFR)

TABLE 9.3 Automated multichannel chemistry panels recognized by HCFA/CMS.

Test	Test abbreviation	Basic metabolic panel	Comprehensive metabolic panel	Electrolyte panel	Renal function panel	Hepatic (liver) function panel	Lipid panel
Panel abbreviation		BMP	CMP	Lytes	RFP	LFP, LFT	
CPT Code		80,047[b] 80,048[c]	80,053	80,051	80,069	80,076	80,061
Creatinine	Cr	X	X		X		
Urea nitrogen	BUN	X	X		X		
Glucose	GLU	X	X		X		
Sodium	Na	X	X	X	X		
Potassium	K	X	X	X	X		
Chloride	Cl	X	X	X	X		
Carbon dioxide	CO_2	X	X	X	X		
Anion gap	AG	[a]	[a]	[a]	[a]		
Calcium, ionized	iCa	X[b]					
Calcium	Ca	X[c]	X		X		
Phosphate	P				X		
Albumin	Alb		X		X	X	
Total protein	TP		X			X	
Alkaline phosphatase	ALP		X			X	
Alanine aminotransferase	ALT		X			X	
Aspartate aminotransferase	AST		X			X	
Total bilirubin	TBili		X			X	
Direct bilirubin	DBili					X	
Triglycerides	TG						X
Total cholesterol	TC						X
High-density lipoprotein cholesterol	HDL-C						X
Low-density lipoprotein cholesterol	LDL-C						[a]
Non-HDL cholesterol (calculated)	Non-HDL-C						[a]
CPT code		80,048	80,053	80,051	80,069	80,076	80,061

[a] *Calculated parameters. LDL-C may also be determined by a direct measurement.*
[b] *Basic metabolic panel (Calcium, ionized).*
[c] *Basic metabolic panel (Calcium, total).*

is calculated by the following formula, where serum creatinine (SCr) is reported in milligrams per deciliter:

$$\text{eGFR}\left(\text{mL/min/1.73 m}^2\right) = 175 \times (\text{sCr})^{-1.154} \times (0.742 \text{ if female}) \times (1.212 \text{ if African American}) \quad (9.2)$$

This formula is valid for individuals 18 years or older. For children, an alternative formula should be used to calculate eGFR—most commonly the revised Schwartz equation [6]:

$$\text{eGFR}\left(\text{mL/min/1.73 m}^2\right) = (0.41 \times \text{height in cm})/\text{sCr} \quad (9.3)$$

To avoid rounding errors, creatinine results should be reported to two decimal places (e.g., 1.25 mg/dL). Note that these formulas use only one laboratory measurement, sCr, and are normalized to the average adult body surface area (BSA) of 1.73 m^2. Although increased BSA is typically associated with larger muscle mass, and therefore higher creatinine production, it also correlates with larger kidney size and thus increased GFR. These two factors tend to cancel each other out, resulting in a relatively linear inverse correlation of serum creatinine levels and BSA-normalized GFR.

This MDRD formula is applicable to isotope dilution mass spectrometry (IDMS)-traceable creatinine assays. IDMS methods are considered the gold standard for creatinine measurement and suffer from less interference than other methods, but they are available only in a few laboratories. However, the use of IDMS assayed calibrators based on an international standard (SRM 967: Creatinine in Frozen Human Serum), created by the National Institute for Standards and Technology, allows different methods to be "traceable" to the gold standard method, thus improving accuracy and standardization of the various creatinine assays. In general, compared to non-IDMS-traceable methods, IDMS-traceable methods show lower results, as expected from higher specificity of mass spectrometry for creatinine. The coefficient of the MDRD formula was changed from 186, which was based on non-IDMS-traceable creatine assays, to 175 to account for the lower creatinine levels measured with IDMS-traceable methods. Because the GFR is inversely proportional to sCr, the revised formula results in comparable eGFR. Currently, most laboratories in the United States use IDMS-traceable calibrators and the revised MDRD formula.

Limitations of the MDRD equation

There are certain limitations of the MDRD equation:

1. The formula underestimates the true GFR in the normal and near normal range. If the eGFR is greater than 60 mL/min/1.73 m^2, laboratories should not report the numeric calculated measurement instead they should report eGFR as ">60 mL/min/1.73 m^2" Note that all creatinine-based screening methods miss patients with mild degrees of renal damage (chronic kidney disease category 2; GFR = 60−89 mL/min/1.73 m^2). In this range, large changes in GFR result in small changes in creatinine, usually within the reference range. In contrast, with severe renal failure, small changes in GFR result in large changes in creatinine levels.
2. As previously stated, this equation applies only to individuals 18 years or older. Although the original study did not include patients older than 70 years, the formula is considered useful in older individuals as a screen for renal disease.
3. In general, the MDRD equation is a more accurate estimation of GFR than directly measured creatinine clearance. However, carefully measured creatinine clearance or an alternative method of GFR estimation, such as inulin clearance, is preferable in patients with the following:
   a. Unusual dietary intake (vegetarian, low-meat diet, Atkins diet, and creatine supplements). Meat creatine is converted to creatinine by cooking.
   b. Unusual creatinine production due to advanced liver insufficiency or in individuals with extreme body size or muscle mass, including body builders, severe obesity, and muscle wasting or loss (e.g., severe malnutrition, cachexia, frailty, amputation, paraplegia, rhabdomyolysis, muscle dystrophy, and other neuromuscular disorders).
   c. Treatment with drugs that inhibit creatinine secretion, such as cimetidine, ranitidine, trimethoprim, pyrimethamine, dapsone, amiodarone, dronedarone, tyrosine kinase inhibitors such as alectinib, crizotinib, gefitinib, imatinib, pazopanib, sorafenib, and sunitinib, and possibly phenacemide, salicylates, and vitamin D derivatives [7]. Normally, tubular secretion accounts for approximately 10% of creatinine excretion and the use of drugs that inhibit secretion can cause small increases in serum creatinine without an actual decrease in GFR. Note that the MDRD equation was derived in chronic renal failure patients with presumably higher degrees of tubular secretion of creatinine; therefore, the underestimation of GFR may be larger than 10%. Tubular secretion of creatinine is particularly elevated in recipients of kidney transplantation, leading to poor performance of the MDRD equation in estimating GFR in these patients [8].
   d. The MDRD equation, like all creatinine-based estimates of GFR, is applicable only to individuals

with stable creatinine levels. It does not apply to patients with rapidly changing creatinine levels, including pregnant women and acutely ill patients, particularly those with acute renal failure.

4. Most currently available drug dosage guidelines are based on GFR estimations using older approaches, such as creatinine clearance or the Cockcroft–Gault formula, which is based on non-IDMS- traceable methods. However, a large study concluded that there is little difference between drug dosages calculated with the various methods of GFR estimation [9], leading the National Kidney Disease Education Program to recommend that for most patients, the eGFR based on the MDRD (or based on the Cockcroft–Gault equation) should be used. If using the MDRD-based eGFR in very large or very small patients, the eGFR (reported in mL/min/1.73 m^2) should be multiplied by the patient's BSA (in m^2) to obtain the patient's individual estimated GFR (in mL/min).

Recently, the new Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula using a 2-slope spline relationship between creatinine levels and GFR [10] has been shown to be more accurate than the MDRD equation at GFR levels >60/mL/min/1.73 m^2 and has been recommended by the National Kidney Foundation for GFR estimation.

Other approaches use cystatin C, a 13.3 kDa polypeptide removed from the plasma by glomerular filtration, to estimate GFR independently of protein intake and less dependent on muscle mass, age, gender, and ethnicity. Formulas for GFR estimation are available but have not demonstrated superiority over creatinine-based formulas, although the best estimation of GFR can be derived using both creatinine and cystatin C together [9].

Creatinine assay methods

Creatinine assays can be classified as chemical, based on the Jaffe reaction, or enzymatic methods.

Jaffe-based methods

In the Jaffe reaction, first described in 1886, picrate ions react with creatinine under alkaline conditions to yield an orange-red product absorbing in the 490- to 520-nm wavelength range. This reaction is not specific to creatinine, and more than 50 compounds have been described that can produce a similar chromogen with alkaline picrate ions. These substances, called non-creatinine chromogens, include proteins, ketoacids such as pyruvate, acetoacetate, ascorbic acid, glucose, cephalosporins, and other reducing substances, and they can account for up to 20% of the apparent creatinine concentration in the plasma of healthy individuals. Some of these compounds are increased in the blood of patients with chronic renal and liver disease, adding to the difficulties of accurately measuring creatinine and eGFR in these patients. Possibly the most clinically significant interferences with the Jaffe reaction occur in patients with diabetic or starvation-associated ketoacidosis, but the exact amount of interference varies with the particular creatinine assay used.

To reduce interference from non-creatinine chromogens, most modern assays in automated analyzers use a kinetic approach. Some non-creatinine chromogens, such as proteins, react quickly (within 20 s) with the picrate ions, whereas others, such as acetoacetate, react slowly and do not form significant amounts of chromogen until 60–100 s after the reaction is initiated. Chromogen formation measured between 20 and 60 s is mostly derived from creatinine; therefore, the rate of chromogen formation in this time period is used for measuring creatinine levels in current Jaffe-based assays. Further minimization of non-creatine chromogen interferences is achieved by careful optimization of reagent concentration, temperature, and measurement wavelengths. Another approach, the "compensated" Jaffe assay, automatically subtracts a fixed number (e.g., 0.20 mg/dL) from the final result to account for the average contribution of non-creatinine chromogens. However, this approach assumes that this contribution is constant and does not account for changes in the concentration of non-creatinine chromogen or sample matrix, such as in elderly individuals with lower protein concentrations and lower creatinine production. Because non-creatinine chromogens do not appear to interfere significantly when creatinine is assayed in urine, the compensation approaches should not be used when assaying creatinine in urine, at the risk of causing significant negative biases in urine creatinine and creatinine clearance calculations.

An often overlooked interference with Jaffe-type creatinine assays is the presence of hemoglobin F in the plasma/serum [11]. Most methods are insensitive to even significant degrees of hemolysis (e.g., 500 mg/dL of plasma-free hemoglobin) because hemoglobin A quickly converts to a brown pigment under alkaline conditions, which is blanked out of the reaction rate measurement. However, hemoglobin F is alkali resistant and slowly changes color in a Jaffe reaction. Individuals with high amounts of hemoglobin F, such as newborns and patients with thalassemias or hereditary persistence of hemoglobin F, can have significant interference in Jaffe-based creatinine assays when a hemolyzed sample is assayed.

Another major source of interference in the Jaffe reaction is bilirubin, in both the conjugated and the unconjugated form, which inhibits the reaction between

picrate and creatinine and results in falsely low creatinine measurements. Attempts to minimize the effect of this interference include deproteinization, which also removes bilirubin; blanked kinetic measurements; and the addition of buffering ions, surfactants, and ferrocyanide or bilirubin oxidase to convert bilirubin to biliverdin, which does not significantly interfere. With these modifications, modern Jaffe-based creatinine assays do not show significant interference from bilirubin levels less than 20–25 mg/dL.

Table 9.4 lists many of the substances reported to interfere with modern Jaffe-based creatinine assays. A complete medication list and consultation with the particular laboratory performing the assay is important to determine the occurrence and magnitude of possible interferences. Note that several of the interfering drugs are accumulated in the urine to levels much higher than in plasma, and therefore urine creatinine determinations may be more susceptible to error from a variety of interferents.

TABLE 9.4 Substances interfering with modern creatinine assays[a].

Interferent	Jaffe	Creatininase
5-Flurocytosine		Yes
Acetaminophen		Yes
Acetoacetate	Yes	
Acetohexamide	Yes	Yes
Acetone	Yes	
Acetylcysteine		Yes
Albumin (>4 g/dL)	Yes	
Ascorbate	Yes	Minimal
Bilirubin (>20 mg/dL)	Yes	Yes
Bromide (from halothane)		Yes
Catecholamines		Yes
Cefaclor	Yes	
Cefoxitin	Yes	
Cefpirome	Yes	
Cephalothin	Yes	
Cephazolin	Yes	
Creatine	Yes	Yes
Dipyrone (methimazole)		Yes
Dobesilate		Yes
Dobutamine		Yes
Dopamine		Yes
Fluorescein	Yes	
Flucytosine		Yes
Furosemide	Yes	Yes
Glucose (>300 mg/dL)	Yes	
Glutamine	Yes	
Glutathione	Yes	
Hemoglobin (>500 mg/dL)	Yes	Yes (Hgb > 900 mg/dL)
Hemoglobin F	Yes	
Hydroxyurea		Yes
Homogentisic acid		Yes
Intralipid (1000 mg/dL)	<10%	<10%
L-Dopa	Yes	Yes
Lidocaine		Yes
Methyldopa		Yes
Paraprotein	Yes	Yes
pCO_2		Yes[b]

Continued

Case reports

A 40-year-old woman with insulin-dependent diabetes mellitus was admitted for ketoacidosis [12]. On admission, her blood pressure was low (100/60 mmHg), heart rate was elevated (124 beats per minute), and respirations were fast (20/min) and deep, suggesting metabolic acidosis with respiratory compensation. Laboratory values at admission included hyperglycemia (592 mg/dL), hyponatremia (134 mmol/L), low chloride (90 mmol/L), low serum bicarbonate (6 mmol/L), and an elevated anion gap of 36 mmol/L, consistent with ketoacidosis. Blood urea nitrogen (BUN) was 18 mg/dL and creatinine was 3.2 mg/dL, resulting in an unusual BUN:creatinine ratio of 5.6. The normal BUN:creatinine ratio is between 10 and 20 and may be increased in cases of prerenal causes of acute renal injury, including gastrointestinal bleeding and dehydration, whereas intrarenal damage, such as acute tubular necrosis, may reduce urea reabsorption, thus lowering the BUN:creatinine ratio to less than 10. This patient had signs of dehydration, with decreased turgor and dry mucous membranes, and the BUN:creatinine ratio did not appear consistent with the clinical presentation. Absorption of creatinine with an exchange resin to purify it from interfering chromogens yielded a true creatinine concentration of 1.0 mg/dL at admission, in contrast with 3.2 mg/dL by an automated Jaffe method. Addition of acetoacetate, but not β-hydroxybutyrate, to normal serum in concentrations exceeding 2 mM resulted in a linear increase in creatinine levels with the automated Jaffe method, implicating acetoacetate in the falsely high creatinine results. In addition, it is possible that high glucose and acetone levels associated with

TABLE 9.4 Substances interfering with modern creatinine assays[a].—cont'd

Interferent	Jaffe	Creatininase
Proline	Yes	Yes
Protein (>12 g/dL or <3 g/dL)	<20%	
Pyruvate	Yes	
Rifampicin		Yes
Uric acid	Yes	

[a]*The various methods are grouped based on the Jaffe reaction or creatininase, and interferences are variable, depending on the specific method used. For complete information, consult the manufacturer's package insert, as well as reference books [1–3].*
[b]*iSTAT only; approximately 5% for each 10-mmHg deviation from 40 mmHg.*

ketoacidosis further contributed to spuriously high creatinine levels.

In addition to diabetes, ketoacidosis can be associated with fasting. A study of five individuals who fasted for 96 h showed an increase in mean serum creatinine levels from 1.0 to 1.7 mg/dL, corresponding to an average increase in acetoacetate levels of 0.03–1.39 mM [13]. The correlation between acetoacetate and creatinine measured by a Jaffe method was very high, with a coefficient of 0.95. No such correlation was seen with an enzymatic creatinine method, which was unaffected by acetoacetate.

Since these cases were reported in the 1980s, improvements have been made in the Jaffe reaction to minimize interference by acetoacetate, mainly by restricting the kinetic measurement to an optimal time range. Current Jaffe-based methods do not show significant interference with acetoacetate levels below 20 mM, although some rapid creatinine methods designed for STAT use, such the Beckman modular creatinine assay (CREAm), can show a positive bias of approximately 0.07 mg/dL for each 1 mM of acetoacetate elevation [14]. Notably, the Jaffe-based creatinine assay performed in the cuvette side of the Beckman DxC analyzer shows no interference with acetoacetate levels below 20 mM. Because acetoacetate levels in diabetic ketoacidosis range from 1.2 to 9 mM, averaging 3.5 ± 1.4 mM [12,15], this interference remains a problem with some creatinine assays.

A 6-year-old female had bone marrow transplant for acute myelogenous leukemia and developed severe graft-versus-host disease and severe hyperbilirubinemia, with plasma bilirubin reaching 55.3 mg/dL [16]. She was treated with hemodialysis for renal insufficiency secondary to a large bladder hematoma with obstructive uropathy. As part of assessing the efficiency of hemodialysis, the sieving coefficient, calculated from creatinine concentration in dialysate of 0.7 mg/dL divided by plasma creatinine of 0.3 mg/dL, equaled 2.33, which is clearly abnormal because 100% efficiency will result in a coefficient of 1.0. When the same calculation was applied to plasma and dialysate urea, the sieving coefficient was 1.0. The creatinine was initially measured with a Jaffe based method, and a repeat measurement using an enzymatic method resulted in both plasma and dialysate creatinine levels of 0.7 mg/dL and therefore a sieving coefficient of 1.0. This case highlights the susceptibility of the Jaffe-based assays to negative interference by high levels of bilirubin, and the potential for overestimation of hemodialysis efficiency, because the measurement in the plasma, but not the dialysate, is susceptible to this interference. It is not clear why bilirubin does not cross the dialysis membrane, although binding of bilirubin to albumin and electrostatic repellence between negative charges in both conjugated bilirubin and membrane are possible explanations.

Enzymatic creatinine assays

To obviate the problems with the Jaffe-based methods and increase specificity for creatinine, several assays use enzymatic reactions to obtain a measurable product. Two enzymes can act on creatinine: creatininase, which converts creatinine to creatine, and creatinine deiminase, which generates *N*-methyl hydantoin and NH_3. To convert one of these products to a photometrically measurable substance, a cascade of other enzymes must be used. For example, in the most commonly used method, creatininase generates creatine and creatinase converts creatine to sarcosine, which in turn generates hydrogen peroxide and formaldehyde after treatment with sarcosine oxidase. Subsequently, peroxidase catalyzes the oxidation of a chromogen to a colored product by H_2O_2.

Whole blood analyzers, such as the Abbott iSTAT, Radiometer ABL800, and Nova StatSensor, use a similar creatininase/creatinase/sarcosine oxidase cascade and electrodes that generate current upon oxidation by H_2O_2 to measure creatinine in whole blood. In the case of the Radiometer, two electrodes are used: One electrode is associated with the full cascade of enzymes and measures H_2O_2 generation from creatinine, whereas a second electrode omits the creatininase enzyme and measures background H_2O_2 generation from creatine and other substances, thus significantly minimizing interferences [17]. Rapid creatinine measurements in whole blood analyzers have become increasingly important in the expedite screening of patients undergoing emergency radiologic procedures using contrast dyes, as a mechanism to prevent contrast-induced nephropathy (CIN), because patients with eGFR less than 60 mL/min/1.73 m^2 are at higher risk of CIN. In addition, these amperometric methods can be used when high bilirubin or hemolysis interferes with photometric Jaffe-based or enzymatic creatinine assays.

Another approach used in automated analyzers to minimize interference by reaction intermediates is to

measure reaction rates with all enzymes but without creatininase and then add creatininase to measure creatinine-dependent chromogen generation. As with other hydrogen peroxide (H_2O_2)-based methods, the results of enzymatic creatinine assays that generate H_2O_2 are subject to interference by strong reducers, such as ascorbic acid and bilirubin, which compete with the chromogen for H_2O_2. Interference by ascorbic acid can be overcome by ascorbate oxidase, whereas bilirubin oxidase or ferrocyanide can minimize interference by bilirubin.

Alternatively to using peroxidase and measuring H_2O_2 generation, another method uses formaldehyde dehydrogenase to oxidize formaldehyde to acetic acid while converting nicotinamide adenine dinucleotide (NAD^+) to NADH, which can be measured by absorption at 340 nm. In another H_2O_2-free approach, creatinine deiminase converts creatinine to *N*-methylhydantoin and ammonia, and either of these compounds can be converted by subsequent steps into a chromogen. The simpler methods use glutamate dehydrogenase to convert ammonia and α-ketoglutarate to glutamate while oxidizing NADH to NAD^+. The use of recombinant creatinine deaminase from the bacterium *Tissierella creatinini* has improved the specificity of the assay because this enzyme is highly specific for creatinine, in contrast to creatinine deiminase from other sources that can react with cytosine and related compounds. However, none of these methods have found widespread routine use in clinical laboratories.

Most interfering substances reported with the Jaffe methods do not significantly interfere with enzymatic creatinine assays at physiologic or therapeutic levels, with the exception of catecholamines, carbidopa, levodopa, α-methyldopa, rifampicin, and calcium dobesilate (Doxium and Dobest), a vasoprotective drug used in the treatment of diabetic retinopathy and chronic venous insufficiency (see Table 9.4). Interference from high levels of homogentisic acid after the laboratory switched from a Jaffe-based to an enzymatic creatinine assay has been recently reported in a case of a patient with alcaptonuria [18].

Despite increased accuracy and decreased susceptibility to interferences, enzymatic creatine assays have not been widely implemented, in part due to their 5−10 fold higher costs compared to Jaffe-based methods. To minimize the extra cost of the enzymatic creatinine test, Lehman et al. proposed a cost-effective strategy involving routine use of the Jaffe-based test with the enzymatic test performed on outpatients deemed at high risk for interferences (e.g., transplant and endocrinology clinics) or reflexed from Jaffe-based eGFR results between 55 and 65 mL/min/1.73 m^2 in low-risk outpatients [19].

Case report

A 36-year-old male with severe heart failure and progressive renal failure had initial creatinine levels of 4.6, 4.4, and 4.3 mg/dL in the first 2 days of admission to the hospital. Given the cardiovascular situation, the patient was transferred to the intensive care unit (ICU) and treated with dopamine to improve blood flow and kidney function. The creatinine values in the next 2 days in the ICU fluctuated more than 25% with no correlation to the patient's unchanged clinical status. When the samples were analyzed by both the Roche enzymatic assay and a Jaffe-based method, spurious decreases in creatinine levels were seen with fresh samples tested with the Roche method but not with samples stored for more than 24 h. When dopamine was added at a molar ration of 0.25:1 to creatinine, the negative bias was −67% with the Roche enzymatic assay, −13% with the Vitros enzymatic assay, and between −3 and −5% with three Jaffe-based methods [20]. It is well recognized that strong reducing substances, such as dopamine, dobutamine, and other catecholamines, can interfere with enzymatic reactions that use H_2O_2 and peroxidase as intermediate steps to color generation. This interference is due to either complex formation with the chromogen, in the case of dopamine and methods using 4-aminophenazone, or reactivity between the catecholamine and H_2O_2, with consequent depletion of the peroxide and falsely low results [21]. Since dobutamine and dopamine are often given through central venous catheters, it is recommended that in these patients monitoring of creatinine levels with enzymatic assays should be performed in blood samples obtained from peripheral veins. Instability of catecholamines explains why samples older than 24 h showed no negative interference by these substances [20].

UREA ANALYSIS

Urea is another substance excreted primarily (90%) by the kidney, and because it was initially measured as urea nitrogen, it is reported in the United States as BUN, even though all modern assays measure urea concentration and not nitrogen. To convert BUN to urea, in milligrams per deciliter, multiply by 2.14. Urea is formed by the urea cycle, the end pathway for disposal of ammonia mostly derived from protein catabolism. The enzymes of the urea cycle are most abundant in the liver, and they are present in the kidney and other tissues in much smaller amounts.

In contrast to creatinine, passive tubular reabsorption of urea is significant, particularly in situations of impaired urinary flow; such as with dehydration and reduced blood

volume, and therefore urea clearance is lower than the GFR. In situations of high urine flow, such as pregnancy, diuretic treatment, and hyperosmolar loads, urea reabsorption is minimal and urea clearance approaches the GFR. A similar situation is seen is advanced renal failure, with high osmotic diuresis. Despite the superiority of creatinine for estimation of GFR, the measurement of urea has a role in the following clinical situations:

1. When creatinine measurements are misleading due to interferences or physiologic situations, such as extremes of muscle mass, that affect the relationship of creatinine and GFR.
2. To help determine the mechanism of renal insufficiency by comparing the elevation in urea and creatinine, as reflected by the BUN:creatinine ratio.
   - **a.** Increased BUN:creatinine ratio
     - i. Prerenal disease, with hypovolemia and/or impaired kidney perfusion, leads to increased urea reabsorption and elevation of BUN disproportionally to the decrease in GFR and increase in creatinine, which often is in the normal range. Therefore, the BUN:creatinine ratio is elevated, typically greater than 20. Examples of systemic conditions associated with prerenal azotemia include bleeding, burns, shock, cirrhosis, congestive heart failure, anaphylaxis, dehydration, prolonged diarrhea, vomiting, and sweating. Examples of localized impairment of renal blood flow include aortic coarctation, aneurism, dissection, and renal artery occlusion by thromboembolism or stenosis.
     - ii. Conditions that increase urea production (high-protein diet, high catabolic states such as fever, infection, burns, hyperthyroidism, acute myocardial infarction, postsurgery (especially gastrointestinal surgery), anti-anabolic drugs such as tetracycline and corticosteroids, and gastrointestinal bleeding with absorption and catabolism of blood proteins) or decrease creatinine production (e.g., low muscle mass) can also increase the BUN:creatinine ratio. Due to these multifactorial effects, the BUN: creatinine ratio is of low value in discriminating the cause of renal insufficiency in acutely ill patients, although a high BUN: creatinine ratio is associated with worse outcomes in these patients [22,23].
     - iii. Postrenal disease, such as obstruction, stenosis, or reflux in the urinary outflow tract (ureters, bladder, and urethra), will also lead to reduced urinary flow and increases in both BUN and creatinine, with the BUN:creatinine ratio usually, but not always, greater than 15.
   - **b.** Decreased BUN:creatinine ratio (<10)
     - i. Conditions that decrease urea reabsorption: high urinary flow (e.g., pregnancy and osmotic diuresis), acute tubular necrosis, interstitial nephritis, and sickle cell disease.
     - ii. Conditions with decreased urea synthesis (negative nitrogen balance): severe liver disease, malnutrition, low protein intake, protein malabsorption (e.g., celiac disease), protein loss (nephrotic syndrome), increased protein synthesis (late pregnancy, childhood, convalescence, acromegaly, anabolic drugs such as androgens, growth hormone).
     - iii. Conditions with increased creatinine production (bodybuilders, rhabdomyolysis, etc.)
   - **c.** Normal BUN:creatinine ratio [10–20].
     - i. Most cases of renal failure
3. Urinary urea measurements, in conjunction with an assessment of protein intake, can be used as a crude estimation of proper nitrogen balance for nutritional purposes. For example, net nitrogen balance can be calculated by the following formula:

$$\text{Nitrogen balance} = \text{protein intake (g/day)}/6.25 - \text{urinary urea nitrogen (g/day)} + 4 \quad (9.4)$$

Urea assay methods

Virtually all current routine methods for urea measurement use urease to convert urea to ammonium and bicarbonate ions. The detection of ammonium formation, which is proportional to the concentration of urea, is achieved by one of the following methods:

1. Change in conductivity: The Beckman modular assay uses the rate of change in conductivity of the diluted sample when ammonium (NH_4^+), bicarbonate (HCO_3^-), and hydroxyl (OH^-) are generated by urease from non-ionic urea.
2. Ammonium-selective potentiometry: In some whole blood analyzers, urease is immobilized on a membrane, and the generation of ammonium is measured by current generated at the ammonium-selective electrode.
3. Reaction with quinolinium dye: The Ortho Vitros dry chemistry method uses an ammonium-permeable membrane to filter the ammonium generated by the urease reaction into the color-forming layer, where it reacts with a quinolinium chromogen, generating color measured by reflectance spectrometry at 670 nm.
4. Glutamate dehydrogenase: This enzyme converts ammonium and α-ketoglutarate to glutamate and in the process oxidizes NADH to NAD^+ with the consequent decrease in absorption at 340 nm.

In all these methods, an obvious interference to control for is endogenous or exogenous (anticoagulant) ammonium, which will cause an increase in urea corresponding to the ammonium concentration. Normally, the concentration of ammonia in plasma (10−200 μM) is much lower than the concentration of urea (1−10 mM), but it may become a problem in certain metabolic disorders, including those associated with liver dysfunction, and in acidic or bacterially contaminated urine. The problem can be solved by taking a sample blank reading with all reagents except urease.

High amounts of fluoride inhibit urease and may cause falsely low BUN results, but the amount present in gray-top tubes (∼2.5 mg/mL) does not interfere significantly. Likewise, ammonium heparin anticoagulant at 14 units/mL shows no significant interference with BUN.

Other interferences reported include levodopa (−3 mg/dL at 40 μg/mL) and methylbenzethonium chloride (−5 mg/dL at 0.5 mg/dL) for the modular Beckman assay. No significant interference is seen with hemolysis (at 500−800 mg/dL hemoglobin), bilirubin (30−100 mg/dL), and lipemia (500 mg/dL Intralipid) in various BUN assays. The Vitros assay shows a bias of 1.1 mg/dL with 500 mg/dL of plasma hemoglobin.

AMMONIA ASSAY

Ammonia is generated in the body predominantly from the action of intestinal bacteria, which deaminate urea and amino acids. In patients with gastric *Helicobacter pylori* infections, the urease activity of this organism can be an important source of circulating ammonia, particularly in patients with cirrhosis [24]. The ammonia generated in the intestine flows into the liver through the portal vein and is metabolized in the urea cycle. In excess, ammonia can be a potent neurotoxin.

Plasma ammonia levels are measured most commonly in two clinical situations: to monitor metabolic disorders in infants, such as deficiencies in urea cycle enzyme and related pathways, and in advanced liver disease to monitor the risk of hepatic encephalopathy. The latter indication is controversial because the correlation between ammonia levels and encephalopathy is poor, and routine measurement of plasma ammonia is not recommended in patients with known liver disease, although it may be useful in patients with encephalopathy of unclear etiology [25].

Pre-analytical factors

Diets with high protein content may result in increased ammonia generation, mainly reflected by increased urinary excretion, whereas a 7 day 33% restriction in calories decreased ammonia by 17% [26]. Exercise increases ammonia generation approximately threefold [25]. Subjects should abstain from smoking because it increases ammonia blood levels by approximately 10 μmol/L/h [25]. Certain drugs, such as ammonium salts, barbiturates, opioids, valproic acid, and asparaginase, can increase ammonia generation. Patients treated with fluids containing glutamine, such as for irrigation post-transurethral prostatectomy, can have significant increases in ammonemia [25].

Clenching fist, prolonged tourniquet application, and improper cleaning of the skin during blood collection can result in spuriously high ammonia. Of particular concern are capillary samples collected from infants, in which sweat contamination and higher frequency of hemolysis and tissue damage can artificially increase ammonia in the sample [25,27]. Arterial ammonia levels are higher than venous and correlate better with liver function; therefore, arterial specimens are preferable in this condition [25].

Blood samples for ammonia analysis should be collected in tubes containing ethylenediaminetetraacetic acid (EDTA) or heparin as anticoagulant, and obviously ammonium heparin or ammonium oxalate should not be used. Serum is not recommended because ammonia is produced by platelet lysis during the clotting process. The tubes containing the blood samples should be placed immediately on ice slurry and centrifuged as soon as possible to prevent generation of ammonia *in vitro*, 90% of which originates from red cells, platelets, and leukocytes [25,28]. In healthy individuals, ammonia accumulates in whole blood at a rate of approximately 4 μmol/L/h, compared to 5 and 25 μmol/L/h at 20° and 37 °C, respectively [28]. Analysis should proceed expeditiously because ammonia levels increase in plasma even at 0 °C. After cellular separation, ammonia accumulates in plasma at a rate of approximately 0.4 μmol/L/h [29]. Conditions associated with elevated levels of γ-glutamyl transferase (GGT) in the plasma, such as alcoholism and hepatobiliary diseases, can have up to a 35-fold increase in the rate of ammonia generated *in vitro* from glutamine and peptide deamidation in the plasma, even at 0 °C [29]. The addition of 2 mM borate and 5 mM serine to inhibit GGT can prevent ammonia generation in patients with high levels of GGT [29]. The Urea Cycle Disorders Conference Group issued a consensus statement suggesting that blood should be collected in a pre-chilled ammonia-free tube, kept on ice, and centrifuged with 15 min of collection [27]. Plasma should be frozen at −70 °C if not assayed immediately. Collection of capillary samples and delay in processing the samples were found to be the most common causes of falsely elevated ammonia results in children, which occurred in approximately

28% of the patients with values higher than the reference range [27].

Assay methodology for ammonia

The most commonly used methods for ammonia determination use glutamate dehydrogenase to convert ammonia and α-ketoglutarate to glutamate while oxidizing NADPH (or NADH) to $NADP^+$ (or NAD^+) with a resulting decrease in absorption at 340 nm. Alternatively, in the Vitros dry chemistry method, ammonia is isolated from the rest of the sample solution with a semipermeable membrane that subsequently reacts with bromophenol blue, causing a change in reflection density measured at 600 nm.

Contamination of laboratory air or containers with ammonia, e.g., from cleaning products, may cause an artificial increase in ammonia levels. Hemolyzed samples are not recommended because of higher rates of spontaneous ammonia generation *in vitro*. Glucose at 600 mg/dL (33.3 mM) can cause a decrease of 8–40 μmol/L in ammonia concentration using the Vitros method. Intravenous treatment with vitamin K can lead to falsely low ammonia results in patients with liver dysfunction [30]. High levels of alanine aminotransferase have been reported to cause a positive interference in enzymatic ammonia assays in patients with liver failure, possibly by catalyzing the conversion of the alanine to pyruvate, which is then converted by LDH into lactate, consuming NADH [31]. Sample blanking and/or deproteinization is recommended to correct for interference caused by dehydrogenases and other substances in the plasma able to oxidize NAP(P)H [32,33]. Interestingly, methods using NADPH are much less susceptible to nonspecific interferences than those using NADH [34].

URIC ACID ANALYSIS

Uric acid is the product of nucleic acid catabolism, as a major breakdown product of purine nucleosides. Accumulation of uric acid in joints leads to inflammatory arthritis known as gout, whereas excessive uric acid excretion can lead to urate nephropathy and kidney stones. The final step in uric acid formation is the conversion of xanthine to uric acid catalyzed by xanthine oxidase, and inhibition of this enzyme by allopurinol can be effective in reducing circulating uric acid levels. Uric acid is excreted by the kidneys by distal tubular secretion, a process that can be enhanced by uricosuric drugs such as probenecid, sulfinpyrazone, azapropazone, tiaprofenate, and several others and inhibited by diuretics and organic acids.

Causes of hyperuricemia include several congenital metabolic disorders, such as Lesch–Nyhan syndrome resulting from deficiency of hypoxanthine-guaninephosphoribosyltransferase, excess purine intake (e.g., red meat, liver, kidney, and sardines), excess nucleic acid turnover (leukemias, myeloma, tumor lysis syndrome, trauma, and excessive exercise), or decreased excretion caused by renal disease or inhibitors of urate secretion (organic acids including lactate and acetoacetate, salicylates, thiazides, and lead poisoning). Interestingly, prolonged starvation and other ketoacidosis states can lead to more than a 20% increase in uricemia, due to a combination of decreased intake and increased catabolism of nucleic acids, and decreased excretion due to inhibition by ketoacids [35]. In the case of acute alcohol ingestion, the associated increased purine catabolism, dehydration, and lactic acidosis further contributes to increased uricemia [36].

Causes of hypouricemia include decreased synthesis (deficiency or inhibition of xanthine oxidase or its molybdenum co-factor, severe liver disease, purine synthesis inhibitors such as 6-mercaptopurine, and azathioprine) and decreased renal reabsorption (Fanconi syndrome, interstitial nephritis, radiologic contrast nephropathy, and overtreatment with uricosuric drugs).

Analytical considerations for uric acid analysis

Virtually all routine assays for uric acid use uricase to convert uric acid to allantoin with generation of H_2O_2 and CO_2 followed by peroxidase-catalyzed oxidation of a chromogen by the H_2O_2. As with other peroxidase-based methods, interference by ascorbic acid and bilirubin is common. For example, the Beckman assay shows a negative bias of 10% with 10 mg/dL of bilirubin and of 0.3 mg/dL with 1.5 mg/dL of ascorbate. The Vitros dry chemistry method obviates bilirubin interference (up to 27 mg/dL) by removal of proteins from the color-forming layer, but it is still affected by high ascorbate levels (−17% bias at 100 mg/dL). In the Roche method, ascorbate oxidase is used to reduce interference by ascorbic acid. Other interferants reported with uricase/peroxidase methods include hemolysis, theophylline metabolites, catecholamines, methylene blue, sulfasalazine, gentisic acid, hydralazine, and other reducing substances (see also the section on creatinine). Borate preservatives, sodium azide, and ascorbate in urine interfere with the urease reaction and are frequent causes of misleading urinary urate measurements [37]. Urine pH less than 8.0 can result in urate precipitation and inaccurate urinary urate measurement.

Case report

A 30-year-old patient with Burkitt's lymphoma was hospitalized for dyspnea, night sweats, fatigue, and early satiety [38]. Tumor staging indicated the patient was in stage D. The patient's admission serum uric acid levels were 14.1 mg/dL, and the patient was treated

for 3 days with allopurinol, resulting in a decline of uricemia to 5.1 mg/dL. After a single dose of cyclophosphamide treatment, resulting in rapid tumor lysis and disappearance of his chest masses in 2 days, uric acid levels increased to approximately 10 mg/dL as measured by a chemical method but only 6 mg/dL as measured by a uricase method. The discrepancy correlated with high serum levels of xanthine, an inhibitor of the uricase reaction, which increased from a pre-chemotherapy level of 10 mg/dL to a peak of 140 mg/dL. Normally, xanthine is rapidly cleared by the kidney, but in cases of rapid tumor lysis in which conversion to uric acid is inhibited by allopurinol, it can reach higher levels, especially if associated with renal insufficiency, and cause falsely low uric acid measurements with uricase methods.

GLUCOSE ANALYSIS

When point-of-care (POC) and home testing assays are included, glucose represents the most frequently measured analyte in clinical settings. As the most important source of energy for most tissues, including the brain, levels of glucose in the blood is highly regulated and deviations from normality have severe consequences. Hypoglycemia is defined as blood glucose levels less than 45 mg/dL or by the Whipple's triad of low glycemia (<70 mg/dL) with symptoms of hypoglycemia (palpitations, sweating, tremors, weakness, headache, fainting, etc.) and disappearance of the symptoms when glucose is normalized. Hypoglycemia can have many causes:

1. Hyperinsulinic hypoglycemia, characterized by low ketones and free fatty acids
   a. High-insulin secretion (associated with high levels of C-peptide), which includes drugs (sulfonylurea, meglitides, quinine, and pentamidine), insulinoma, islet cell dysplasia, and very rarely β cell-stimulating autoantibodies.
   b. Low-insulin secretion (low C-peptide): excessive exogenous insulin administration, anti-insulin antibodies, insulinomimetic antibodies, and ectopic insulin-like growth factor production by tumors.
2. Non-hyperinsulinic hypoglycemia
   a. With elevated free fatty acids and ketones: Failure to produce or obtain glucose or excess consumption of glucose leads to increased fatty acid oxidation and ketonemia—drugs (ethanol, salicylates, and propranolol), severe liver disease, hormone deficiency (growth hormone, cortisol, thyroid, and pituitary), extrapancreatic malignancy, and malnutrition, inborn errors metabolism (galactosemia, fructosemia, glycogen storage disorders, gluconeogenesis defects, various organic acidurias, etc.).
   b. Inborn diseases of fatty acid oxidation: Compensatory increase in glucose consumption results in hypoglycemia, low ketone levels, normal to elevated free fatty acids, and low acylcarnitine levels.

Hyperglycemia is usually a chronic disease caused by insulin deficiency with pancreatic β cell destruction (diabetes mellitus type 1) or peripheral insulin resistance, often associated with partial insulin deficiency (diabetes type 2). Rare inherited forms of diabetes include defects in pancreatic genesis, insulin secretion, and glucose sensing in the β cells, termed maturity-onset diabetes of the young and often involving transcription factors such as HNF1, HNF4, KLF1, PAX4, and IPF1; defects in insulin or its receptor genes; and as part of complex genetic disorders such as Down, Klinefelter, Turner, and Prader–Willi syndromes. Chronic hyperglycemia is also often associated with diseases of the exocrine pancreas (hemochromatosis, chronic pancreatitis, and cystic fibrosis), endocrinopathies (Cushing's syndrome, acromegaly, glucagonoma, pheochromocytoma, and hyperthyroidism), infections (congenital rubella or cytomegalovirus, coxsackievirus B, mumps, sepsis, and meningitis), drugs (thiazides, steroids, dilantin, statins, etc.), toxins (streptozotocin and other fungal toxins), and, rarely, anti-insulin receptor antibodies or the stiff-person syndrome with anti-glutamate-decarboxylase autoantibodies [39].

The American Diabetic Association (ADA) has defined criteria for diagnosis of diabetes mellitus, to include any of the following [39–41]:

1. Hemoglobin A1c (HbA1c) $\geq$ 6.5%
2. Fasting plasma glucose $\geq$126 mg/dL (fasting defined as no caloric intake for $\geq$8 h)
3. Oral glucose tolerance test (OGTT), with 75-g load of glucose, plasma glucose $\geq$ 200 mg/dL at 2 h
4. Random plasma glucose $\geq$ 200 mg/dL with classic symptoms of hyperglycemia (polyuria, polydipsia, or unexplained weight loss)

It is important to note that criteria 1–3 should be confirmed by repeated testing on a different day, unless the patient has two or more of the criteria or in cases of obvious diabetes, defined by plasma glucose > 500 mg/dL, HbA1c > 7.0%, ketoacidosis, nonketotic coma, and classic symptoms of hyperglycemia. In most cases, the OGTT is unnecessary to diagnose or exclude diabetes, as a combination of fasting glucose, random glucose, and HbA1c offers sufficient sensitivity and specificity.

Patients with intermediate values between the upper limit of the reference range and the ADA criteria are at risk for developing diabetes and its complications:

1. Hemoglobin A1c (HbA1c): 6.0−6.5%. Some experts advocate an HbA1c of 5.7−6.5% to define prediabetic risk [39].
2. Impaired fasting glucose: 100−125 mg/dL.
3. Impaired glucose tolerance—random plasma glucose or 2-h OGTT: 140−199 mg/dL.

For diagnosing gestational diabetes, more stringent criteria have been defined [39,41]:

1. Fasting plasma glucose ≥92 mg/dL.
2. OGTT with 75 g load: glucose ≥180 mg/dL at 1 h and ≥153 mg/dL at 2 h.
3. OGTT with 100 g load: glucose ≥ 180 mg/dL at 1 h, ≥ 155 mg/dL at 2 h, and ≥ 140 mg/dL at 3 h.

Pre-analytical considerations for glucose measurement

The ADA guidelines recommend fasting glucose as one of the screening tests for diabetes. Patients should abstain from solid food for at least 8 h but should maintain normal liquid intake, otherwise dehydration and hemoconcentration could artificially increase the measured glucose level. It is recommended that patients abstain from alcohol, smoking, caffeine, and any herbs or medications known to affect glucose metabolism, and that collection of blood is performed in the morning to avoid the effects of diurnal variation. For the OGTT, the patient should consume an unrestricted diet containing at least 150 g/day of carbohydrates for 3 days and fast for 10−16 h before the test is performed [42]. Anxiety, recumbence, inactivity, acute illness, incomplete ingestion of the glucose solution, and performance of the test in the afternoon can cause misleading results with the OGTT.

Perhaps the most common source of error in glucose measurements in inpatients is contamination by intravenous fluids containing a high concentration of glucose [43]. For example, 50% dextrose in water (DW) contains 50 g/dL of glucose, which is 500-fold higher than the normal glycemia. Therefore, even a 0.1% contamination with 50% DW could increase apparent glucose levels by 50 mg/dL. Sampling should never be done proximal to the infusion site and preferably should be done in the contralateral site. The infusion should be stopped for 1 or 2 min prior to collection. If it must be collected from the catheter, it should be flushed with an isotonic saline volume a minimum of 10 times the intraluminal catheter volume, and the first 5 mL of blood should be discarded or diverted. Contamination of the skin with sugar, e.g., from fruit peeling, can also lead to falsely high glucose measurements, especially when a small amount of capillary blood is used for POC glucose testing [44].

Prolonged tourniquet application and fist clenching and unclenching can result in glucose consumption by the underperfused tissues, leading to lower glucose levels. For example, tourniquet application for 6 min resulted in a drop of plasma glucose by nearly 4% [2].

Serum or plasma should be separated from cells within 30 min to minimize consumption of glucose by blood cells, which is a source of variability in glucose results much higher than analytical imprecision. If separation cannot be done within 30 min, it is recommended that the sample be placed on ice or an appropriate glycolysis inhibitor be present [40]. In the past, sodium fluoride was recommended, usually in combination with an anticoagulant such as potassium oxalate (gray-top tubes), to prevent glycolysis because fluoride is a potent inhibitor of the enolase enzyme in the glycolytic pathway. However, inhibition does not occur during the first 1 or 2 h due to poor permeability of the red cell membrane to fluoride, and consumption of 2−10 mg/dL per hour of glucose may be seen for up to 3 or 4 h. Consensus guidelines for diagnosis of diabetes mellitus recommended the use of tubes containing citrate, EDTA, and fluoride for effective inhibition of glucose consumption at 24−37 °C [40]. However, these tubes are not widely available in the United States, and one alternative is to keep gray-top tubes on ice if centrifugation is delayed more than 30 min, particularly in situations associated with increased *in vitro* glucose consumption, including polycythemia (e.g., in newborns), leukocytosis (>60,000/μL), thrombocytosis, sepsis, and the use of nonsterile blood collection tubes. Other body fluids, such as cerebrospinal fluid, pleural fluid, and peritoneal fluid, should be immediately analyzed for glucose because of possible glucose consumption by cells. Urine should be treated with acetic acid, borate, thymol, or sodium azide and refrigerated to avoid bacterial glucose consumption.

Fasting glucose levels in arterial blood are 2−5 mg/dL higher than those in capillary blood and 5−10 mg/dL higher than those in venous blood. However, after a meal, increased glucose utilization by peripheral tissues stimulated by the postprandial insulin surge can result in capillary or arterial blood glucose levels 20−70 mg/dL higher than those in venous blood measured about 30 min after the meal [42]. Low perfusion associated with shock, hypotension, and vascular disorders can also cause similar discrepancies between arterial, capillary, and venous blood as slow blood flow leads to increased glucose extraction by the tissues. These discrepancies are frequent sources of confusion for clinical providers because glucose can be measured nearly simultaneously in hospitalized patients as part of an arterial blood gas profile, with POC glucose meters sampling

capillary blood, and in the central laboratory high-throughput analyzers using venous blood serum or plasma. Note that the ADA criteria for diabetes and pre-diabetes diagnoses apply to venous blood glucose and appropriate adjustments should be made for capillary samples.

Often, the discrepancy between whole blood analyzers, such as the blood gas instruments or the glucose meters, and plasma or serum glucose is attributed to the difference between glucose levels in whole blood and in the plasma fraction. This is true if glucose is measured in lysed blood. Whereas glucose is freely permeable and equilibrates at similar concentrations in plasma and the intracellular water fraction, the water content in red blood cells is only 71% of the total volume compared to 93% in the plasma, resulting in a 10−15% decrease in glucose concentration compared to plasma, depending on hematocrit. However, most meters measure glucose in unlysed whole blood (i.e., in the water fraction of plasma), and correct for the sample hematocrit; therefore, the glucose concentrations are equivalent to those measured in plasma. In true whole blood analyzers using lysed blood, a correction factor is typically applied to report glycemia in terms of milligrams per deciliter of plasma [45]. However, large amounts of substances that displace water in the plasma, such as very high triglycerides, will result in falsely low glucose measurements.

Methodology for glucose testing

Virtually all glucose assays use enzymatic approaches to generate a measurable product proportional to the glucose concentration. The following enzymes can act on glucose and have been utilized to measure glucose levels:

1. Glucose oxidase: β-D-glucose + O_2 → gluconate + H_2O_2
2. Hexokinase: D-glucose (and other hexoses) + ATP → glucose (hexose)-6-phosphate; usually coupled with glucose-6-phosphate dehydrogenase:
   Glucose-6-phosphate + $NAD(P)^+$ → 6-phosphogluconolactone + NAD(P)H
3. Glucose dehydrogenase: glucose + $NAD(P)^+$ → gluconolactone + NAD(P)H

As described previously, the change in concentration of $NAD(P)^+$ versus NAD(P)H can be assayed spectrophotometrically at 340 nm and can form the basis for most methods using hexokinase or glucose dehydrogenase. Alternatively, NAP(D)H can be used to reduce a tetrazolium chromogen to a colored product or to reduce an electron donor, such as flavin adenine dinucleotide (FAD) or pyrrol-quinoline quinone (PQQ), resulting in measurable current. Careful calibration and quality control is required because of instability of co-factors, particularly ATP, NAD^+, and $NADP^+$, in solution.

Glucose oxidase methods rely on one of the following approaches to produce a measurable signal:

1. Measuring oxygen consumption with an O_2-specific electrode: This approach is used in the Beckman DxC/LX20 modular assay. To minimize spontaneous O_2 generation from H_2O_2, molybdate, iodide, and ethanol are included to enhance catalase-mediated inactivation of the peroxide.
2. Measuring H_2O_2 formation by using peroxidase to convert a chromogen to a colored product: Many chromogens have been used, including *o*-dianisidine and 4-aminoantipyrine.
3. Measuring H_2O_2 formation by oxidation of a platinum electrode, generating current proportional to the glucose levels: This method can be used in whole blood analyzers because it avoids optical readings as well as issues with oxygen consumption by blood cells.

Interferences in glucose assays

In this section issues of interferences in various glucose methods are discussed.

Glucose oxidase

Interference by strong redox agents is expected in glucose oxidase methods using peroxidase, similarly to other methods (e.g., uric acid methods). These include endogenous substances such as uric acid, bilirubin, hemoglobin, glutathione, and acetoacetate and exogenous chemicals such as ascorbic acid, acetaminophen, tetracycline, and catecholamines.

With glucose oxidase methods relying on oxygen consumption, high oxygen levels in the blood ($pO_2 > 150$ mmHg) can cause falsely low glucose results; conversely, low oxygen levels ($pO_2 < 45$ mmHg) associated with high altitude can cause falsely high results [46]. This method should not be used in whole blood due to highly variable oxygen levels and consumption by red cells. The reaction is also dependent on pH levels, with acid pH causing increased glucose levels and high pH causing low glucose results.

Hexokinase

These methods are more susceptible to interferences that affect absorption at 340 nm, including turbidity due to lipemia, hyperbilirubinemia, and hemolysis. In addition to spectral interference by hemoglobin, hemolysis is associated with the release of several interfering substances from blood cells, including phosphates that inhibit hexokinase, glucose-6-phosphate, glucose-1-phosphate, fructose-6-phosphate, and enzymes

competing for glucose-6-phosphate and NADP. High levels of fructose (e.g., in some OGTT preparations) also interfere with hexokinase methods: Ingestion of 2 g/kg body weight of fructose raises glycemia by 10 mg/dL for 1 or 2 h [42].

Glucose dehydrogenase

Glucose dehydrogenase (GDH) is not entirely specific for glucose, and other sugars can interfere in a manner related to the particular formulation of the assay and the co-factor used. In particular, GDH-FAD reacts to xylose, and GDH-PQQ can react with maltose, galactose, xylose, and mannose, in addition to glucose.

Case report

An 86-year-old male with a diagnosis of diabetes was admitted for rapidly progressive cellulitis of the foot with necrotizing fasciitis and sepsis [47]. He was treated with above-the-knee amputation, dialysis for oliguria and progressive renal insufficiency, insulin, antibiotics, and intravenous immunoglobulin (Octagam™) for off-label sepsis treatment. The Octagam was administered over a 7-h period, during which a POC device used to measure glucose levels showed increasing glycemia peaking at 535 mg/dL 4 h into the infusion. The patient was then treated with 12 units/h of intravenous insulin, which was titrated toward 24 units/h by the next day. The patient then became increasingly unresponsive, and a POC glucose level reading was 115 mg/dL, whereas a central laboratory assay resulted in glucose of 12 mg/dL. The patient remained unresponsive despite 50% dextrose and insulin administration, and the patient eventually died due to irreversible neurological brain damage.

Several other similar cases reported to the U.S. Food and Drug Administration (FDA), including 7 fatalities, led this agency to issue a safety warning in October 2005 alerting patients and health care providers to the possibility of falsely elevated glucose results with several blood testing devices due to interference from intravenous products containing maltose, galactose, or xylose. This problem is unique to devices using glucose dehydrogenase (GDH) coupled with oxidation of PQQ for production of a colored product. Subsequently, the FDA reported 13 deaths associated with GDH−PQQ glucose testing, 10 of which occurred in patients who received Extraneal (icodextrin) peritoneal dialysis solution and 3 that occurred in patients who received maltose infusions, and issued a public health notification in August 2009 reiterating the possibility of fatal errors with GDH−PQQ glucose meters [48]. The problem is particularly severe when large amounts of interfering sugars are being infused in the patient; for example, a total dose of 25 g maltose can result in peak blood maltose levels of approximately 100−150 mg/dL, which decay with a half-life of approximately 90 min [49]. Table 9.5 shows some pharmaceuticals containing substances interfering with the GDH−PQQ glucose assays. A recent modified mutant PQQ GDH assay was supposed to react only with glucose but interference by maltose [50] and galactose [51] was observed.

Tang et al. investigated several POC glucose meters for possible interference by a list of 30 commonly used drugs, and they established that ascorbic acid (3−9 mg/dL), acetaminophen (2−10 mg/dL), dopamine (2−4 mg/dL), and mannitol (500 mg/dL) caused interference with various glucose meters. With the exception of acetaminophen and mannitol, these levels are much higher than typical therapeutic concentrations. However, in certain situations associated with rapid drug infusion or decreased excretion, such as in critically ill patients with renal failure, interferences by these drugs may become significant [52].

ANALYSIS OF ELECTROLYTES

Electrolytes are substances that form ions in aqueous solutions, therefore conducting current. In clinical biochemistry, this term usually refers to clinically significant small ions that account for most of the conductivity of body fluids, and it excludes other charged substances such as amino acids, peptides, proteins, and nucleic acids. The most frequently measured electrolytes have critical physiologic roles and tightly regulated concentrations. These include the cationic metals sodium (Na^+), potassium (K^+), magnesium (Mg^{2+}), and calcium (Ca^{2+}) and the anions chloride (Cl^-), phosphate (HPO_4^{2-}), and bicarbonate (HCO_3^-), of which Na^+, K^+, Cl^-, and HCO_3^- are the most commonly assayed, usually as an "electrolyte panel" (see Table 9.3). It is beyond the scope of this chapter to discuss the clinical implications of electrolyte measurements, and the reader is referred to excellent textbooks on the topic [42]. However, a few key concepts are important to understand physiopathologic and pre-analytical considerations of relevance to avoid misinterpreting electrolyte levels.

With the exception of calcium and magnesium, which are albumin-bound, the major electrolytes are present almost exclusively as free ionized species in body fluids. Calcium and magnesium can be measured as the total concentration in the fluid or as the free ionized fraction. The other electrolytes are measured as free ions.

Cellular membranes, which are predominantly hydrophobic bilayers, do not allow for free movement of electrolytes between the intracellular and extracellular compartments. As a result, movement of electrolytes occurs mostly through membrane-bound protein complexes called pores, which can have open or close

TABLE 9.5 Pharmaceuticals containing sugars that cause false-positive elevations in glucose tests with GDH-PQQ assays.

Manufacturer	Trade name	Product	Interfering substance	Interferent concentration (g/dL) or maximum dose
Talecris Biotherapeutics	Gamimune N 5%[a]	Immune globulin intravenous (human)	Maltose	9–11
Octapharma Pharmazeutika Produktionsges m.b.H.	Octagam	Immune globulin intravenous (human)	Maltose	10
Cangene Corporation	WinRho SDF Liquid	Rh (D) immune globulin intravenous (human)	Maltose	10
Cangene Corporation	None	Vaccinia immune globulin intravenous (Human)	Maltose	10
Cangene Corporation	HepaGam B	Hepatitis immune globulin (human)	Maltose	10
GlaxoSmithKline	Bexxar	Tositumomab and 131I-tositumomab	Maltose	10
Baxter	Extraneal	Peritoneal dialysis solution	Icodextrine	7.5
Baxter	Adept	Adhesion reduction solution	Icodextrine	4
Bristol-Myers Squibb	Orencia	Abatacept	Maltose	2-g dose
NERL Diagnostics	D-Xylose USP	D-Xylose	Xylose	25-g dose
		Galactose tolerance test	Galactose	40-g dose

[a]*Discontinued.*

states and can be subject to regulation. In addition, ions can be actively moved across pores by active transporters called pumps to maintain physiologic important gradients that are associated with electric charges critical for many cellular and organ functions, such as muscle contraction and nerve conduction. For example, sodium is actively pumped out of cells, whereas potassium is actively transported into the intracellular compartment mostly by Na^+/K^+-ATPase, which exchange three Na^+ for two K^+ and therefore create the membrane resting potential with net negative charges intracellularly. This process alone is responsible for approximately one-third of energy consumption in most cells and even higher in neurons. Interference with this active mechanism can lead to perturbations in nerve conduction or muscle contraction, such as when digoxin inhibits the Na^+/K^+-ATPase, resulting in decreased membrane potential and release of calcium from intracellular stores.

Active transporters also play a critical role in the kidney tubules, where K^+ and H^+ are actively transported into the tubular lumen in exchange for Na^+, thus regulating blood levels of sodium and potassium and acid–base balance. In addition, Cl^- ions move freely with Na^+ to maintain electrical balance. As sodium and chloride ions move across cell membranes, they carry water molecules and thus regulate cellular water content and volume. As the major ions in the extracellular compartment, Na^+ and Cl^- are responsible for most of the osmotic pressure across cell membranes, which can be calculated by the following formula:

$$\begin{aligned}&\text{Plasma osmolality (in mOsm/kg)}\\&\quad = 2 \times Na^+ + \text{glucose}/18 + \text{BUN}/2.8\end{aligned} \tag{9.5}$$

Where urea and glucose (expressed in mg/dL) are the major non-ionic molecules contributing to osmotic pressure, and $2 \times Na^+$ represents the combined effect of sodium and its partner anions expressed in mM. This formula is useful to calculate the osmolal gap, which is the difference between measured osmolality (usually assayed by freezing point depression osmometers) and calculated osmolality. Differences greater than 10 mOsm/L indicate the presence of unmeasured osmotically active substances, such as alcohols, ethylene glycol, acetone, mannitol, hypergammaglobulinemia, and hypertriglyceridemia, or point to a measurement error in sodium, glucose, BUN, or osmolality.

As with regulation of osmotic pressure in cells, sodium transport in the kidney is a major factor in water retention and blood volume. Conversely, the balance between water and sodium content in the extracellular compartment determines sodium concentration. Therefore, the kidney transporters are major targets of hormonal systems that affect plasma sodium concentration and water content, as well as blood volume and blood pressure, including antidiuretic hormone (ADH), natriuretic peptides, and the renin–angiotensin–aldosterone system.

An important factor in interpreting plasma electrolyte concentrations is the effect of non-electrolyte substances, such as glucose, that do not freely cross the plasma membrane. Elevated glucose levels in the plasma can cause increased osmotic pressure in the extracellular fluids because glucose does not quickly equilibrate with the intracellular fluid in the absence of insulin. Therefore, high glycemia can cause a shift of water from the intracellular to the extracellular fluid, resulting in dilution of all extracellular solutes. Particularly in the case of sodium, which has a narrow range in plasma, it is advisable to correct the plasma sodium for dilution due to hyperglycemic osmotic expansion in order to avoid misinterpreting the measured hyponatremia as either an excess of total body water or a salt deficiency. The following formula can be used to correct dilutional hyponatremia due to hyperglycemia (Na^+ in mM and glucose in mg/dL):

$$\begin{aligned}Na^+(\text{corrected}) &= Na^+(\text{measured}) + 0.016\\&\quad \times \text{glucose} - 1.6\end{aligned} \tag{9.6}$$

In contrast to tight regulation across cellular membranes, electrolyte ions move freely between the intravascular and extravascular compartments. Intercellular junctions in capillaries can regulate the distribution of macromolecules, such as proteins, between the extravascular and the intravascular compartments. Usually, the concentration of proteins in the plasma is higher than that in the extravascular compartment and contributes to pulling water into the intravascular compartment, forming a pressure gradient called oncotic or colloid osmotic pressure. If there is a reduction of plasma protein content or an increase in vascular permeability, fluid moves out of the intravascular compartment and causes edema.

In general, electrolyte concentrations are not significantly affected by changes of plasma proteins or intravascular volume, with the exception of total calcium and magnesium. For example, a shift from the supine to the upright position with increased intravascular hydrostatic pressure causes flow of intravascular fluid to the interstitial space, resulting in a decrease in plasma volume of approximately 12%. Under normal endothelial permeability conditions, molecular complexes greater than 4 nm in diameter (e.g., most proteins, including albumin, which has a 7-nm diameter) will be retained in the intravascular compartment; therefore, their concentration will be increased proportionally. Most electrolytes are not affected, but calcium, being approximately 40% protein bound, will increase by 5–10% (0.2–0.8 mg/dL) when moving from the

supine to the upright position, whereas magnesium, being less protein bound, will increase by approximately 4% [2]. A similar situation with shift to interstitial space and hemoconcentration occurs when the tourniquet is kept for more than 1 min, especially with repeated fist clenching, during blood collection from the arm. In this case, in addition to total calcium and magnesium, potassium will increase as much as 2 mM due to muscle activity [53], whereas glucose and phosphate will decrease due to intracellular consumption.

Free ionized calcium comprises 48−50% of the total calcium, whereas 40% is protein bound (32% bound to albumin and 8% to globulins) and 10−12% complexed with other anions, such as phosphate, bicarbonate, and lactate. Approximately 25−30% of plasma magnesium is complexed with albumin, whereas 15−20% is complexed with anions and 50−55% exists in the free form. Only the free ions are physiologically active. Therefore, allowances should be made for the albumin concentration to correctly evaluate measurements of total calcium and magnesium. Because total levels of divalent cations linearly correlate with albumin concentrations below the reference range (<4 mg/dL), several formulas have been used to estimate what the levels of total calcium and magnesium would be with albumin in the normal range, such as follows (electrolytes in mg/dL, albumin in g/dL) [54]:

$$\text{Calcium (corrected)} = \text{total calcium (measured)} - 0.8 \times \text{albumin} + 3.2 \quad (9.7)$$

$$\text{Magnesium (corrected)} = \text{total magnesium (measured)} - 0.123 \times \text{albumin} + 0.492 \quad (9.8)$$

However, the reliability of these formulas has been questioned, particularly in renal failure and critically ill patients with several factors affecting electrolyte levels, including altered pH and anion levels. Excess H^+ causes a decrease in negative charges in calcium-binding proteins, leading to dissociation of protein-bound calcium and increased free Ca^{2+}. For each decrease of 0.1 pH units, free Ca^{2+} increases by 0.2 mg/dL. The reverse changes occur in alkalemia. Other factors affecting the accuracy of these formulas include the effect of substances that complex with calcium or compete for binding to albumin, such as free fatty acids, bilirubin, heparin, drugs, and several organic anions including lactate and ketoacids. In the case of renal failure patients, phosphate levels showed a significant effect in the proportion of free calcium and should be taken into consideration [55]. In the case of critically ill patients, calcium correction formulas showed very poor sensitivity to detect hypocalcemia, and direct free Ca^{2+} measurements should be done [56]. Direct measurement of free Ca^{2+} is also preferable in cases of liver transplantation, malignancy, especially myeloma, and in neonates [42].

Some studies indicate a strong correlation of total and free Mg^{2+}, but there is poor correlation between plasma total magnesium or free Mg^{2+} and total body magnesium because only 0.3% of total body magnesium is present in plasma. Whole body magnesium may be depleted up to 20% with maintained normal magnesemia. Because total Mg assays have better specificity for magnesium and are cheaper, more widely available, and better standardized, they may be preferable to free Mg^{2+} assays for assessment of plasma magnesium. The most accurate way to diagnose magnesium deficiency is to perform an Mg tolerance test, in which 24-h urine Mg excretion is measured before and after the patient is injected with 1.2 mmol of Mg/kg body weight. A recovery of less than 80% of the injected magnesium in the urine is indicative of magnesium deficiency in the absence of diuretic treatment, renal disease, or drugs, such as cyclosporine and tacrolimus, that cause urinary Mg wasting [54].

A similar situation occurs with calcium because plasma calcium correlates poorly with total body calcium—99% of the body calcium is trapped as calcium phosphate (hydroxyapatite) in the bone. In contrast with magnesium, intracellular concentrations of calcium are much lower than plasma concentrations; therefore, hemolysis does not lead to an increase in calcium levels, although hemoglobin can cause spectral interference with colorimetric methods.

Physiologic pre-analytical issues

Circadian variation in hormones such as ADH, corticotrophin (ACTH), cortisol, aldosterone, renin, and epinephrine along with individual biorhythms associated with meals, exercise, and sleep affect electrolyte excretion, the movement of ions and water across cell membranes, and the distribution of fluid between the intra- and extravascular compartments. For example, potassium levels in the plasma can vary by more than 0.6 mM, with peaks between 8 a.m. and 4 p.m. and nadirs between 8 p.m. and 1 a.m [57]. This variation may have more to do with shifts to the intracellular compartment than rates of excretion because urinary levels tends to parallel serum K^+ concentration [57]. Circadian variation in the plasma levels of phosphate is also observed, with peaks in the afternoon and evening, immediately after major meals, and the nadir in the fasting morning specimens.

As major intracellular electrolytes, the levels of both potassium and phosphate increase after organic food ingestion, with phosphate levels in plasma increasing by approximately 15% and potassium by approximately 5% 2 h after a standard meal [2]. Standard meals do not have a significant effect on sodium, chloride, calcium, or

magnesium, although magnesium levels tend to be higher in vegetarians and vegans.

Exercise stimulates release of potassium mainly from skeletal muscles, leading to rapid increases in venous potassium averaging 0.8–1.2 mM and peaking as high as 8 mM in arterial blood during intense exercise [56]. Well-trained athletes have increased sodium pump density in their muscles and show lower increases in plasma K^+ during exercise, whereas patients with heart failure and muscular dystrophies have lower muscle Na^+/K^+ pump density [56]. Higher catecholamine levels can induce intracellular potassium influx by stimulating the Na^+/K^+-ATPase via β_2-adrenergic receptors, causing a quick drop in serum potassium levels post-exercise and during stress (e.g., in patients with acute myocardial infarction). The hypokalemic effect of β-adrenergic receptors can be attenuated by caffeine and stimulation of α-adrenergic receptors in the liver [58]. Similarly, peak insulin levels after meals or glucose infusion tend to reduce potassium levels because insulin leads to increased activity of the Na^+/K^+ pump and increased cellular potassium influx. Due to their synergistic effects in activating the Na^+/K^+ pump, insulin and β_2 agonists are used in the treatment of hyperkalemia.

The levels of plasma phosphate also reflect the net movement of phosphate ions across cell membranes as well as dietary intake. For example, phosphate levels increase immediately after meals and start declining after 2 or 3 h as insulin stimulates glucose and phosphate intracellular influx. Metabolic or respiratory acidosis leads to hydrolysis of intracellular organic phosphates with release into the extracellular fluid. On the other hand, high intracellular phosphate consumption with associated hypophosphatemia is common in states of enhanced cell growth, such as during childhood, acromegaly, and tumor proliferation. Hypophosphatemia is frequent in the postoperative phase due to multiple factors, such as respiratory alkalosis, insulin, and diuretics. Catecholamines also cause decreases in phosphatemia, which tends to parallel serum K^+ during exercise [59]. Magnesium is released from muscles leading to mild increases during intense exercise, whereas well-trained athletes tend to have decreased magnesemia with endurance exercise. Although total calcium does not change with exercise, the lactic acidosis associated with intense exercise can result in increases in free Ca^{2+} as the lower pH dissociates calcium from proteins.

Several drugs can affect electrolyte levels. For a comprehensive listing of drug effects on clinical testing, see Young [3]. For example, angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor blockers, and potassium-sparing diuretics are associated with higher risk of hyperkalemia, whereas β_2 agonists, theophylline, chloroquine, thiopental, thiazides, and other loop diuretics can lead to hypokalemia. Interestingly, consumption of caffeine-containing sugary sodas has been associated with hypokalemia due to inhibition of the Na^+/K^+ pump by the caffeine metabolite theophylline, insulin secretion, and osmotic diarrhea [58].

Acute ingestion of ethanol can also have significant effects on electrolytes. First, ethanol inhibits secretion of hypothalamic ADH, resulting in increased free water diuresis, which can lead to dehydration and hypernatremia; it also significantly increases aldosterone, leading to sodium retention and loss of potassium. Local effects at the kidney level may also lead to increased reabsorption of sodium and chloride. Second, alcohol is metabolized to acetate, with resulting metabolic acidosis with low plasma bicarbonate, compounded by ketoacidosis related to inhibition of glucose utilization. In chronic alcoholics, phosphate and magnesium deficiencies are common.

Bicarbonate in plasma is in equilibrium with dissolved carbon dioxide and hydrogen ion (pH):

$$CO_2 \leftrightarrow HCO_3^+ + H^+ \tag{9.9}$$

Situations with acidosis generate an excess of hydrogen ions and shift the previous equation to the left with decrease in plasma bicarbonate, whereas alkalosis has the opposite effect. Similarly, an increase in carbon dioxide concentrations (e.g., due to respiratory insufficiency) leads to a shift to the right, with an increase in bicarbonate level with compensatory decrease in pH. The lungs can contribute to acid–base regulation by controlling the amount of CO_2 excretion. In addition to the short-term buffering and respiratory mechanisms, acid–base balance is highly regulated by the kidney, where the excretion of hydrogen ions and bicarbonate can be modified by several mechanisms, including alteration of the rate of absorption of Na^+ in exchange for H^+ and K^+ and production of ammonia from glutamine to trap H^+ for excretion.

In order to maintain ionic balance, the loss of bicarbonate in the body leads to an increase in chloride anions, whereas bicarbonate retention results in loss of chloride. The exception to this rule is when the loss of bicarbonate is caused by metabolic acidosis resulting from accumulation of organic acids such as lactate or ketoacids. In this case, the organic acids balance the cations, and the chloride concentration is minimally affected. Conversely, changes in plasma chloride levels result in inverse changes in bicarbonate concentration. For example, loss of chloride by vomiting of gastric fluid will result in an increase in plasma bicarbonate concentration and metabolic alkalosis, whereas ingestion of chloride will result in metabolic acidosis with low bicarbonate. Measurement of chloride and bicarbonate (or total CO_2) even in venous plasma is

useful to identify and classify possible acid–base disturbances.

In addition to renal regulation, chloride responds to changes in plasma CO_2 in the following manner: As CO_2 accumulates in plasma, it moves freely into the red cells, where it is rapidly converted into HCO_3^- by carbonic anhydrase. Because there is no such enzyme in plasma, the rate of conversion to HCO_3^- is much lower in plasma, creating a huge gradient that forces bicarbonate out of the cells. Because the plasma membrane in impermeable to H^+ and hydrogen ions are tightly captured by red cell proteins, particularly deoxy hemoglobin, chloride, which can move freely across the membrane, must move intracellularly to maintain ionic balance (chloride shift). This phenomenon is relevant to measurement of chloride in blood samples: With improperly capped whole blood samples stored for prolonged periods before cell separation, the loss of CO_2 is followed by conversion of intracellular bicarbonate to CO_2, which diffuses out of the cells; as intracellular bicarbonate concentration decreases, chloride shifts intracellularly to maintain ionic balance, causing artificially low plasma chloride measurements.

The balance between the four major electrolytes is best illustrated by the anion gap formula:

$$Na^+ + K^+ = Cl^- + HCO_3^- + \text{anion gap} \qquad (9.10)$$

The anion gap is caused by unmeasured anions such as proteins and organic acids. In a healthy individual, unmeasured anions account for approximately 12–15 mEq/L. The anion gap will increase if unmeasured anions are in excess, such as with lactic acidosis, and will decrease if unmeasured cations, such as immunoglobulins, are significantly increased. It is good laboratory practice to monitor the anion gap for possible analytical errors in the measurement of components of the electrolyte panel.

Note that the anion gap is also particularly sensitive to pre-analytical issues affecting the measurement of its components (see Table 9.6). These pre-analytical effects can be caused by prolonged storage of unseparated blood at room temperature and also by evaporation that occurs in uncapped samples, e.g., when aliquots are made into sample cups that are not expeditely processed by the analyzers. As the water in separated plasma evaporates, all the electrolytes increase by the same proportion. However, the absolute change in sodium is higher than other electrolytes, e.g., a 10% increase in sodium of 140 mM is 14 mM whereas a chloride level of 100 mM will increase by 10 mM. To compound the effect, the loss of CO_2 in uncapped samples is greater than the concentration due to loss of water, resulting in progressively lower bicarbonate in the plasma. The result is a significant spurious increase in anion gap.

In addition to the changes in HCO_3^- and Cl^-, acidemia causes intracellular shifts of K^+, as H^+ and K^+ compete for intracellular anions. As H^+ moves intracellularly, K^+ moves out; on average, plasma K^+ will rise approximately 0.6 mM (range, 0.2–0.7 mM) for each 0.1 unit of fall in plasma pH [60]. This increase in K^+ is much lower with organic acidemia, such as in lactic- or ketoacidosis, perhaps because these anions will buffer H^+ intracellularly. The reverse phenomenon is true because hyperkalemia will cause a mild metabolic acidosis. On the other hand, metabolic alkalosis and hypokalemia are similarly interconnected, with a drop of approximately 0.4 mM K^+ per 0.1 unit of pH increase. Also, it is important to note that in situations associated with peripheral ischemia, such as in hypothermia, vascular disease, thrombosis, and shock, a combination

TABLE 9.6 Changes in electrolytes caused by prolonged storage of improperly capped blood samples at room temperature before centrifugation and separation from cells and by prolonged exposure of plasma or serum to air in uncapped containers. The changes are represented by arrows followed by a summarized mechanistic explanation.

	Prolonged whole blood	**Uncapped plasma/serum**
tCO_2 (HCO_3^-)	⇓ Loss of CO_2 leads to $HCO_3^- \rightarrow CO_2$ which diffuses out of cells	⇓ Loss of CO_2 due to evaporation > increase from solute concentration
Cl^-	⇓ Intracellular shift to balance HCO_3^-	⇑ Solute concentration
Na^+	Minor changes	
K^+	⇑ Moves out of cells as pH ⇓	
Anion Gap	⇑ Glucose → lactate in cells	⇑ (⇓ HCO_3^- and absolute ⇑ in Na^+ > absolute ⇑ in Cl^-)
pH	⇓ Glucose → lactate in cells	⇑ Due to ⇓ CO_2 and HCO_3^-
HPO_4^{2-}	⇑ Phosphatase activation → HPO_4^{2-} diffusion out of cells as pH ⇓	⇑ Solute concentration
Ca^{++}	⇑ Dissociates from proteins as pH ⇓	⇓ Associates with proteins as pH ⇑

of acidosis and hypoxia with insufficiency of the Na^+/K^+ pump will result in higher potassium levels in peripheral vein samples, and a more physiologic assessment of kalemia should be done from central vein sampling if available. Phosphate levels also tend to respond to pH in the same manner as K^+, with acidosis causing hydrolysis of intracellular phosphates and increased phosphatemia, whereas alkalosis results in activation of glycolysis and consumption of phosphate by the cell.

As mentioned previously, pH also has an important effect on the fraction of free Ca^{2+}, with every change in 0.1 unit of pH resulting in the opposite change in free Ca^{2+} of approximately 0.2 mg/dL. It is important to concurrently report the pH of the sample for proper interpretation of Ca^{2+}. The clinician must distinguish *in vivo* changes in pH causing true shifts in free Ca^{2+} from spurious changes in free Ca^{2+} resulting from sample pH adulteration. Most commonly, samples lose CO_2 upon air exposure, with a corresponding increase in pH and falsely decreased Ca^{2+}. Prolonged storage of uncentrifuged whole blood can also lead to glucose consumption by blood cells, with corresponding lactic acid production resulting in pH drop and spurious Ca^{2+} increase. Every effort should be made to avoid these sources of misleading results in free Ca^{2+} determination. Ideally, specimens should entirely fill a minimally heparinized and tightly capped plastic blood gas syringe and be analyzed within 15–30 min.

Specimen issues

Specimens for electrolyte analysis include whole blood, plasma, serum, and urine. Sometimes, assessment of electrolytes in other body fluids is important. For example, chloride levels in sweat are used to diagnose cystic fibrosis. Because the levels are lower than in plasma, the methods have to be validated for detecting levels as low as 10 mM in sweat. Likewise, other body fluids, such as gastrointestinal fluids, may be analyzed to identify electrolyte disturbances specific to the organ of interest, but the results are not as well standardized or comparable, and matrix effects need to be taken into considerations when applying different methods to the analysis. For urinary and fecal fluids, timed collections are indicated to assess rates of electrolyte excretion.

Arterial and capillary samples differ significantly from venous blood in calcium (−2.4 to 4.6%), sodium (0 to −2.3%), and chloride (−1.8 to 2.0%) measurements [42]. It is important to avoid contamination by infusion fluids, one of the most common causes of misleading laboratory results in hospitalized patients. In particular, contamination with sodium citrate infusions has a significant effect on free Ca^{2+} and pH measurements, even after discarding the first 9 mL of blood from the catheter [57]. In addition to binding Ca^{2+}, citrate contamination can also lower Na^+ by forming weak complexes with Na^+ [58]. Other less obvious sources of contamination that interfere with sodium measurements include sodium or lithium heparin (which weakly chelates Na^+ and reduces Na^+ levels), benzalkonium heparin-coated catheters, benzalkonium antiseptic cleaning agents, and ticarcillin, which contains a high concentration of sodium [58].

The clotting process leads to release of intracellular fluid, mostly from platelets, and therefore the serum contains higher levels of intracellular electrolytes than plasma, such as potassium (+0.3 to 0.4 mM or 5–15%) and phosphate (+2 to 8%), whereas other electrolytes are only minimally affected (<1%) [42]. There is a linear relationship between the serum-plasma difference in potassium levels and the platelet count (~0.15 mM per 100,000/μL) [61]. Similarly, patients with high leukocytosis, particularly with fragile cells such as in chronic lymphocytic and chronic myelomonocytic leukemias, commonly have pseudohyperkalemia. In patients with high platelet or leukocyte counts, collection of samples in heparinized tubes with avoidance of hemolysis is indicated to avoid spurious hyperkalemia due to the clotting process. However, in certain cases, heparin-induced lysis of fragile leukemic cells can result in higher K^+ levels in plasma than in serum ("reverse pseudohyperkalemia").

Centrifugation at very high speeds or in fixed-angle rotors can induce cell rupture and cause pseudohyperkalemia (see Chapter 3 for case report). Recentrifugation of blood samples after prolonged storage is discouraged because the small amount of serum remaining below the gel separator is rich in potassium and will move to the original serum layer, falsely increasing K^+ [62].

Contamination from intracellular electrolytes is much magnified if there is hemolysis, leading to a significant increase in potassium and phosphate, with very minor increases in magnesium. Because the potassium concentration in red cells is 105 mM, even a small amount of hemolysis results in significant increases in plasma potassium. On average, the amount of potassium released by *in vitro* hemolysis correlates with free hemoglobin, increasing approximately 0.2–0.5 mM for each 0.1 g/dL of plasma hemoglobin [63]. However, formulas for correcting potassium based on estimation of free hemoglobin are highly variable and should not be used. Instead, laboratories should establish a hemolysis threshold, by visual or spectrophotometric inspection, above which the artifactual increase in potassium is unacceptable, leading to specimen rejection. Hemoglobin levels of 0.1 g/dL or greater (corresponding to lysis of 0.7% of the red cells in an individual with a hemoglobin of 15 g/dL) are usually associated with clinically significant spurious increases in potassium. More problematic are whole blood analyzers, such as blood gas and POC

devices, where hemolysis cannot be easily recognized. A recent study showed that up to 13% of arterial blood gas samples collected in the emergency department and 4% of samples from intensive care units were significantly hemolyzed and resulted in spuriously elevated K^+ levels [64]. Whole blood potassium levels should be regarded with suspicion if inconsistent with the clinical situation, and in these cases centrifugation of the specimen to assess hemolysis is indicated. In particular, in the absence of renal insufficiency, hyperkalemia is rare, and elevated K^+ should prompt a search for causes of spurious hyperkalemia [53]. Table 9.7 summarizes the various causes of pseudohyperkalemia.

In contrast to potassium, the intracellular magnesium concentration is only two- or threefold the plasma levels; therefore, common amounts of hemolysis do not significantly increase magnesium levels in plasma. Calcium levels are also not significantly affected by hemolysis, although free Ca^{2+} can be affected by binding from intracellular proteins and changes in specimen pH. For colorimetric calcium, phosphate, and magnesium assays, spectral interferences with the analytical measurement are possible with significant hemolysis, hyperbilirubinemia, or lipemia.

Anticoagulants may significantly affect electrolyte measurements. Obviously, anticoagulants such as sodium or potassium heparin, citrate, or oxalate should not be used for electrolyte measurements. Likewise, divalent cation chelators such as EDTA, citrate, and oxalate should not be used for calcium or magnesium determinations. Collection of blood for electrolyte determination should precede the use of anticoagulants containing sodium, potassium, or chelators due to the possibility of contamination of the needle. Less obviously, heparin may bind calcium and magnesium unless all binding sites are saturated, and therefore balanced heparin should be used for measurement of free calcium or magnesium in plasma or whole blood. While unexpected hypocalcemia is a clue to contamination by potassium EDTA in cases of suspected pseudohyperkalemia, the best approach is to directly assay for serum EDTA in practices where high rates of EDTA contamination are observed [65,66].

TABLE 9.7 Causes of pseudohyperkalemia[a].

***RELEASE FROM CELLS* IN VITRO**
Prolonged tourniquet time (up to 3 mM)
Forearm exercise and fist clenching (1–2 mM)
Delayed transportation and centrifugation
Cold storage/transportation of uncentrifuged samples
Familial pseudohyperkalemia: Autosomal dominant hereditary stomatocytosis with leakage of K^+ at <37 °C; immediate separation is required.
Leukocytosis (can also temorarily cause low K^+ at 25–30 °C)
Thrombocytosis (serum K^+ > plasma K^+ by ~0.15 mmol/100,000 platelets/μL)
Hemodialysis: Up to 50% have serum K^+ > plasma K^+, even with normal cell counts.
Reverse pseudohyperkalemia: Plasma K^+ > serum K^+ in leukocyte malignancies fragilized by heparin.
HEMOLYSIS (AST AND LDH SHOULD BE ELEVATED)
Ethanol contamination
Difficult venipuncture
Inappropriate needle diameter
Syringe and needle collection
Excessive storage/transport temperature (<30 °C)
Excessive centrifugation speed
Prolonged contact with wooden applicator sticks used to remove fibrin (up to 0.5 mM)
CONTAMINATION
Collection through catheter or above potassium-infusion site
K_2-EDTA contamination, wrong tube type or order of tube collection (also low calcium)
DILUTION
Blood sample from arm with infusion lacking K^+ (e.g., DW5) causes dilution.

[a]*Suspect if patient has no predisposing factors, signs, or symptoms of hyperkalemia, such as numbness, chronic renal failure, abnormal electrocardiogram, treatment with loop diuretics, spironolactone, and ACE inhibitors.*

Case report

A primary care physician was called in the evening because one of her patients was contacted to go urgently to the hospital due to a potassium level of 7.2 mM [53]. This 72-year-old male was being treated with lisinopril for hypertension, and his previous potassium levels ranged from 4.5 to 5.0 mM. A repeat specimen in the hospital showed a level of 4.8 mM and normal BUN and creatinine, an electrocardiogram was normal, and the patient was discharged home. Subsequent investigation indicated that the primary care practice was sending the uncentrifuged samples in a metal container to the central laboratory and exposing the specimens to near freezing temperatures for hours during transportation.

Storage of blood samples before serum or plasma separation leads to progressive leakage of potassium from cells. In contrast with the attenuating effect on glucose consumption, storage at 4 °C inhibits the Na^+/K^+-ATPase responsible for keeping potassium inside the cell and magnifies the artificial increase in blood K^+ levels. Plasma kept in contact with blood cells for 8 h at 4 °C shows an average increase in K^+ of

approximately 1 mM, minimal change at 23 °C, and a decrease of approximately 1 mM at approximately 30 °C [2]. Exposure to temperatures higher than 30 °C for several hours can lead to consumption of glucose and cell disruption, with consequent leakage of potassium into the plasma. Samples with high leukocyte counts can exaggerate these effects, with increased potassium leakage at low or high temperatures and increased intracellular shifts at 25–30 °C. Because of the possibility of either pseudohyperkalemia or pseudohypokalemia with changes in ambient temperature, centrifugation of whole blood samples with a gel separator is indicated within 1 h of collection if prolonged storage or transportation is expected before analysis.

In addition to potassium, phosphate levels tend to increase upon unrefrigerated storage of whole blood due to the action of cellular phosphatases on organic phosphates. In contrast, levels of HCO_3^- will decrease as the tubes lose CO_2 after being exposed to air (partial pressure of carbon dioxide (pCO_2) decreases ~15–20% per hour at room temperature in de-capped tubes) [67]. Because CO_2 accounts for only 3–10% of the total CO_2 in plasma, this loss of CO_2 results in only moderate decreases in serum HCO_3^- or total CO_2 (~0.6–1 mM per hour) [67]. However, in large-volume laboratories, the loss of HCO_3^- may become significant because the tubes may sit open for more than 1 h. This loss of CO_2 is accentuated at temperatures higher than room temperature and attenuated in refrigerated serum specimens. The loss of CO_2 occurs even in capped tubes if the filling is not complete. A study of 10-mL Vacutainer tubes showed that for each extra milliliter of air above the sample in an underfilled tube, the HCO_3^- will decline by 0.5 or 0.6 mM and the anion gap will increase by 0.2 or 0.3 mM [68].

Analytical issues

There are many analytical issues concerning analysis of electrolytes. In this section, such issues are discussed.

Ion-specific electrodes

Virtually all major high-throughput and POC analyzers utilize ion-specific electrodes (ISEs) to measure Na^+, K^+, and Cl^-. Furthermore, free Ca^{2+} and Mg^{2+} are measured with ISEs. A special type of ISE is a pH meter, which determines the concentration of H^+ ions. In general, ISEs contain an electrolyte-selective membrane that allows only the ion of interest to move across the membrane, thus creating an electromotive force proportional to the ion concentration. There are two main types of ISEs: Indirect ISEs dilute the sample 1:20 to 1:34 before measurement, whereas direct ISEs use undiluted samples. Whereas direct ISEs measure the electrolyte concentration in the plasma water fraction, indirect ISEs measure electrolyte concentrations in the diluted sample and multiply the results by the dilution factor. In 2011, more than 80% of the laboratories in the United States used indirect ISEs for measuring sodium, potassium, and chloride. In samples with significantly altered plasma water fraction, results with indirect ISEs can be misleading. POC and whole blood gas analyzers, as well as the thin-film electrode technology (Ortho/Vitros) and the Roche Integra systems, use direct ISEs and can be an alternative for rare samples with significantly altered plasma water fraction. An evaluation of a tertiary care hospital showed disagreements between direct and indirect ISE measured sodium in 25% of intensive care and 8% of hospitalized patients, mostly attributable to increased plasma water due to hypoproteinemia [69].

Adjustment for plasma water with indirect methods

In healthy individuals, plasma water occupies approximately 93% of the plasma volume. When solute concentrations are measured in plasma water, an adjustment factor of 93% is applied to report measurements in units of plasma. For example, a measured sodium concentration in plasma water of 156 mM is corrected to 145 mM, reported as plasma sodium concentration. Methods that measure solute concentration only in plasma water without dilution (direct methods) yield the same results independently of the true plasma water fraction and true plasma concentration of the solutes. Because the physiological activity of electrolytes and other solutes is proportional to their concentration in plasma water, this is an accepted convention and an appropriate approach to measuring the solutes. The problem occurs with methods that measure analytes after significant dilution of the plasma sample because that solute is now diluted in a much larger volume of water (indirect methods). For example, a change in plasma water fraction from 93% to 80% will result in a reduction in sodium levels measured with indirect methods of 12% relative to the measurement with direct methods (Fig. 9.1). To calculate the amount of change in plasma water fraction, and consequently in sodium and other solutes, several formulas are useful.

Plasma water fraction (PWF) can be estimated as follows [70]:

$$\begin{aligned}\text{PWF\%} = {} & 99.1 - 0.001 \\ & \times (\text{triglycerides} + \text{cholesterol in mg/dL}) \\ & - 0.73 \times \text{total protein (g/dL)}\end{aligned} \tag{9.11}$$

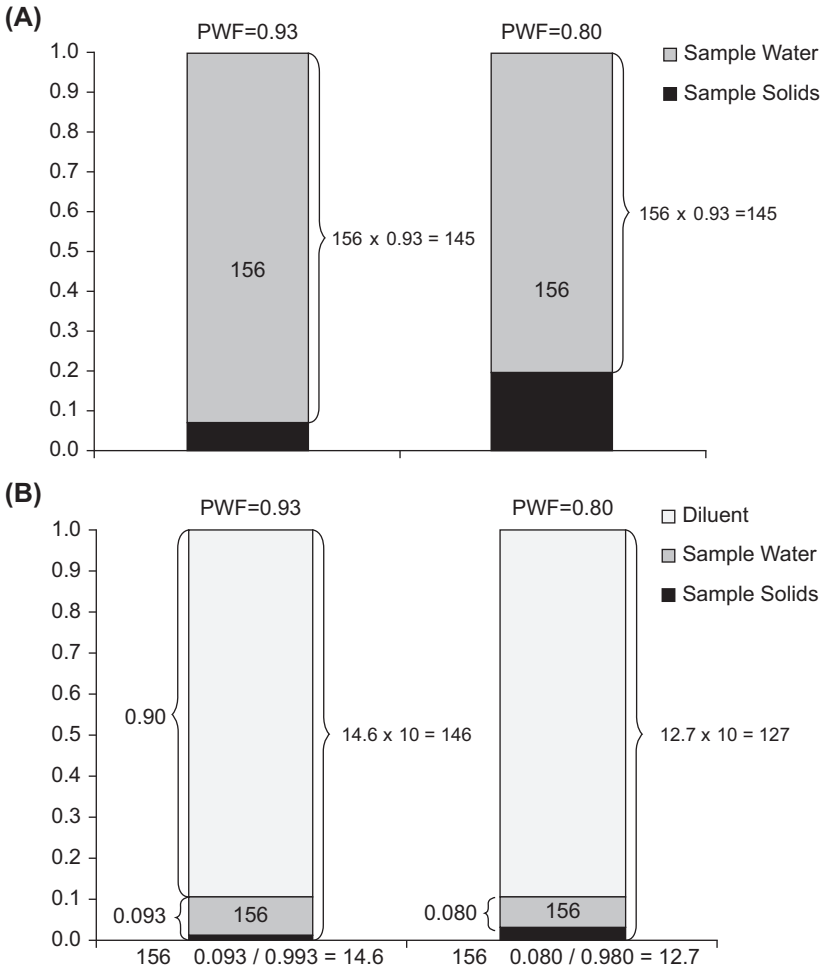


FIG. 9.1 Sodium concentrations in the plasma water fraction (PWF) of normal individuals (A) and in patients with high amounts of plasma solids (B). Note that a PWF of 80% is shown for illustrations purposes because it would need highly unusual lipid concentrations >14,000 mg/dL, total protein >25 mg/dL, or a combination of these (e.g., triglycerides = 3000 mg/dL with cholesterol = 500 mg/dL and total protein = 12 mg/dL).

If both direct and indirect sodium measurements are available and the diluent volume (in ml/mL of plasma) is known, PWF can be better estimated by the following formula [71]:

$$\text{PWF\%} = 93.3 \times \text{Diluent} \times \frac{\text{Indirect Na}^+}{\text{direct Na}^+ \times (0.933 + \text{Diluent}) - 0.933 \times \text{Indirect Na}^+} \quad (9.12)$$

Assuming a normal PWF of 93%, any plasma solute concentration can be calculated as follows:

$$\text{True concentration} = \frac{\text{Measured Concerntration} \times 93\%}{\text{Calculated PWF\%}} \quad (9.13)$$

Table 9.8 shows different values of calculated PWF depending on the plasma levels of triglycerides, cholesterol, and total protein. It can be seen that according to this formula, only extremes of protein concentrations have a significant water exclusion effect and that triglycerides and cholesterol even at the highest pathological levels have a minimal effect on plasma water and true solute concentrations. The error of less than 3% caused by plausible plasma lipid concentrations is below the total allowable error for sodium (±4 mM) and therefore only a minor source of inaccuracy. A rare exception is hypercholesterolemia caused by high levels of lipoprotein X in patients with severe cholestasis [72]. In one case report, a sodium level of 145 mM measured by direct potentiometry was reported as 124 mM by an indirect method [73]. The patient's total protein was 5.1 g/dL, triglycerides 208 mg/dL, and cholesterol 1836 mg/dL, resulting in calculated plasma water of 93%. However, high levels of lipoprotein X appear to occupy a larger volume than other lipoproteins and result in disproportional water exclusion. Using the direct − indirect measurement difference and a dilution factor of

TABLE 9.8 Calculated plasma water fraction (PWF) and true sodium concentration for a measured sodium cConcentration of 140.0 mM assayed with an iIndirect method in plasma containing different levels of protein, triglycerides, or cholesterol.

Protein level (g/dL)	Triglycerides (mg/dL)	Cholesterol (mg/dL)	Calculated PWF (%)	Estimated true sodium (mM)	% Error
7	60	200	93.7	138.9	0.8
20	60	200	84.2	154.6	−10.4
10	60	200	91.5	142.2	−1.6
4	60	200	95.9	135.7	+3.1
7	3000	200	90.8	143.4	−2.5
7	60	1000	92.9	140.1	−0.1

1/33, plasma water was calculated as 80%. More common causes of pseudohyponatremia (or pseudonormonatremia in patients with true high plasma Na^+) are elevated protein levels, whereas pseudohypernatremia occurs in patients with hypoproteinemia [69].

If protein abnormalities are thought to be the major cause of spurious sodium levels, an adjustment for total protein can be simplified in the following formula, with protein in g/dL and Na^+ in mM [74]:

$$\text{Protein Corrected Na} = \text{Na(indirect ISE)} - 10.53 + (1.3) \times \text{total protein} \tag{9.14}$$

A good approach to investigate hyponatremia is to directly measure serum osmolality: In the presence of pseudohyponatremia, the measured plasma osmolality will be normal, resulting in a gap to calculated osmolality; true hyponatremia will have a corresponding decrease in plasma osmolality.

In general, these free water correction or estimation formulas should be applied with caution, as even small differences among sodium measurements by different manufacturers of ISEs can result in errors of clinical impact when precise correction of hyponatremia is needed [75].

Direct ISEs are more susceptible to protein buildup and need frequent washing and calibration. In a patient with extremely high glucose levels (2916 mg/dL), a positive interference (+9 mM) with sodium measurement was seen with a direct sodium ISE but not with indirect ISE methods [76]. On the other hand, a different study did show reduced Na^+ and Cl^+ measurements by indirect ISE compared to direct ISE in the presence of $>$ 2000 mg/dL of glucose, particularly in urine samples [77]. These *in vitro* effects must be distinguished from true dilutional hyponatremia caused by hyperglycemia *in vivo*.

In whole blood analyzers, interference by the red blood cells in potentiometric readings may occur. In one study, sodium levels measured by the Radiometer ABL800 direct ISE decreased by 0.15 mM for each 1 g/dL increase in blood hemoglobin [78].

The Cl^- electrodes use an organic polymeric salt-exchanger membrane to achieve selectivity for Cl^-. However, other lipophilic anions, including the halides bromide and iodide, thiocyanate (increased in smokers, consumers of milk and vegetables such as broccoli, and after nitroprusside treatment), nitrates, salicylates, heparin, lactate, bicarbonate, and β-hydroxybutyrate can also interact with the membrane and cause falsely elevated Cl^- results [58,79]. These high-polarity membranes are particularly susceptible to protein precipitation and need careful maintenance.

High levels of ascorbic acid (~12 mM) can interfere with Na^+ (+43%), K^+ (+58%), Ca^{2+} (+103%), and Cl^- (−33%) measured by the Beckman Synchron LX20 ISEs, possibly by redox reactivity at the ISE membrane [80]. Other strong redox agents, such as *N*-acetylcysteine, cysteine, glutathione, guanidine, procainamide, and sodium azide, show similar interferences.

Case Report: An 18-year old woman with a history of immune thrombocytopenic purpura presented to the emergency room with severe headache and seizure activity and was diagnosed with acute subarachnoid hemorrhage [81]. The patient was treated with platelet transfusions and boluses of 3% normal saline and subsequently developed uncal herniation, loss of brain stem reflexes and diabetes insipidus with severe hypernatremia. During the next 3 days, the patient was treated with a total of 130 g of intravenous immunoglobulin (IVIG). In the second day, her sodium levels were 175 mmol/L with the Radiometer ABL800 FLEX whole blood direct ISE, 170 mmol/L with the Siemens epoc point of care whole blood direct ISE analyzer, and 160 mmol/L with the Siemens Dimension EXL indirect ISE analyzer. Her total protein levels during IVIG therapy ranged from 8.0 to 9.6 g/dL. The corrected sodium by Eq. (9.14) was 4 mmol/L below the direct ISE sodium level, highlighting the limitations of correction formulas and the need to use alternative measurement methods when interferences are suspected.

Total CO_2

Two main methods are in use:

Potentiometry: The plasma is diluted, acidified to convert all HCO_3^- to CO_2, and the pCO_2 is measured with a pCO_2 electrode. Most instruments use indirect potentiometry, whereas Vitros analyzers use direct potentiometry in thin-film electrodes. These ISE methods are subject to interferences by strong redox substances (see Table 9.2). Note that whole blood gas analyzers do not measure HCO_3^- directly; rather, pCO_2 and pH are measured by potentiometry, whereas HCO_3^- is calculated from the equilibrium equation of Henderson–Hesselbach:

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+ \quad (9.15)$$

Enzymatic methods: The plasma is alkalinized to convert all CO_2 to HCO_3^-, and the HCO_3^- is used by phosphoenolpyruvate (PEP) carboxylase to convert PEP to oxaloacetate, which is then reduced to malate by malate dehydrogenase, while generating NAD^+ from NADH. The decrease in NADH concentration, measured by absorbance at 340 nm, is proportional to the amount of HCO_3^-. Interferences are minimal and only seen at very high levels of hemolysis (free hemoglobin > 800 mg/dL), hyperbilirubinemia (>30 mg/dL), or with specimen turbidity caused by paraprotein precipitation [82] or extreme hyperlipemia (triglycerides > 3,500 mg/dL) [83].

Calcium assays

Total calcium is measured by chemical methods in most analyzers, except in the Beckman Synchron instruments, which use an indirect ISE to measure ionized calcium after diluting the sample with strong calcium complexing reagents. These reagents dissociate Ca^{2+} from proteins and anions, resulting in a standardized molar ratio of ionized and total calcium in the solution at equilibrium. The chemical methods use a colorimetric approach after releasing protein-bound calcium by acidification followed by complexing calcium with a chromogen. Some methods use an arsenazo III dye under alkaline conditions, whereas others use *o*-cresolphthalein in acidic pH to produce a colored complex with calcium. Reaction conditions and buffers have been optimized to enhance selectivity for calcium over magnesium and improve linearity.

Interferences with calcium methods include calcium chelators in the sample, either as inappropriate anticoagulants such as EDTA, citrate, and oxalate, or *in vivo* as a result of transfusion with citrate containing blood products or apheresis with citrate anticoagulant infusion. Heparin reduces free ionized calcium (~0.01 mM per unit/mL of heparin) but has no significant effect on total calcium. The use of balanced or calcium-titrated lyophilized heparin reduces but does not eliminate this effect, whereas lithium–zinc heparin does not show significant interference with free Ca^{2+} determinations [42,58]. Spectral interferences by high levels of hemolysis, bilirubin, and lipemia are common with colorimetric methods and minimal with ISE methods. In case of significant spectral interference, it is possible to treat the samples with ethyleneglycol-tetraacetic acid (EGTA) to chelate all the calcium and subtract the results from the initial measurement. Contamination by calcium compounds can occur in glass tubes or cork-capped tubes, but these are rarely used in clinical practice.

Magnesium assays

As with calcium, total magnesium is commonly measured by spectrophotometry after forming complexes with dyes, such as arsenazol, calmagite, magon, or methylthymol blue, and therefore is susceptible to chromogenic interferences, such as high levels of bilirubin and hemolysis. Specificity for magnesium is not absolute, and calcium interference is reduced by a chelator such as EGTA. Free Mg^{2+} can be measured by magnesium-specific electrodes, although these are not as reliable as calcium ISEs and suffer from interference from free calcium, heparin, certain tube additives and silicone lubricants, and thiocyanate. To correct for calcium interference, Ca^{2+} is simultaneously measured by a parallel calcium-specific electrode, and the results are used to adjust the Mg^{2+} measurement. As with calcium, albeit to a lower extent, changes in pH should be avoided to minimize disturbing the proportion of free and total Mg^{2+}. For most clinical purposes, assays for total magnesium are more reliable and cost-effective than free Mg^{2+} measurements. The newer heparins developed to minimize interferences with free Ca^{2+}, such as zinc or lithium heparin, can cause spurious increases in total magnesium and should be avoided.

Phosphate Assays

Inorganic phosphate is commonly assayed by reaction of phosphate ions with ammonium molybdate to form a phosphomolybdate complex that is then measured spectrophotometrically, either by direct UV absorption at 340–363 nm or after reaction with a reducing agent to increase stability and color formation, measured at 600–700 nm [42]. Methods using UV absorption are more susceptible to interference by lipemia, hemolysis, and hyperbilirubinemia. Analyzers that utilize dry-film slides to remove bilirubin, lipids, hemoglobin, and other proteins from the analytical layer are less susceptible to these interferences.

A common cause of interference in phosphate assays is the presence of monoclonal immunoglobulins and

paraproteins associated with multiple myeloma and Waldenström macroglobulinemia, which interfere with the colorimetric reaction and cause spurious hyperphosphatemia and, rarely, false hypophosphatemia [84,85]. Hyperphosphatemia in the presence of normal calcemia and renal function and in the absence of other causes of hyperphosphatemia, such as hypoparathyroidism and acromegaly, should prompt suspicion for a monoclonal gammopathy [86]. Other less common interferences include Alteplase, a catheter flushing solution containing high phosphorus excipient, liposomal amphotericin B, heparin, mannitol, fluoride, and tissue plasminogen activator.

BLOOD GASES ANALYSIS

Whole blood gas analyzers provide rapid assessment of the patient's acid–base and ventilation status, which are essential in the treatment and monitoring of critically ill patients, as well as some patients with chronic metabolic and respiratory diseases. Typically, these analyzers offer direct measurements of the partial pressures of oxygen (pO_2) and carbon dioxide (pCO_2) in blood, as well as pH and electrolytes (Na^+, K^+, Cl^-, and Ca^{2+}), hemoglobin (with or without co-oximetry for oxy-, deoxy-, carboxy-, met-, and fetal-hemoglobin measurement), and a variety of calculated parameters, including HCO_3^- concentration and hematocrit. Many blood gas analyzers also offer measurements of other substances of interest in critical care medicine, including glucose, lactate, creatinine, urea, and bilirubin. In this section, the methodologies and sources of error in pH, blood gas, and co-oximetry measurements are discussed; electrolytes and metabolites are reviewed in other sections.

Pre-analytical issues

Arterial blood samples are necessary for evaluation of oxygen status because the pO_2 and derived calculated parameters are significantly different between arterial and venous blood. In most patients, arterial pO_2 is 60–70 mmHg higher than venous pO_2. However, in many patients, assessing the oxygen status is not as critical and can be estimated from pulse oxymetry or transcutaneous monitoring of oxygen saturation (SaO_2), and venous blood can effectively be used to assess the acid–base status, avoiding the difficulties and complications of arterial puncture. Several studies examined the differences between venous and arterial blood gases and concluded that in mildly to moderately ill patients without severe hemodynamic or cardiorespiratory failure, pH, pCO_2, and calculated HCO_3^- were sufficiently accurate to estimate acid–base status [87–90]. Arterial–venous differences for pH averaged +0.032 (range, 0.009–0.050), whereas pCO_2 averaged −4.9 (range, −1.6 to −7.0 mmHg) and calculated HCO_3^- averaged +1.40 (range, −0.15 to +1.88 mM).

Samples for blood gas analysis should be collected in gas-impermeable syringes, coated with lyophilized heparin, and sealed after collection to prevent gas exchange. Syringes should be held tip up, lightly tapped, and a drop of blood ejected to remove all air bubbles before placing a tight cap. Drawing blood through a butterfly infusion set can increase pO_2 by diffusion through the plastic lines [91]. Liquid heparin, which equilibrates with ambient air, can have a significant effect on blood gas analysis, leading to decreases in pCO_2 and increases in pO_2. Likewise, large bubbles or entrapped air will cause pO_2 to equilibrate with ambient air (with $pO_2 = 160$ mmHg at sea level) and pCO_2 to decrease (pCO_2 of ambient air $= 0.25$ mmHg). The change in pCO_2 is usually mild because of the capability of the plasma bicarbonate to buffer any changes in pCO_2. However, even a small bubble comprising 0.5% of the blood volume will quickly cause an increase of 8 mmHg in pO_2 [92]. Due to rapid changes in air pressure and agitation, this problem is exacerbated if the samples are transported in pneumatic tube systems, even when no bubbles are visible [93]. Therefore, samples should be hand-delivered to the laboratory when accuracy of pO_2 is essential.

Samples should be analyzed as soon as possible. If the analysis is delayed more than 15–30 min, the samples should be collected in gas-impermeable syringes and kept on ice to minimize cellular respiratory activity. Samples kept at room temperature in gas-impermeable containers show an average decrease in pO_2 of 2–4 mmHg/h, whereas pCO_2 increases approximately 1 mmHg/h and pH decreases 0.02–0.03 units/h [42,94]. These changes are accentuated at higher temperatures and in patients with markedly elevated leukocyte, platelet, or erythrocyte counts; for example, patients with greater than 100,000 leukocytes/μL can have a drop in pO_2 of 40 mmHg in 5 min. When the specimen is placed on ice, these changes are reduced by more than 50%, except in samples collected in plastic syringes, which have increased permeability to oxygen in ice water compared to ambient air. Plastic syringes allow some gas exchange on ice resulting in changes in pO_2 of 2–15% (either increase or decrease depending on whether the patient's pO_2 is lower or higher than ambient pO_2 of 150 mmHg) [94,95]. Plastic syringes should be kept at room temperature and analyzed in less than 30 min. If delays are unavoidable, gas-impermeable glass syringes kept on ice should be used.

Prolonged application (more than 1 min) of a tourniquet for collection of venous blood, especially when coupled with fist clenching, can lead to oxygen consumption and lactic acidosis with a drop in pH. Application of tourniquet for 30 s followed by release before collection is recommended [96].

Blood gas sampling of venous and arterial cord blood is widely used to assess the respiratory and acid-base status of newborns. The small umbilical artery and the large umbilical veins are sampled, but only the arterial cord blood reflects the physiologic status of the newborn; the venous sampling is done only for confirmation of the arterial sampling by showing a significance arterial-venous difference in pH and pCO_2. In the umbilical cord the venous blood carries oxygen to the fetus and the arterial blood carries CO2 from the fetus, therefore the pCO2 should be > 3.75 mmHg higher and the pH lower by more than 0.02 units in cord arterial blood.

Severe cord arterial blood acidemia ($pH < 7.00$) is associated with intra-partum hypoxia and conversely, a normal pH is good evidence against intrapartum events and complications such as cerebral palsy. In addition, a calculated base excess (BE) < -12 mM or < -16 mM is a good marker of metabolic acidosis, and in contrast to pH which is logarithmic, BE changes with a linear correlation to the degree of acidosis. See section below for issues related to BE calculation.

In addition to those pre-analytic issues discussed above, such as the importance of avoiding bubbles, incomplete mixing, and analysis delays of more than 30 min, the following should be considered for accurate cord arterial blood gas interpretation:

(1) Since the placental gas exchange ceases after delivery, delayed clamping of the cord even by 45 s can result in significant decreases in pO_2, pH and BE, and increases in pCO_2 and lactate. However, once the newborn pulmonary ventilation is established, the pO_2 increases and the pCO_2 decreases rapidly, but the accumulated organic acids such as lactate persist and the pH and BE will continue to decrease [97]. Compared to baseline at time 0, 60 min of delayed clamping can reduce pH by 0.2 pH units and BE by 9.2 mM [98]. If delayed clamping greater than 20 min is desired, cord blood sampling should be done within 30 s of delivery in the un-clamped cord [97].

(2) Delayed sampling post-clamping of the cord has a significant effect on cord arterial blood results: pH decreases about 0.05 and BE decreases by about 4 mM for each 30 min delay in sampling after cord double clamping [99]. These values fall at the similar rates irrespective of intra-partum events and the newborn status, therefore adjustments can be made for proper interpretation of pH and BE results if sampling is delayed.

(3) As pH and BE are relatively stable in gas-impermeable syringes after collection, analytical delays of up to 60 min are acceptable for arterial cord blood gas interpretation.

Analytical issues

Methods for blood gas analysis rely on electrochemical measurements, either by potentiometry, in the case of the pCO_2 and pH electrodes, or by amperometry, in the case of the pO_2 electrode. The pH electrode has a glass membrane that exchanges metal ions with protons on both sides of the membrane. A change in H^+ concentration on the sample side of the membrane generates a potential difference across the membrane that is proportional to the pH. The pCO_2 electrode has a membrane permeable only to uncharged molecules such as CO_2, O_2, and N_2. When CO_2 diffuses across the membrane into the electrolyte solution, it dissociates into HCO_3^- and H^+, which generate a potential difference across the H^+-impermeable membrane that is proportional to the pCO_2. In the pO_2 electrode, oxygen diffuses across an oxygen-permeable membrane and is reduced to water on the cathode (usually a platinum rod), consuming electrons and H^+ ions. To complete the current, in the anode silver ions are oxidized to Ag^+ with electron release and subsequently complexed with Cl^- to form a deposit of AgCl on the anode. The size of the current generated from the consumption of electrons during oxygen reduction is proportional to the amount of oxygen in the sample (pO_2).

Bicarbonate concentration is calculated from the measurement of pH and pCO_2 using the following Henderson–Hesselbach equation (pCO_2 in mmHg and HCO_3^- in mM):

$$\mathrm{pH} = \mathrm{pK}' + \log\frac{\mathrm{HCO_3^-}}{\alpha \times p\mathrm{CO_2}} \tag{9.16}$$

In most patients, $\mathrm{pK}' = 6.103$ and $\alpha = 0.0306$, but these factors depend on temperature and the ionic strength of the solution. Marked changes in ionic strength of the sample, as well as extremes of protein and lipid concentration, can affect the CO_2 solubility coefficient [42]. Therefore, in some patients, particularly acutely ill children, calculated HCO_3^- based on pCO_2 and pH measurements in blood gas analyzers is less reliable than total CO_2 measured by the routine chemistry analyzers.

Oxygen parameters are calculated from directly measured pO_2 and hemoglobin parameters; the following are examples (Hgb in g/dL and pO_2 in mmHg):

$$\text{Oxygen saturation } (SaO_2) = \text{Oxyhemoglobin}/(\text{deoxyhemoglobin} + \text{oxyhemoglobin}) \tag{9.17}$$

$$\text{Fraction of oxygenated hemoglobin } (FO_2Hb) = \text{oxyhemoglobin/total hemoglobin} \quad (9.18)$$

$$\text{Blood oxygen content } (ctO_2) = 1.36 \times FO_2Hb \times Hgb + 0.0031 \times pO_2 \quad (9.19)$$

It is important to understand the difference between FO_2Hb and SaO_2 because they differ significantly in patients with significant amounts of abnormal hemoglobins, such as carboxy- or methemoglobin, which cannot carry oxygen. Oxygen saturation is commonly measured by pulse oxymetry, which simply measures oxyhemoglobin (O_2Hb) and deoxyhemoglobin (HHb) by transdermal spectrophotometry at two wavelengths. SaO_2 can also be calculated from empirical formulas using pH, pO_2, and hemoglobin, but these formulas assume normal oxygen binding by hemoglobin and are unreliable in patients with changes in the O_2–hemoglobin dissociation curve. In contrast, the more sophisticated co-oxymeter analyzers scan absorbance of a whole blood hemolysate at all wavelengths between 535 and 670 nm and calculate the fraction of each hemoglobin fraction by their characteristic absorption profile.

In most patients, FO_2Hb and SaO_2 are roughly interchangeable. For example, in a patient with 94% O_2Hb and 3% HHb, $SaO_2 = 94/97 = 96.9\%$. However, in a patient with 85% O_2Hb, 3% HHb, and 12% carboxyhemoglobin (COHb), $SaO_2 = 85/88 = 96.6\%$, which is significantly different from FO_2Hb of 85%. With pulse oxymetry alone, this patient may be erroneously assumed to have normal blood oxygen content, whereas co-oxymetry would demonstrate the impairment in oxygen content and therefore the decreased oxygen delivery to the tissues.

Base excess (BE) is a measure of the non-respiratory component of acid-base disturbances and can help distinguish e.g., acidemia with pure respiratory acidosis, with normal BE, from metabolic acidosis with decreased BE (equivalent to increased base deficit). BE is commonly used to assess the acid-base status of the newborn (see section above). BE corresponds to the amount of base or acid necessary to titrate one liter of blood to a normal pH of 7.40. Calculations for BE are available for the blood BE_{blood} and for the extracellular fluids BE_{ecf} (hemoglobin − Hb in g/dL and bicarbonate in mM)

$$BE_{blood} = (1 - 0.014 \times Hb) \times \left([HCO_3^+] - 24\right) + (1.43 \times Hb + 7.7) \times (pH - 7.400) \quad (9.20)$$

$$BE_{ecf} = [HCO_3^-] - 24.8 + 16.2 \times (pH - 7.400) \quad (9.21)$$

Eqs. (9.20) and (9.21) may be slightly different in different analyzers and it is important to consult the procedure manual if one wishes to compare results from different manufacturers.

The International Federation for Clinical Chemistry no longer recommends BE_{blood} for clinical evaluation of acid-base status, because the BE_{ecf} better represents the available buffering capacity in the patient. In adults, the differences are small, but in the neonate, with a larger extracellular fluid volume and high hematocrit, it is important to ensure that base excess is calculated with the BE_{ecf} formula.

One source of inaccuracy in BE calculations is the Haldane effect, which represents the effect of oxygen saturation in the buffering capacity of hemoglobin [100]. This can be corrected as follows (SaO_2 in % and Hb in g/dL):

$$BE_{ecf-corrected} = BE_{ecf-} - 0.3 \times Hb \times (100 - SaO_2) \quad (9.22)$$

Measurement of blood gases is highly dependent on atmospheric pressure and ambient temperature, which can vary during the day. Therefore, frequent calibration, usually every 30 min, is performed to account for these fluctuations. In some electrodes, exposure of the patient to other gases, such as NO and halothane, can spuriously increase pO_2 measurements.

Co-oxymetry depends on photometric scan and therefore is affected by substances that absorb in the range of 535–670 nm, such as methylene blue, which is used in the treatment of methemoglobinemia and absorbs strongly in the 550- to 700-nm region. A concentration of 25 mg/L of methylene blue will spuriously increase FO_2Hb and reduce methemoglobin results by approximately 4–8% points, which may lead to misleading interpretation of the efficacy of methylene blue treatment [101]. Other dyes, such as Patent Blue V, Evans Blue, and Cardio Green, show variable amounts of interference with co-oxymetry, usually less than 1% point. Highly lipemic samples can also be affected; for example, a 4% concentration of Intralipid will increase carboxy- and methemoglobin by 0.5–0.9%. Some co-oxymeters measure hemoglobin F fraction (HbF), which is more susceptible to interference by abnormal pH, blue dyes, and lipemia. At high levels seen in neonates and hereditary persistence of fetal hemoglobin, HbF can cause decreases in HHb and SaO_2 results. Other colored substances causing significant interference with co-oxymetry are vitamin B_{12} preparations. For example, a concentration of 200 μg/mL of cyanocobalamin (more than 10-fold maximum attainable serum levels) can cause a 2% point decrease in FO_2Hb measured by the Radiometer ABL800. Similarly, high doses of hydroxycobalamine, a

red chromophore used to treat cyanide poisonings, can cause interference in several spectrophotometric methods, including carboxyhemoglobin and methemoglobin by co-oxymetry, alanine aminotransferase, amylase, bilirubin, cholesterol, creatinine, creatine kinase, lactate, magnesium, total protein, and uric acid [102,103].

LACTATE ANALYSIS

Evaluation of lactic acidosis is important in the assessment of many critically ill patients. Lactic acid production increases when tissues are subject to anaerobic conditions (tissue hypoxia), such as with hemodynamic impairment or respiratory failure. Lactic acidosis due to tissue hypoxia is classified as type A, whereas the rarer type B is due to metabolic diseases interfering with mitochondrial function (e.g., diabetes, drugs such as ethanol, methanol, and salicylates, and several inborn errors of metabolism). Although L-lactate is the predominant isomer generated by human cells, intestinal bacteria can produce large amounts of D-lactate, which is slowly metabolized by human tissues. In certain situations, such as with jejunoileal bypass or other causes of short bowel syndrome, impaired carbohydrate absorption leads to increased production of D-lactate by colonic gram-positive anaerobic bacteria and accumulation in the systemic circulation with metabolic acidosis. D-Lactate is not measured by assays using enzymes specific for L-lactate.

Several studies associate lactic acid levels with mortality. For example, in patients admitted to the ICU, elevated lactate at admission was associated with a 24% mortality, and patients with persistent lactic acidosis after 24 h of treatment had 82% mortality [104]. Routine monitoring of lactate is indicated in acutely ill patients at risk for tissue hypoxia due to either decreases in oxygen delivery or increases in oxygen consumption, and recent sepsis protocols using lactate-guided resuscitation insist on measuring lactate initially and remeasuring it in 2–4 h if initial results are >2 mM [105]. Note that to minimize false positive or negative calls, it is preferable to use the upper reference range limit provided by the laboratory rather than the 2 mM cutoff because different methods have slightly different ranges and normal lactate concentrations differ in venous and arterial blood.

Even minor exercise can rapidly increase lactate levels, and a minimum resting period of 30 min is indicated before sample collection. Hyperventilation, catecholamine stimulation, and infusion of intravenous glucose or bicarbonate also lead to increased lactate production.

Lactate measurements are typically available in whole blood gas analyzers and reported with arterial blood gas profile. However, if assessment of oxygen status is not essential, venous blood is preferable for assessment of lactic acidosis to avoid the complications of an arterial puncture. Several studies show that venous lactate levels are slightly higher and correlate strongly with arterial lactate levels. For example, a study in emergency department patients showed a regression R^2 for the equation arterial lactate $= 0.996 \times$ venous lactate $- 0.259$ [106]. This equation can be used to predict arterial lactate, or clinical cutoff values can be slightly adjusted for venous lactate. In cases of impaired hemodynamics (e.g., in shock), the arterial–venous lactate difference will increase and can actually be used as a measure of tissue perfusion.

A study in healthy volunteers showed that application of a tourniquet for up to 5 min before blood collection had no effect on lactate levels [107], however, prolonged tourniquet application together with repeated fist clenching will increase lactate and should be avoided. The same study also determined that storage on ice is not necessary if the sample is analyzed within 15 min. However, in cord arterial whole blood, lactate levels rise quickly (about 0.012 mM/min) at room temperature, perhaps because of high hematocrit and anaerobic glycolysis, and therefore cord blood samples for lactate analysis should always be immediately refrigerated [108]. Samples should be stored on ice if separation from cells cannot be achieved in less than 15 min. Alternatively, samples may be collected in gray-top tubes containing sodium fluoride and potassium oxalate, which show a minimal increase (averaging 0.3 mM) after 8 h at room temperature [109].

Analytical issues

Lactate measurements are typically performed either in whole blood by amperometry or in serum by colorimetric methods. The whole blood analyzers and some high-throughput instruments use an electrode separated from the sample by a multilayer membrane. Lactate crosses the outer membrane and is oxidized to pyruvate by lactate oxidase immobilized between the outer and inner membranes. The reaction consumes O_2 and produces H_2O_2, which crosses the inner membrane and is oxidized at the platinum anode, whereas the silver cathode is reduced to produce a current proportional to the amount of lactate. Potent redox agents such as cysteine, fluoride, bromide, iodide, thiocyanate, isoniazid, acetaminophen, and hydroxyurea may show interference with this method.

The colorimetric methods use either L-lactate oxidase or L-lactate dehydrogenase. The lactate oxidase generates H_2O_2, which then oxidizes a chromogen in a reaction catalyzed by peroxidase to generate a product measurable by spectrophotometry. Lactate dehydrogenase methods rely on the conversion of lactate to

pyruvate at high pH, with generation of NADH and increased absorption at 340 nm. D-Lactate is not measured by these assays and requires a specific method using D-lactate dehydrogenase.

Because the lactate oxidase is not entirely specific for lactate, some methods based on this enzyme (i.e., the Radiometer ABL) are subject to positive interference by citrate, oxalate, glycolate, and glyoxylate [110]. The latter three substances are ethylene glycol metabolites and may result in spuriously high lactate levels in patients who ingested ethylene glycol. For a list of household products containing ethylene glycol, see the National Institutes of Health reference [111]. Other lactate oxidase methods (e.g., iSTAT, Abbott Architect, Ortho Vitros, Siemens Advia, Roche Modular, and Beckman Synchron) show less interference, whereas LDH-based methods (e.g., Siemens Dimension) are not subject to interference by these metabolites. Based on these differences, a "lactate gap" between the Radiometer measurement and methods unaffected by glycolate and glyoxylate may be indicative of ethylene glycol poisoning [110,112]. The magnitude of the interference may be variably dependent on the method and the age of the lactate electrode [113]; therefore, it should not be used as a quantitative approach to estimating amounts of ethylene glycol.

Patients with elevated LDH may show spurious decreases in lactate concentrations due to its conversion to pyruvate *in vitro*. To avoid this interference, plasma or serum samples should be frozen or analyzed rapidly after collection. Not all assays show this interference; for example, up to 4000 IU/L of LDH does not interfere with the Beckman SYNCHRON lactate assay.

Case report

A 72-year-old male was admitted to the ICU for cardiac failure and accidental ingestion of a large amount of propylene glycol. Laboratory results included an anion gap of 27 mEq/L, metabolic acidosis with pH = 7.16, and low amounts of propylene glycol in the blood. An arterial blood gas measurement using the Radiometer ABL 700 analyzer revealed 39 mM lactate, whereas a serum sample measured with the Vitros analyzer showed normal lactate levels. Measurement with a D-lactate-specific kit resulted in D-lactate levels greater than 100 mM [114]. This case highlights that propylene glycol can be metabolized to D-lactate in the absence of intestinal malabsorption, and that the Radiometer method is not entirely specific for L-lactate and shows positive interference by D-lactate.

BILIRUBIN ANALYSIS

Bilirubin is an end product of heme metabolism. The major source for heme production is hemoglobin; therefore, bilirubin generation depends on the rates of hemoglobin synthesis and degradation. Bilirubin originating from heme metabolism in the various tissues, predominantly from the phagocytic system and ineffective erythropoiesis, binds noncovalently to albumin and is carried by the circulation to the liver, where it is further metabolized by conjugation to glucuronic acid, a reaction catalyzed by the uridine diphosphate glucuronyltransferase enzyme coded by the UGT1A1 gene. Excretion of conjugated bilirubin then occurs via the biliary system into the intestines, where 95% of it is reabsorbed into the circulation while the remainder is further metabolized by intestinal bacteria into urobilinogen, which can also be reabsorbed (~20%). Circulating urobilinogen and conjugated bilirubin are soluble in the plasma and can be excreted by the kidneys.

Main causes of hyperbilirubinemia include the following:

1. Defects in conjugation, including severe liver insufficiency, genetic defects in UGT1A1 (Crigler–Najjar and Gilbert syndromes), and inhibition of UGT1A1, leading to increased blood unconjugated bilirubin levels
2. Increased heme catabolism—for example, associated with severe hemolysis—leading to elevated blood levels of unconjugated bilirubin
3. Defects in biliary excretion ("cholestasis"), caused by diseases interfering with liver or biliary architecture, which cause increases predominantly in conjugated bilirubinemia

Accurate measurement of unconjugated bilirubin is of particular importance in neonates because this substance penetrates the blood–brain barrier and can cause severe encephalopathy, known as kernicterus. Determination of conjugated bilirubin is also critical in the evaluation of neonatal jaundice because severe diseases such as sepsis, biliary atresia, and hepatitis are associated with elevated conjugated bilirubin. In adults, measurement of total bilirubin is a relatively sensitive test for liver and biliary dysfunction. In hepatobiliary diseases, discrimination between conjugated and unconjugated hyperbilirubinemia can help distinguish pure cholestastic conditions, which elevate conjugated bilirubin, from those involving hepatocytes, which typically lead to increases in both conjugated and unconjugated bilirubin. However, testing for both total and unconjugated bilirubin is often unnecessary because other markers of hepatic or biliary disease will be abnormal.

Pre-analytical issues

Starvation and caloric malnutrition can be associated with large increases in unconjugated bilirubin,

especially in patients with Gilbert syndrome. Several substances affect bilirubin glucuronidation, and unconjugated bilirubin can rise as a result of elevated thyroid hormones, estrogens, oral contraceptives, fatty acids in breast milk, antibiotics such as novobiocin or gentamicin, or anti-retroviral agents such as indinavir and atazanavir. Other drugs, such as sulfonamides and ibuprofen, raise unconjugated bilirubin by displacing it from albumin binding sites. Recent alcohol ingestion, smoking, meals, and exercise have all been associated with mild increases in bilirubin [1,2].

Prolonged tourniquet application or standing can increase unconjugated bilirubin by approximately 10% due to its binding to albumin [2]. Because bilirubin is sensitive to degradation by light, samples should be shielded from exposure to light during pre-analytical processing if analysis is delayed. A decrease of as much as 50% can occur with 1 or 2 h of exposure to light [1].

Analytical issues

Fractionation by chromatography shows that bilirubin circulates in four main forms (Fig. 9.2) [115]:

1. Unconjugated bilirubin, loosely bound to albumin (α-bilirubin)
2. Bilirubin monoglucuronide (β-bilirubin)
3. Bilirubin diglucuronide (γ-bilirubin)
4. Bilirubin conjugates covalently bound to albumin (δ-bilirubin)

In normal individuals, only unconjugated bilirubin (α) is present in the serum. The delta fraction occurs by substitution of albumin for the glucuronide moiety of conjugated bilirubin and is increased in neonates and in patients with prolonged hyperbilirubinemia. Whereas the half-life of fractions α−γ is short (minutes in normal individuals), δ-bilirubin has the half-life of albumin (2 or 3 weeks) and complicates assessment of recovery of jaundice due to cholestasis. Bilirubin is determined most commonly by a colorimetric reaction between diazotized sulfanilic acid and bilirubin—the so−called "diazo reaction." Initially described by Erlich in 1883, the reaction has been subject to several modifications to increase reactivity with unconjugated bilirubin. Most current methods are derived from the diazo method of Jendrassik and Grof (J/G), in which caffeine benzoate is added to the reaction to displace unconjugated bilirubin from albumin and accelerate its reaction with the diazo chromogen. Other less commonly used accelerators include methanol, dyphylline, and various surfactants. When the accelerator is present, all of the bilirubin fractions (α−δ) react quickly with the diazo dye, and the result is labeled as "total bilirubin." When the accelerator is omitted, unconjugated bilirubin reacts very slowly, the conjugated fractions (β, γ) react quickly but incompletely, and δ-bilirubin reacts to a variable extent; the result is labeled "direct bilirubin" (Fig. 8.2). Direct methods can variably measure between 1 and 10% of unconjugated bilirubin, so their specificity for conjugated fractions is more important than their accuracy [116] because unconjugated bilirubin is estimated from "indirect bilirubin," calculated by subtracting "direct" from "total" bilirubin.

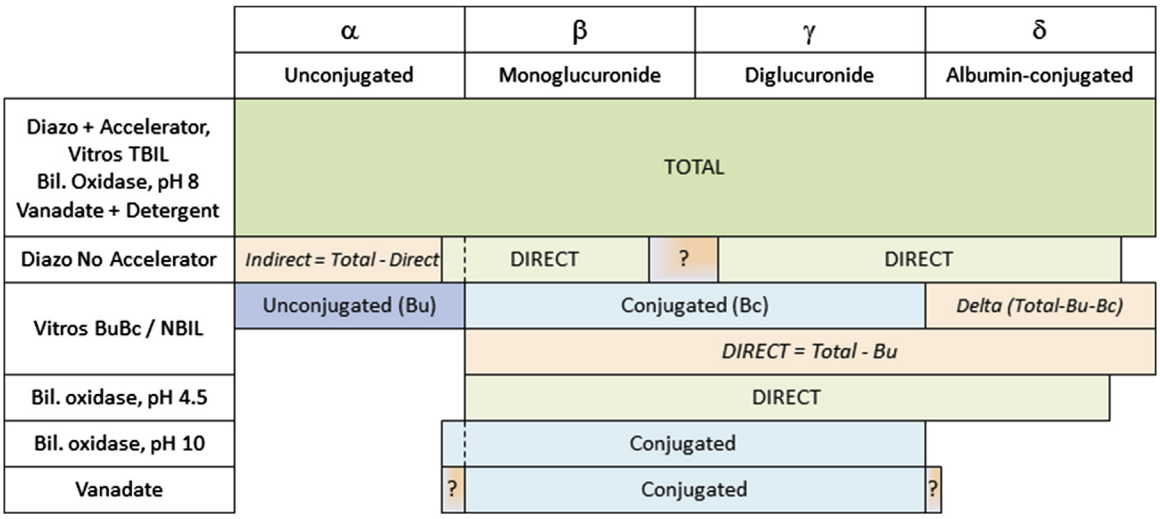


FIG. 9.2 Distribution of bilirubin fractions measured with diazo assays derived from the Jendrassik and Grof method, from the Vitros BuBc dry chemistry method, or from the vanadate and bilirubin oxidase methods. Calculated fractions are depicted in italics. The graph shows that 10−25% of δ-bilirubin may not react with direct methods [131], whereas 1−10% of unconjugated bilirubin may react with diazo or bilirubin oxidase methods. "Var" refers to a variable portion of conjugated bilirubin that is not measured by diazo direct methods. The question mark reflects the unknown reactivity of vanadate oxidation methods with α and δ fractions.

More recent methods include spectrophotometric determination of the oxidation of conjugated bilirubin by vanadate or bilirubin oxidase to biliverdin, with a resulting decrease in absorbance at 425–460 nm. In both of these methods, only conjugated bilirubin fractions (β and γ) are oxidized. Manipulation of pH or addition of detergents allows unconjugated bilirubin to also be oxidized for total bilirubin determination (Fig. 8.2). In the Ortho "dry chemistry" method (BuBc slide), the absorbance of conjugated (β and γ) bilirubin is differentiated from that of unconjugated (α) bilirubin by spectrophotometric scanning in the presence of a cationic mordant (Fig. 9.2). Because δ-bilirubin is not detected in these recent methods, the conjugated (Bc) and unconjugated (Bc) fractions better represent the functional status of bilirubin production, conjugation, and excretion and respond quickly to changes such as removal of biliary obstruction. One disadvantage of spectrophotometric scanning methods is the susceptibility to spectral interference by substances such as amphotericin B, levodopa, methotrexate, nitrofurantoin, sulfasalazine, hemoglobin, and bilirubin isomers originating from light exposure.

The classic diazo method is particularly susceptible to interferences due to the presence of serum substances such as free hemoglobin, immunoglobulins (paraproteins), lipemia, acetoacetate, and ascorbic acid [1,85]. More recent methods using different diazo reagents with serum blanking, bilirubin oxidase, or vanadate are less susceptible to such interferences.

Case report

A 37-year-old male was admitted for a suicide attempt by ingestion of naproxen in combination with ethanol. A liver profile showed only mild elevation in aspartate aminotransferases (66 U/L) and γ-glutamyl-transpeptidase (53 U/L), with normal alanine aminotransferase and alkaline phosphatase, and a total bilirubin concentration of 20.5 mg/dL [117]. There was no past medical history or physical evidence of liver disease. Naproxen serum levels were138 μg/mL—well above the therapeutic level. Measurement of bilirubin with the Vitros BuBc method shows levels of Bu and Bc below 0.1 mg/dL. Another similar case showed a naproxen level of 167 μg/mL, and total bilirubin determinations with two classic J/G methods were 13.9 and 10.7 mg/dL, whereas an alternative diazo method using 2,4-dichlorophenyl diazonium tetrafluoroborate showed only 0.5 μg/mL of total bilirubin. In both these patients, direct bilirubin was 0.1 mg/dL or less. The interference appears to be mostly due to the naproxen metabolite *O*-desmethylnaproxen reacting with the classic J/G diazotized sulfanilic reagent in the presence of caffeine [118].

Methods have been developed to measure free, non-protein-bound unconjugated bilirubin levels, which better correlate with the severity of acute bilirubin encephalopathy in neonates [119]. However, these are not well standardized or widely used.

LIPID PROFILES ANALYSIS

Lipid profiles are commonly used in the routine evaluation of cardiovascular risk, given the high correlations of hypercholesterolemia and hypertriglyceridemia and cardiovascular risk. A standard lipid profile includes determination of serum or plasma total cholesterol (TC), high-density lipoprotein-associated cholesterol (HDL-C), low-density lipoprotein-associated cholesterol (LDL-C), and total triglycerides (TG). In many laboratories, LDL-C is calculated from the Friedewald equation (LDL-C = TC − HDL-C − TG/5), which correlates well with gold standard determinations of LDL-C by ultracentrifugation when TG are less than 400 mg/dL. This calculation is based on the following assumptions [3]: The vast majority of cholesterol is transported by LDL, HDL, and very low-density lipoproteins (VLDL) in fasting plasma [1]; most TG are associated with VLDL in fasting specimens; and [2] the normal ratio of TG to cholesterol in VLDL is 5. However, these assumptions are not always true.

Fasting versus nonfasting lipid profiles

Cardiovascular risk assessment and monitoring of lipid lowering therapy based on LDL-C and TG should be performed in specimens collected after a period of 12–14 h of overnight fasting without any dietary intake except for water and medication because TG (predominantly in the form of chylomicrons) remain elevated after meals for several hours. In nonfasting specimens and other conditions with elevated TG, non-VLDL particles carrying TG change the ratio of TG to cholesterol and invalidate the Friedewald formula, usually leading to overestimation of LDL-C. In these cases, a direct assay for LDL-C can be used, although direct LDL-C results in nonfasting specimens may not be as predictive of cardiovascular events in women [120]. Recently, emphasis has been placed on non-HDL-C (TC − HDL-C) as a secondary target of therapy because it includes not only LDL-C but also other atherogenic lipoproteins, such as intermediate-density lipoproteins [121]. Importantly, TC, HDL-C, and non-HDL-C can be accurately determined in nonfasting specimens. Some studies also indicate that nonfasting TG, which peak approximately 4 or 5 h postprandially, may be better predictors of cardiovascular events [122], but the exact cutoffs and postprandial intervals have not been well-defined. Together with determination

of apolipoproteins A1 and B100, it is possible that in the future, nonfasting lipid profiles will be acceptable for cardiovascular risk assessment.

Other pre-analytical considerations

Although cholesterol and triglycerides are considered small molecules, they circulate as components of large lipoprotein complexes; therefore, plasma levels are subject to dilutional variation in protein concentration induced by a change to the upright position (~7–16% increase) and prolonged tourniquet application (5–20% higher). Pregnancy is associated with a decrease of cholesterol and triglycerides in the first trimester but a gradual increase afterwards [1]. The nephrotic syndrome and hypothyroidism are associated with elevated TC, predominantly due to LDL-C. Stress, inflammation, infections, and other acute disorders are associated with increases in triglycerides and cholesterol, whereas acute coronary syndromes result in decreases of TC and LDL-C and 20–30% increases in TG within 24–48 h, leading to the recommendation that lipid profiles should be avoided after 24 h of hospitalization or initiation of an acute event [123]. A study using fasting samples indicated that the magnitude of change after an acute myocardial infarction is small, with a mean decrease in TC and LDL-C of 2% at 24 h of admission and a subsequent increase of 6% in the next 2 days, whereas TG and HDL-C showed even smaller changes [124]. However, other studies showed larger reductions in TC, non-HDL-C, direct LDL-C, TG, and apolipoprotein A1 in the first 18–42 h postadmission, whereas apolipoprotein B100 and HDL-C did not change significantly [123,125]. The best recommendation is to perform lipid profiles at admission of patients with suspected acute coronary syndromes.

Analytical issues

Virtually all currently available methods for measuring cholesterol rely on the production of hydrogen peroxide from cholesterol in an enzymatic reaction catalyzed by cholesterol oxidase. Interestingly, this enzyme is not specific for cholesterol, and plant sterols can also be reacting. However, even in individuals ingesting large amounts of plant sterols, the plasma concentration (μg/dL) is minuscule compared to cholesterol (mg/dL) [126].

In order to measure both free cholesterol and esterified cholesterol, samples are treated with cholesteryl ester hydrolase to release cholesterol from fatty acid esters. The H_2O_2 produced by the oxidase reaction is then typically measured by a peroxidase catalyzed reaction. Therefore, all previously described interferences with peroxidase-based methods (e.g., hemoglobin, bilirubin, ascorbic acid, and uric acid) also affect cholesterol measurements, although these interferences are minimized by current approaches, such as the use of multiple wavelengths, kinetic measurements, and bilirubin oxidase.

For the determination of HDL-C, either HDL is physically separated from other lipoproteins with precipitation methods or in homogeneous assays the cholesterol associated with HDL is distinguished from the cholesterol associated with other lipoproteins by a variety of different approaches. Separation methods include precipitation of non-HDL cholesterol with dextran sulfate or a heparin–manganese complex, followed by centrifugation and analysis of the supernatant. These methods are labor-intensive and have been largely replaced with homogeneous methods. Approaches used in homogeneous methods, often used in combinations, include [3] detergent solubilization of the cholesterol in HDL while inhibiting release from other lipoproteins with polyanions [1]; steric inhibition of the reaction of non-HDL cholesterol with the enzymes by the use of antibodies, for example, to apoB100 contained in LDL, VLDL, and chylomicrons [2]; modification of cholesterol esterase and oxidase by linking polyethylene glycol moieties to the enzymes, which excludes access to cholesterol in non-HDL lipoproteins; and [4] selective oxidation of cholesterol from non-HDL with subsequent destruction of H_2O_2 by catalase, followed by addition of a catalase inhibitor and a detergent releasing cholesterol from HDL. Similar approaches are used to measure LDL-C by "direct" homogeneous assays. The main issue with homogeneous methods is the specificity for HDL-C or LDL-C and the degree of reactivity with other lipoproteins, particularly in patients with abnormal amounts of unusual lipoproteins (e.g., in patients with type III hyperlipidemia with excess remnant lipoproteins, cholestasis, and chronic liver or kidney disease). These methods are also subject to varying degrees of interference in samples with high levels of TG [127,128]. A comparison of various homogeneous methods for LDL-C and HDL-C determination showed that most methods met the acceptable total error goals in healthy individuals, but all methods failed to attain these goals in patients with cardiovascular disease, dyslipidemias, and hypertriglyceridemia [128].

Case report

A 64-year-old male presented with anemia (hemoglobin, 11.7–12.0 g/dL) first noted 4 months earlier [129]. Other abnormal laboratory results included serum HDL-C of 10 mg/dL (46 mg/dL 3 years earlier) and a total protein of 9.5 g/dL with albumin of 3.8 g/dL. Total cholesterol was 98 mg/dL, with 75 mg/dL in the LDL-C fraction. Measurement of HDL-C in a slide chemistry

system showed levels of 44 mg/dL. The patient was diagnosed with Waldenström's macroglobulinemia with biclonal IgM-κ and IgM-λ paraproteins. Interference from paraproteins in HDL-C has been noted with precipitation, liquid homogeneous, and also solid-phase homogeneous assays [85].

Like cholesterol analysis, triglyceride assays use a mix of enzymes to produce a measurable product. The first reaction involves hydrolysis of the triglycerides with lipase, releasing free fatty acids and glycerol. The next reaction involves glycerol kinase, which uses ATP to phosphorylate glycerol to glycerophosphate. The measureable product can then be produced by several approaches [3]: measuring ADP produced from ATP with pyruvate kinase and LDH, with production of NAD^+ [1]; using glycerophosphate oxidase to produce dihydroxyacetone and H_2O_2, followed by a typical peroxidase reaction; and [2] using glycerophosphate dehydrogenase to produce dihydroxyacetone phosphate and $NADH^+$. The major problem with these methods is the presence of endogenous glycerol in the sample. In most patients, particularly outpatients, the amount of free glycerol in the plasma is negligible, but it can be significantly increased in patients with severe hypertriglyceridemia, diabetes, chronic kidney disease, total parenteral nutrition, heparin treatment, and congenital deficiency of glycerol kinase, which can be asymptomatic in adults [130]. Contamination of the sample with glycerol from the collection tubes or hand creams and soaps can also lead to falsely high TG [1]. This problem can be obviated by use of serum blanks, in which glycerol is measured by omitting the lipase enzyme, although at the cost of reduced sample throughput. Note that lipolysis can occur in samples stored at room temperature or contaminated with heparin (which induces lipase); in these samples, falsely low TG results would occur with glycerol blanking assays.

CONCLUSION

Pre-analytical sources of variation are the most frequent causes of inaccurate results in clinical chemistry. For many analytes, awareness of physiologic variation, proper patient preparation, and consistent collection practices—including time of the day, fasting, posture, tourniquet application, and collection techniques—is essential to obtain reliable results and correctly interpret any changes observed. Although good manufacturing practices, availability of reference standard materials, quality control, and proficiency testing have minimized errors and inaccuracies in the analytical phase, many methods remain subject to interferences, especially in unusual clinical situations. It is important to question all results that do not fit the clinical picture [131]; consult laboratory specialists, manufacturer's instructions, and reference literature about possible sources of interference; and attempt to eliminate interferants by appropriate sample treatment or by retesting with another methodology that may not be subject to the same interferences.

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CHAPTER

10

Challenges in routine clinical chemistry analysis: proteins and enzymes

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INTRODUCTION

In this chapter, the source of inaccurate results in commonly ordered enzymes and other protein biomarker assays, excluding hormones and tumor markers, is addressed. In this chapter, issues with albumin and total protein measurements, commonly ordered as part of the liver panel or the comprehensive metabolic pane, as well as gamma-glutamyltranspeptidase, lactate dehydrogenase and creatine kinase, are addressed. In addition, two protein biomarkers used in the evaluation of cardiovascular disease—cardiac troponin and B-type atrial natriuretic peptide—are also discussed. Finally, we will address iron studies, which routinely involve two proteins (transferrin and ferritin) as well as iron measurements.

ALBUMIN AND TOTAL PROTEIN

Measurement of total protein and albumin is useful in several clinical situations, including the following:

- Diagnosing hypoalbuminemia resulting from (a) decreased intake of amino acids (protein malnutrition), (b) decreased absorption of amino acids (intestinal disease), (c) decreased protein synthesis (liver insufficiency, acute or chronic inflammation), and (d) increased losses (renal or gastrointestinal).
- Albumin levels can be of value in calculating free electrolyte (calcium), drug (phenytoin), and hormone (testosterone and cortisol) concentrations.
- Screening for monoclonal gammopathies by detecting an increase in globulins as measured by the difference between total protein and albumin. For more sensitive detection of monoclonal gammopathies, direct measurement of various immunoglobulins, serum and urine protein electrophoresis, and immunofixation techniques are indicated (see Chapter 19).
- High albumin, total protein, and hematocrit levels can suggest acute dehydration, whereas a proportional decrease of albumin, total protein, and hematocrit can suggest hemodilution.

Pre-analytical issues

As mentioned previously (Chapter 9), recumbence leads to fluid shifts from the extravascular to the intravascular compartment, resulting in mild hemodilution and a decrease in protein concentrations up to 10%. This decrease can be more pronounced in patients predisposed to edema (e.g., cardiac and liver insufficiency). Similarly, prolonged application of a tourniquet can lead to hemoconcentration with an up to 8% increase in albumin and total protein. Similar effects are seen with all the protein biomarkers described in this chapter. Pregnancy is also associated with hemodilution and a decrease in total protein by as much as 1.3 g/dL in the third trimester.

Analytical issues and interferences

Total protein is commonly measured in plasma or serum by the biuret method, which takes advantage of the reaction of peptide bonds in proteins with cupric ions at alkaline pH to form a colored product that absorbs at 540−560 nm. Therefore, this method assumes a constant ratio of peptide bonds to mass of proteins,

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which is not true for all proteins present in serum but is sufficiently accurate for clinical use. The range of measurement is typically from 1 to 10 g/dL; therefore, it cannot be used to measure low levels of protein in body fluids such as urine and cerebrospinal fluid. For these situations, turbidimetric methods using protein precipitation with trichloroacetic acid, sulfosalicylic acid, or benzethonium chloride or dye-binding methods using Coomassie blue or pyrogallol red-molybdate are employed.

Dextran, carbenicillin, hemoglobin, and bilirubin can have mild positive interferences with some total protein methods, whereas lipemia at high levels (>3+), methylbenzethonium, aminophenazone, sulfasalazine, and fluorescein may cause negative interference. Total protein and albumin measurements are particularly susceptible to error due to poor mixing of thawed samples.

Albumin is usually measured in automated analyzers by its capacity to bind a dye with resulting change in absorption. Two dyes are commonly used—bromocresol green (BCG) and bromocresol purple (BCP). Although generally comparable, albumin values measured with BCG tend to be falsely elevated (as much as 1 g/dL) in hypoalbuminemic patients undergoing hemodialysis, compared to an albumin immunoassay [1], and falsely low above the normal range due to nonspecific binding to globulins [2]. On the other hand, for most patients, BCP results are more comparable to immunoassay. In a few individuals with advanced renal failure, a uremic toxin (CMPF; 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid) can bind to albumin and compete with BCP [3], thus causing artificially low results. This interference appears to be present particularly in patients undergoing hemodialysis and is not observed in patients undergoing peritoneal dialysis or patients with renal failure who are not subjected to dialysis [4]. Similarly, albumin-bound bilirubin interferes with the BCP method [5]. Despite these problems, the BCP method is recommended for general use because it is more specific for albumin and better correlates with immunoassay or gold standard nephelometry, even in hemodialysis patients. The following formula for converting BCP to BCG results works well in both renal insufficiency and nonrenal patients:

$$\mathrm{Alb_{BCG}} = 0.55 + \mathrm{Alb_{BCP}}\ (\text{in g/dL})$$

This formula is applicable even when serum albumin is less than 3 g/dL [6]. Formulas for calcium correction using BCP-measured albumin are different from the standard formula described in Chapter 9; for example (calcium in mg/dL, albumin in g/dL) [7,8],

$$\text{Calcium(corrected)} = \text{total calcium(measured)} - 0.4 \times \text{albumin} + 1.2$$

The BCG method, but not BCP, is negatively affected by the presence of high levels (280 U/mL) of heparin [9], clofibrate, and phenylbutazone in the sample. High-dose penicillin-G causes negative interference in the BCP method [10]. Some BCP methods show up to 25% positive interference by 400 mg/dL of free hemoglobin, whereas others do not show this interference.

Urinary albumin measurements to detect microalbuminuria

In glomerular disease and other kidney pathologies including diabetic nephropathy, small amounts of protein, predominantly albumin, are lost in urine, a condition termed microalbuminuria. Microalbuminuria is defined as urinary albumin excretion of 30–300 mg per 24 h, 20–200 μg/min or 30–300 μg/mg of creatinine, confirmed in at least 1 of 2 additional specimens. Macroalbuminuria is when protein excretion exceeds microalbumin levels, and nephrotic range proteinuria is when total urinary protein exceeds 3 g/24 h or 2 mg/mg creatinine.

Since these small amounts are usually below the limits of detection by colorimetric methods (either automated high throughput methods or using dipstick reagents), sensitive immunoassays are required. Measurement of small amounts of urinary albumin has been colloquially termed microalbumin, in which the term "micro" refers to the amount and not any different property of the analyte, which is identical to serum albumin. Most commonly used methods to detect microalbuminuria use immunoturbidimetry, although recently other automated methods and even point-of-care dipstick lateral flow immunoassays have been developed.

Microalbuminuria has been shown to be a strong predictor of poor outcomes in diabetes and hypertension, even in patients without overt nephropathy. Annual measurement of urinary albumin to detect microalbuminuria is indicated in all patients with diabetes mellitus, renal disease and optionally in hypertension, unless the patient has established proteinuria. Microalbuminuria measurements are also indicated for monitoring the effectiveness of treatment with angiotensin-converting enzyme (ACE) inhibitors in reducing proteinuria, even in renal patients without hypertension or diabetes.

Given the variability in the concentrating ability of the kidneys and therefore the ratio of excreted albumin to urine volume, it is important to either carefully collect a timed urine specimen, most commonly for 24 h. Alternatively, given the inconvenience and difficulties in proper timed urine collection, calculating the ratio of urinary albumin to creatinine in a spot urine sample is recommended. The microalbumin/creatinine ratio

on a spot urine has been shown to be as accurate as carefully measured 24 h urinary albumin measurements to screen for diabetic nephropathy. However, this calculation involves compounding the cost and analytical variability of two assays (albumin and creatinine) and creatinine has inter-individual and gender-related differences in excretion rates. Spot measurement of microalbumin without correction for creatinine concentration has been shown to have similar accuracy as the albumin/creatinine ratio and may be preferable in screening programs [11]. In contrast, serial monitoring of the same patient may be best performed with the albumin/creatinine ratio since inter-individual variability is not a factor in this case.

For spot urine measurements, it is preferable to use first morning urine specimens as they will be more concentrated. Also, the supine position has been associated with less variability in albumin excretion than ambulatory activity, but this variability is reduced with the albumin/creatinine ratio [12].

Any specimens contaminated with blood (e.g., in menstruating women) will have falsely increased urinary albumin. Dipstick testing for hemoglobin will detect most of these cases. Urinary tract infections, fever, inflammatory conditions, active exercise and prolonged upright position should be avoided as they can result in increased proteinuria. Turbidimetric methods may be affected by turbid urine specimens. Dilution of the specimen and specimen blanking may resolve these cases. In cases of massive proteinuria (>1−40 g/L, depending on the test), microalbumin assays may suffer the prozone effect and result in falsely low albumin levels, although manufacturers have developed techniques to deal with this issue, including measurement of turbidity kinetics, and the frequency of prozone effects is below 0.2% in patients from primary care and diabetic clinics [13]. For this reason, microalbumin methods should not be used on undiluted urine when urinary protein exceeds 4 g/L as demonstrated by strongly positive urine dipstick or urinary total protein measurements.

ALANINE AND ASPARTATE AMINOTRANSFERASES ANALYSIS

Aspartate aminotransferase (AST), also known as serum glutamate-oxaloacetate transaminase (SGOT), and alanine aminotransferase (AST), also known as serum glutamate-pyruvate transaminase (SGPT), are important enzymes commonly measured to identify and follow-up liver damage. Aminotransferases are key enzymes in amino acid metabolism, and they work by transferring amino groups between the various amino acids and 2-oxoglutarate (α-ketoglutarate), which functions as an acceptor of the amino group to yield glutamate in a reaction involving pyridoxal-5′-phosphate (P5P), derived from vitamin B_6, as a coenzyme. The reaction is reversible, and glutamate can function as an amine donor to various ketoacids to form amino acids—for example, alanine from pyruvate with AST and aspartate from oxaloacetate with ALT.

The AST enzyme is coded by two different genes, *GOT1* and *GOT2*. The *GOT1* gene codes for c-AST, which is present in the cytosol and most abundant in cardiac and skeletal muscle and red blood cells, whereas the *GOT2* gene codes for m-AST, a mitochondrial isoform of AST most abundant in hepatocytes. The catalytic activities of the two AST isoforms are similar, but they can be differentiated by immunoassays or by modified enzymatic assays because m-AST is highly susceptible to proteolysis by proteinases K. The m-AST isoform has a longer half-life in serum (~87 h) than does c-AST. Whereas c-AST predominates in the plasma of healthy individuals (~88−95% of the AST activity) and patients with mild liver disease, m-AST is predominant in liver parenchyma (~80% of total AST) and increases more than c-AST in acute hepatitis and more severe liver disease [14]. It is likely that the cytoplasmic location allows c-AST to leak into the systemic circulation with mild, reversible cellular damage, whereas the more abundant m-AST requires injury to the mitochondria (e.g., associated with liver ischemia or severe necrosis). In alcoholic hepatitis, the ratio of m-AST to c-AST is also typically elevated, perhaps indicating a particular susceptibility of mitochondria to ethanol-induced damage, whereas in uncomplicated viral hepatitis the predominant lesion is in the cell membrane, and therefore c-AST predominates.

The *GPT* gene codes for ALT1, the cytosolic isoform of ALT (c-ALT), whereas the related gene *GPT2* codes for the mitochondrial isoform, ALT2 (m-ALT). ALT1 is expressed mainly in liver and kidney, and it is weakly expressed in intestine, heart, skeletal muscle, and salivary glands. It is the predominant isoform in serum from healthy individuals, whereas ALT2 is the predominant ALT isoform expressed in skeletal muscle and heart [15,16]. In rodents, ALT2 appears to be present in liver and seems to undergo a more significant increase than ALT1 after liver damage or steatosis [17,18], but it does not appear to be expressed in human liver [15,16,19]. ALT2 is also expressed in adrenal gland, adipose tissue, neurons, endocrine pancreas, and prostate, where it is induced by androgens [16]. ALT1, but not ALT2, is inducible in the liver by peroxisome proliferator-activated receptor (PPAR) α agonists, such as fenofibrate [20]. A similar induction of ALT activity is seen in conditions associated with increased neoglycogenesis, such as fasting, insulin resistance, type 2 diabetes, and the metabolic syndrome [21,22]. Obesity can increase levels of AST and ALT by approximately

40–50%. It is unclear whether the available enzymatic assays show any differential reactivity with ALT1 versus ALT2, although recombinant purified ALT1 had 15-fold higher activity than ALT2 in one study [23]. The different cellular localization, tissue specificity, and inducibility of the two ALT isoforms raise the possibility that isoform-specific immunoassays may have better clinical usability than the currently used enzymatic assay. Despite the presence in other tissues, isolated elevations of ALT are rarely seen in the absence of liver disease.

Both AST and ALT are sensitive markers of liver damage, with ALT being more specific due to its restricted tissue expression and AST being more sensitive because it is approximately two or three times more abundant in liver than ALT. However, this increased sensitivity of AST does not appear clinically relevant in the primary care setting and recent studies have suggested that measurement of AST should only be performed in cases of elevated ALT (e.g., >2 × upper limit of normal), with reporting of the AST/ALT ratio [24]. The ratio of AST/ALT (de Ritis ratio) is commonly used to help differentiate various causes of liver damage (Table.10.1). Various parameters affect the de Ritis ratio:

- Extent of extra-liver disease: Because AST is widely expressed and ALT is predominantly from liver, any conditions involving systemic injury, especially involving skeletal muscles, heart, kidney, lung, pancreas, intestine, or erythrocytes, will increase the ratio. Hemolysis occurring *in vitro* will also increase AST with little effect on ALT.
- Severity of damage: In normal individuals and patients with reversible injury involving the cellular membranes, thought to occur through cytoplasmic blebbing, liver c-AST and c-ALT predominate in roughly equal amounts. Severe injury to mitochondria, such as in liver necrosis or degeneration due to drug toxicity or ischemia, leads to a massive release of the much more abundant m-AST, which increases the de Ritis ratio.
- Elimination of ALT compared to AST: In general, the half-life of ALT is much higher (~2 days) than that of AST (<24 h); therefore, although AST may predominate during an acute episode of toxic or ischemic liver injury, during the recovery phase the ratio may become less than 1. Diseases leading to m-AST release also prolong the elevation of AST activity because m-AST has a longer half-life than c-AST.
- The extent of liver synthetic function affects ALT more than AST because ALT is more liver-specific. Situations with reduced liver function, such as in chronic cirrhosis, tend to have elevated de Ritis ratios due to lower ALT production. In some cases of chronic liver disease, as well as in fulminant liver failure, ALT concentrations may decrease below the reference range.
- Any induction of ALT expression—for example, treatment with PPARα agonists or insulin resistance: Obese patients and those with the metabolic syndrome tend to have higher levels of ALT (40–50%) compared to AST [25].
- The amount of P5P in circulation: Using assays that do not supplement the reaction with exogenous P5P, this becomes a rate-limiting factor, with the ALT enzyme being more susceptible to low levels of P5P

TABLE 10.1 Conditions affecting AST and ALT levels.

Condition	Level of AST	AST/ALT ratio	Comment
Healthy	<40 U/L	0.7–1.4	
Acetaminophen toxicity	>10,000 U/L	>1[a]	Slower increase in 24–48 h
Ischemic injury	>10,000 U/L	>1[a]	Rapid rise in <24 h
Alcoholic hepatitis	<300 U/L	>2	
Autoimmune hepatitis	<10× ULN	<2	
Biliary obstruction	<5× ULN	<2	Return to normal in 72 h
Cholangitis	<10× ULN	<2	
Cirrhosis	<10× ULN	>1	Levels may be normal or decreased
Hemochromatosis	<10× ULN	<2	
Hepatitis, viral, acute	>300 U/L, 10–100× ULN	<1	AST remains elevated for 7–14 days; ALT decays ~10%/day, remains elevated 27 ± 16 days
Hypo- and hyperthyroidism	<3× ULN	<2	
Infectious mononucleosis	50–800 U/L	<2	
Liver metastases	<5× ULN	>1	
Nonalcoholic steatosis	<10× ULN	<2	
Primary biliary cirrhosis	<10× ULN	>2	
Reye's syndrome		>2	
Wilson's disease	<10× ULN	>2	

ULN, upper limit of reference range.
[a]*AST/ALT > 1 initially, then reverts to <1.*

seen, for example, with chronic alcoholism, malnutrition, renal failure (due to P5P binders in the plasma), and presence of xenobiotics such as isoniazid and lead. Also, m-AST is more susceptible to loss of P5P than is c-AST, and assays using P5P supplementation tends to have higher m-AST/c-AST ratios.

Typically, patients with alcoholic hepatitis or severe alcohol-induced liver disease such as cirrhosis have a de Ritis ratio greater than 2 [26], whereas in uncomplicated acute or chronic liver disease the ratio is usually less than 2. Possible mechanisms involved in elevation of the ratio in severe alcoholic liver disease include mitochondrial oxidative damage, decreased liver ALT production, decreased P5P, and extrahepatic damage such as alcoholic myopathy. The ratio is also helpful in distinguishing alcoholic from non-alcoholic liver steatosis, especially when coupled with other determinations such as red cell mean corpuscular volume and γ-glutamyl transferase [27], although the ratio tends to be high in advanced cases of alcoholic and non-alcoholic cirrhosis [28]. In contrast, acute viral hepatitis tends to be associated with de Ritis ratios less than 1 and aminotransferase levels greater than 300 IU/L. Patients with a de Ritis ratio less than 0.6 in acute viral hepatitis had better prognosis than those with ratios greater than 1.2 [29].

Many drugs affect AST and ALT levels, and monitoring of transaminase elevation is routine when evaluating or following therapy with potentially hepatotoxic drugs. Some drugs also increase ALT and AST levels by inducing its expression. Commonly used drugs that may affect ALT and AST levels include acetaminophen, aminoglycosides, angiotensin-converting enzyme (ACE) inhibitors, anticonvulsants, calcium dobesilate, cephalosporins, clofibrate, fenofibrate, fluconazole, furosemide, ganciclovir, griseofulvin, isoniazid, macrolides, nicotinic acid, nonsteroidal anti-inflammatory drugs, omeprazole, opiates, statins, sulfonamides, and sulfonylureas.

The following are additional patient-related factors to take into consideration when evaluating ALT or AST measurements:

- ALT and, more important, AST levels in normal individuals tend to parallel muscle mass and therefore are 10–20% higher in males, African Americans, and athletes.
- Intense exercise can increase AST and ALT levels, usually less than threefold, subsiding within 1–7 days [30].
- Both hypothyroidism and hyperthyroidism tend to increase AST levels.
- Levels of AST and ALT increase with age until approximately 40 years and then decline approximately 10–20% to the age of 60 years. Both AST and ALT tend to be higher in elderly individuals—approximately 10% after age 70 years [31].
- Circadian variation affects ALT by approximately 45%, peaking in the afternoon and a nadir at night, whereas AST varies by less than 10%, although some studies show no significant variation [31].
- Both AST and ALT are elevated in obese patients (40–50% higher) and those with metabolic syndrome, hypertriglyceridemia or high-sugar or high-fat diets [31,32].
  - Hospitalization by itself and even placebo treatment may be associated with mild elevations in transaminases, particularly ALT [33,34].

Case report

A 32-year-old female was referred to a hepatologist for possible liver biopsy [35]. She had a history of AST levels between 120 and 500 U/L persisting for more than 7 years without any symptoms, family history, or significant past medical history of liver disease, except for a limited acute Epstein–Barr virus infection at age 14 years, which caused a reversible 10-fold elevation of ALT and AST. Medical examination was normal, an abdominal ultrasound was unremarkable, and serum levels of other liver enzymes including ALT, hepatitis and autoimmune serology, ferritin, ceruloplasmin, and thyrotropin were normal. The patient denied taking any medications or herbal supplements, except for oral contraceptives. Discontinuation of the contraceptive resulted in no change in AST levels. Before performing the liver biopsy, the patient's serum sample was treated with protein A Sepharose, which binds immune complexes, resulting in a change in AST activity from 459 to 57 U/L, whereas another healthy control sample showed no change. The elevation in AST was attributed to macro-AST, and the patient was subject to no further studies.

Although rarely seen, macro-AST is more common in patients with liver disease, particularly those with autoimmune, viral, or neoplastic liver disease, but it can be found in healthy individuals and persist for several years [35,36]. Isolated increases in AST without ALT or creatine kinase elevations suggest macro-AST, which can be eliminated by polyethylene glycol precipitation, ultrafiltration, dialysis, and exclusion or affinity chromatography.

Specimen processing

Serum or plasma should be separated from cells to avoid leakage of enzymes from red blood cells, which increases markedly after 24 h at room temperature.

The ALT and AST activities tend to decay when specimens are stored at room temperature, particularly in specimens with low P5P levels, because apoenzymes are more susceptible to degradation than are holoenzymes [37]. Because the enzymes are irreversibly degraded, lower activities may be seen even with methods using P5P supplementation. However, several studies show only minimal decay of the enzymes at room temperature, when refrigerated, or when frozen for at least 3 days [31,38–40]. Repeated freezing and thawing may decrease ALT activity.

Methodology

Transaminase levels are measured by supplying 2-oxoglutarate and the specific amino acid (L-aspartate for AST and L-alanine for ALT), which generate glutamate and the respective ketoacids. In several methods P5P is supplied to enhance the activity and remove circulating P5P levels as an analytical variable. Care must be exercised when comparing P5P-supplemented with non-supplemented methods, since the ratios of holoenzymes and apoenzymes varies in different specimens. A signal is generated by dehydrogenases specific to the ketoacid generated in the transaminase reaction. For AST, the oxaloacetate is converted to malate by malate dehydrogenase, with generation of NAD^+, whereas the pyruvate resulting from the ALT reaction is converted to lactate by lactate dehydrogenase (LDH). Because the method uses serum blanking and all the components are in vast excess to usual plasma levels of the corresponding endogenous substances, interference by pyruvate, lactate, and LDH is uncommon. In rare cases with very high levels of LDH (>12,000 U/L), the pre-incubation step during serum blanking may consume all the NADH substrate and cause a very low result or an analyzer flag. A 20-fold dilution of the sample will mitigate this problem. In the Beckman SYNCHRON assay, pyruvate levels of less than 6 mg/dL (normal levels are <1 mg/dL) show minimal interference. However, pyruvate can be generated from lactate and cause interference with ALT measurements in samples in which plasma was in contact with cells for more than 48 h, especially in samples with high LDH levels, which converts lactate to pyruvate [40]. Other interferences include heparin greater than 50 U/L with the Vitros method [41] and also significant lipemia, hyperbilirubinemia, and hemolysis (particularly AST, which is 10–30 times more abundant in red cells than in plasma) [31]. Interestingly, samples with high levels of ethanol (>50 mg/dL) show falsely decreased ALT and LDH (but not AST) activity, possibly by competing with lactate for LDH and reversing the reaction [42].

γ-GLUTAMYL TRANSFERASE AND ALKALINE PHOSPHATASE ANALYSIS

γ-Glutamyl transferase (GGT) and alkaline phosphatase (ALP) are often measured to determine if a patient has intra- or extrahepatic cholestasis because these enzymes are abundant in the plasma membrane of hepatocytes, particularly in the biliary canaliculi and the basolateral surface of biliary epithelia. ALP is the product of one of three genes—*ALPI*, expressed in the intestine; *ALPP*, expressed in placenta; and *ALPL*, expressed in most tissues, particularly in liver, kidney, and bone, in which it can be differentially glycosylated resulting in changes in protein conformation, stability, and clearance. For example, ALPP is heat stable at 65 °C, the bone form of ALPL, derived from osteoblasts, is extensively glycosylated and heat labile, and liver ALP is heat stable at 56 °C. Heterogeneity of glycosylation affects clearance of the various forms of ALP by the liver galactose receptor [43].

Most plasma ALP activity derives from liver, with up to 50% from bone and a small amount of intestinal form. The release of ALP into the circulation during cholestatic disease may be mediated by increased phospholipase activity induced by bile acids because the enzyme is anchored to the membrane by a glycosyl phosphatidyl inositol (GPI) moiety [44]. The various isoforms of ALP can be separated by electrophoresis, isoelectric focusing, wheat germ lectin precipitation, high-performance liquid chromatography, or specific immunoassays; however, it is simpler to determine GGT levels because this enzyme is increased in liver but not bone disease. The liver form of ALPL is elevated in cholestatic disease (up to 12 times normal), particularly in extrahepatic obstruction, whereas parenchymal liver disease, such as viral, alcoholic, or autoimmune hepatitis, usually increases ALP less than 3-fold. Liver metastases can also result in high levels of ALP, depending on the extent of the infiltration. The bone ALPL is elevated in growing children (2- to 5-fold), and in disorders with osteoblastic activity and bone remodeling, such as Paget's disease (up to 25-fold), rickets and osteomalacia (2- to 4-fold), and in osteogenic bone cancer (very high levels). Elevated ALPP is seen in second and third trimester pregnancy (2- or 3-fold) and some tumors (Regan isoenzymes), and ALPI is increased in liver cirrhosis and blood group O or B secretors.

GGT is abundantly expressed in kidney, liver, pancreas, and intestine, but most of the circulating activity in healthy individuals is of hepatic origin. Like ALP, GGT is cleared by the liver, and various glycosylation isoforms are cleared at different rates by the liver asialoglycoprotein receptor [45]. Interestingly, GGT is inducible in the liver by a variety of xenobiotic agents, most

notably microsomal inducers such as the anticonvulsants phenytoin and phenobarbital, oral contraceptives, cimetidine, furosemide, and methotrexate, and in patients with chronic ethanol exposure, who can have hepatic GGT levels approximately 3-fold higher than normal [46]. Chronic alcoholism may also be associated with increased release of GGT into the circulation because serum levels can be as high as 5–30 times the upper limit of normal. In general, it is thought that GGT levels reflect oxidative stress and glutathione consumption in the liver, which makes sense given the role of GGT in the metabolism of glutathione, a key antioxidative agent in the liver. The same liver and biliary diseases that cause increased ALP will also cause GGT elevations to a similar extent, although GGT tends to be more sensitive than ALP to cholestasis, with elevations averaging 12-fold in greater than 93% of cases compared to 3-fold for ALP [47]. Elevations of GGT originating from other organs can be seen in acute and chronic pancreatitis or pancreatic cancer and mildly in chronic kidney disease, diabetes, acute myocardial infarction, hyperthyroidism, rheumatoid arthritis, and lung disease [47,48].

The following additional pre-analytical factors should be taken into consideration when interpreting ALP and GGT measurements [31,38,47]:

- Normal levels of ALP increase in growing children with a peak approximately fivefold the adult levels at approximately age 13 years, mostly due to the bone isoenzyme. The levels then decay until age 25 years when they reach relatively stable levels, similar in men and women. GGT levels increase linearly with age until approximately 40–50 years and are approximately 40–50% higher in men than in women.
- In African Americans, GGT activity is approximately twice that of Caucasians, whereas ALP is 10–15% higher.
- Obesity increases ALP and GGT by approximately 20–30%, and in individuals with a body mass index greater than 30 m^2, levels of GGT may be 50% higher.
- Diurnal variation of less than 10% in ALP and GGT levels has been reported with activities highest in the morning and lowest in the afternoon.
- Ingestion of fatty meals can increase intestinal ALP as much as 30 U/L for approximately 12 h, especially in Lewis-positive secretors of blood type O or B.
- Starvation can decrease GGT, whereas meals cause an initial decrease followed by a small increase.
- Smoking increases GGT by 10–50% and ALP by approximately 10%.
- Oral contraceptives increase GGT and decrease ALP by approximately 20%, whereas pregnancy increases ALP 200–300% and decreases GGT by approximately 20–30%.
- ALP and GGT activities are generally stable in separated serum or plasma at room temperature, 4C or frozen for at least 7 days. Frozen serum or plasma should be thawed for at least 18 h a room temperature to achieve full ALP enzyme reactivation.

Analytical issues

Most commercially available methods for ALP analysis use the chromogenic substrate 4-nitrophenyl phosphate, which upon loss of the phosphate group catalyzed by ALP under alkaline conditions forms a yellow product that absorbs at 405–410 nm. Massive blood transfusion, plasmapheresis, or citrate anticoagulant inhibits ALP activity because citrate chelates the required zinc and magnesium ion co-factors. Citrate, oxalate, and fluoride also inhibit GGT activity up to 15% and should be avoided. The GGT assay also uses a chromogenic substrate such as γ-glutamyl-*p*-nitroaniline, which is cleaved by GGT into *p*-nitroaniline, measured at 405–120 nm, and a glutamyl group that is transferred to an acceptor such as glycylglycine.

Hemolysis and heparin may interfere with some ALP and GGT methods, whereas others show no such interferences. In patients with hepatobiliary diseases, macro-GGT may occur as complexes with IgA autoantibodies and apolipoproteins A and B [49]. Binding to lipoproteins may increase GGT half-life from approximately 9 to 20 h [45]. IgM paraproteins in heparinized plasma may also interfere with some GGT assays [50]. Macro-ALP has also been rarely observed, without clear clinical significance, although increased prevalence in patients with inflammatory bowel disease and chronic peritoneal dialysis has been suggested [51,52].

AMYLASE AND LIPASE ANALYSIS

These enzymes are measured to assess pancreatic disease, and there is considerable debate regarding the utility of amylase versus lipase for this purpose. In general, lipase appears more sensitive and specific for pancreatic disease and is elevated for a longer period of time, therefore guidelines now recommend that only lipase be used to diagnose pancreatitis [53]. Amylase is highly expressed in salivary glands from the *AMY1* gene (S-amylase), whereas pancreatic amylase (P-amylase) originates from the *AMY2A* and *AMY2B* genes. There is high inter-individual variation in the levels of amylase in part due to copy number variations of the *AMY1* gene (from 2 to 15 diploid copies), which may be related to the ability to adapt to high-starch diets and minimize the risk of insulin resistance and diabetes [54]. The amylase enzyme hydrolyzes the α-1,4-glucosidic linkage between two glucose residues in polysaccharides such as starch and amylose.

Pancreatic lipase is expressed from the *PNLIP* gene, specifically in exocrine pancreas. Other lipase genes code for enzymes, such as intestinal or hepatic lipase

and lipoprotein lipase, which are not measured appreciably with current pancreatic lipase assays. Pancreatic lipase hydrolyzes triglycerides, releasing the fatty acids at positions 1 and 3 and resulting in a 2-monoacylglycerol, and it requires activation by bile acids and the presence of a small protein co-factor secreted by the pancreas, colipase.

Amylase is also weakly expressed in a variety of other tissues, and elevations can be seen in a variety of conditions, including the following:

- Abdominal lesions, leading to increased absorption of P-amylase from the intestinal lumen, including various gastric and intestinal lesions, appendicitis, peritonitis, abdominal trauma, and abdominal aortic aneurysm.
- Ovary and fallopian tubes express S-amylase, and elevations can be seen in serous and mixed serous and mucinous ovary cysts and tumors, pelvic inflammatory disease, and ruptured ectopic pregnancy.
- Miscellaneous other conditions can lead to hyperamylasemia, including pregnancy, head injury, septic shock, burns, bulimia, anorexia nervosa, acute alcohol or organophosphate intoxication, diabetic ketoacidosis, cardiac surgery, pheochromocytoma, thymoma, and some lung and colon tumors. Elevations can also be seen in approximately 20% of patients subjected to a variety of abdominal or extra-abdominal surgical interventions.

In healthy individuals, approximately 40–50% of serum amylase activity is of pancreatic origin, whereas 55–60% derives from salivary glands. Amylase is a small protein (54–62 kDa) and is partially eliminated by glomerular filtration; therefore, it is present in normal urine and elevations can be seen in renal failure.

Peter Wilding and colleagues [45] first described persistent elevations of amylase due to the formation of a high-molecular complex with immunoglobulins, thus avoiding glomerular filtration and significantly increasing the half-life of plasma amylase activity. Macroamylase is probably the most common of the macro-enzymes causing abnormal test results, often without any subjacent clinical abnormalities, and it is present in 2–5% of patients with elevated amylase, usually of S-type. One helpful indication for the presence of macroamylase is calculation of the amylase-to-creatinine clearance ratio (ACR):

$$\mathrm{ACR} = \frac{\text{Urine amylase} \times \text{Serum creatinine}}{\text{Serum amylase} \times \text{Urine creatinine}}$$

An ACR ratio less than 1% suggests macroamylasemia. Whereas ratios greater than 5% suggest acute pancreatitis, renal disease, or diabetic ketoacidosis, measurement of urinary amylase and calculation of the ACR ratio is too nonspecific for clinical use and should be restricted to cases of suspected macroamylasemia. Although much rarer than macroamylasemia, cases of macrolipase have also been reported, including complexes with IgG, IgA, and α_2-macroglobulin [55].

In contrast, pancreatic lipase measurements offer increased specificity and sensitivity for pancreatic disease and in general should replace amylase determinations for the diagnosis and monitoring of pancreatic disease. At least one prominent institution has discontinued offering amylase for pancreatic disease evaluation [56]. In acute pancreatitis, both amylase and lipase typically rise 4–8 h after the onset of the symptoms and peak at 24–48 h. Due to its shorter half-life, amylase returns to normal range within 3–5 days, whereas lipase tends to persist for 1 or 2 weeks. In most studies, the sensitivity of lipase for pancreatitis varies from 85 to 100%, whereas amylase averages 85–95%. In cases of chronic pancreatic disease with pancreatic insufficiency, production of pancreatic enzymes may be impaired. Because levels of lipase in pancreatic tissue are approximately four times higher than amylase, normal or decreased levels of amylase are seen more often than lipase in chronic pancreatic insufficiency.

Although much less common than with amylase, non-pancreatic sources of lipase also exist, mainly related to conditions involving the gastrointestinal and biliary tracts, such as cholecystitis and gastroenteritis. Lipase, a 48-kDa protein, is also filtered by the glomeruli and may be increased in renal insufficiency, but in contrast to amylase, it is fully reabsorbed by the renal tubules and not normally found in urine.

In general, extensive investigation of elevations of amylase or lipase less than threefold the upper limit of normal in patients with nonspecific abdominal pain is of low diagnostic yield and probably not cost-effective [57]. Some individuals show levels of amylase and lipase that range from 1 to 16 times the upper limit of normal, with marked day-to-day fluctuations, without any symptoms or evidence of pancreatic disease. This condition has been termed Gullo's syndrome and in some case seems to have a hereditary component [58]. In approximately 50% of cases of asymptomatic hyperamylasemia, a significant pathological pancreatic or extrapancreatic disease can be present; therefore, a thorough diagnostic workup may be indicated [59].

Analytical issues

Amylase is usually measured by a variety of methods. One of the simplest methods, the Ortho Vitros amylase slide, uses the natural substrate starch covalently linked to a dye, dried in the spreading layer,

which upon addition of the sample is hydrolyzed by amylase to release the dye into the reagent layers, where it is measured by reflectance spectrophotometry. Some liquid chemistry methods use a series of enzymatic reactions to convert the product of an amylase reaction (usually one or more maltose residues) to a measureable product (usually NADH from a glucose-6-phosphate dehydrogenase reaction). One problem with these assays is that the stoichiometry of the reaction may shift depending on the different maltose oligomers present. Other assays use a variety of chromogenic maltose oligomers to release a measurable dye (typically 4-nitrophenyl) upon hydrolysis by amylase with or without further hydrolysis by α-glycosidases. The International Federation of Clinical Chemistry (IFCC) reference method uses a heptamaltose substrate covalently linked to a protecting ethylidene residue to avoid slow hydrolysis of the intact substrate by α-glycosidase in the reagent mix.

A variety of substrates and reaction conditions have also been used for pancreatic lipase measurement. For example, one Beckman SYNCHRON method uses the following sequence of reactions leading to formation of a quinone dye from the oxidative coupling of *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-toluidine (TOOS) with 4-aminoantipyrine (4-AAP):

1. Pancreatic lipase: 1,2-Diglyceride + H_2O → 2-monoglyceride + fatty acid
2. Monoglyceride lipase: 2-Monoglyceride + H_2O → glycerol + fatty acid
3. Glycerol kinase: Glycerol + ATP → glycerol-3-phosphate + ADP
4. Glycerophosphate oxidase: Glycerol-3-phosphate + O_2 → dihyroxyacetone-phosphate + H_2O_2
5. Horseradish peroxidase: $2H_2O_2$ + 4-AAP + TOOS → quinone diimine dye + $4H_2O$

The Ortho Vitros method uses a similar sequence of enzymes, but the substrate is 1-oleoyl-2,3-diacetylglycerol and the final acceptor of H_2O_2 oxidation is a leuco dye. The Vitros substrate may be more specific for intestinal than pancreatic lipase and may be interfered by lipoprotein lipase in heparinized samples [60], but the addition of colipase and bile salts in the current assay makes it very specific for pancreatic lipase. The Panteghini method (e.g., Roche analyzers) uses a completely different approach resulting in direct formation of a colored dye (methylresorufin) from a synthetic substrate (1,2-*O*-dilauryl-rac-glycero-3-glutaric acid-(6′-methylresorufin)-ester) [61].

Few analytical interferences have been reported for amylase and lipase assays. Icodextrin, a polysaccharide used in peritoneal dialysis, may inhibit amylase and interfere with some amylase assays, particularly those using 4-nitrophenyl substrates. Notably, other assays unaffected by icodextrin showed true amylase levels to be reduced in patients undergoing peritoneal dialysis [62]. High levels of hydroxocobalamin, used in therapy of cyanide poisoning, impact a strong red color to the serum that increases the hemolysis index provided by the analyzer and interferes with various measurements, including amylase, creatine kinase, carboxyhemoglobin, creatinine, bilirubin, cholesterol, magnesium, phosphate, uric acid, and lactate [63,64].

An inhibitor present with pancreatitis associated with severe hypertriglyceridemia interfered with starch-iodine amylase assays, but most modern assays, including the starch-based Vitros method, are unaffected by triglyceride levels of at least 800 mg/dL. Very high levels of glycerol, greater than 160 mg/dL (normal levels <0.6 mg/dL), can cause interferences in some lipase methods, particularly those that use diglyceride substrates [31,65].

LACTATE DEHYDROGENASE ANALYSIS

LDH is a ubiquitously expressed enzyme with limited clinical sensitivity and specificity for a variety of diseases involving tissue damage. The main clinical indications for measuring LDH include monitoring of disease activity in cases of hemolytic and megaloblastic anemias, thrombotic thrombocytopenic purpura, lung disease, and some tumors, particularly lymphomas and germinal cell cancers. Some clinicians use LDH elevations as an early indicator of tissue damage. However, due to its nearly ubiquitous expression, LDH is elevated in a large number of conditions and shows poor specificity for any particular clinical situations. For example, intense exercise can result in elevations in LDH of 30−50% [31]. LDH fractionation can be used to somewhat improve specificity because there are five isoforms with some tissue specificity: LDH1 predominates in heart, kidneys, and red cells; LDH5 predominates in liver and skeletal muscle; and LDH2, -3, and -5 are present in a variety of different tissues. Fractionation can occasionally be helpful to identify the source of an LDH elevation, but due to considerable overlap of tissue expression, it does not provide sufficient diagnostic accuracy for significant clinical use. Measuring LDH in other body fluids may help point to exudates due to infectious, inflammatory, necrotic, or malignant processes. Because body fluids typically contain much lower amounts of LDH than serum, a ratio of body fluid/plasma LDH greater than 0.6 or an fluid LDH level greater than 200 IU/L is more consistent with severe hemorrhage or an exudate [66]. For example, increased LDH levels in the cerebrospinal fluid may indicate bacterial or viral meningitis if

hemorrhage or loss of blood–brain barrier function can be excluded.

LDH catalyzes the conversion of lactate to pyruvate, whereas NAD^+ is reduced to NADH or vice versa. Reaction rates are monitored by measuring absorbance at 340 nm. Either the forward (lactate to pyruvate) or the reverse reaction (pyruvate to lactate) can be used, although the IFCC reference assay uses the forward reaction because it is less susceptible to substrate exhaustion and loss of linearity.

The most important pre-analytical consideration is avoidance of hemolysis because red cells contain LDH levels approximately 50- to 150-fold higher than those of plasma, and even minor amounts of hemolysis can significantly increase measured plasma LDH levels. For this reason, LDH is a sensitive marker of *in vivo* hemolysis. Similarly, serum contains up to 40% higher levels of LDH than plasma due to release from platelets during clotting. However, serum may be a preferred specimen, especially if platelet-poor plasma cannot be consistently achieved. Plasma or serum should be separated from cells in less than 1 h to avoid leakage of LDH from cellular elements. The LDH activity is unstable in refrigerated or −20 °C frozen serum; therefore, it is preferable to store specimens at room temperature, which maintains LDH activity for up to 3 days. Storage of specimens on heparinized tubes with gel-based plasma separator results in an approximately 5% loss of activity [67]. LDH can be interfered by high amounts of ethanol (>50 mg/dL), possibly by competing with lactate [42].

CREATINE KINASE ANALYSIS

Creatine kinase (CK) catalyzes the transfer of phosphate between ATP and creatine to generate creatine phosphate and ADP or vice versa. Creatine phosphate serves as an important reservoir of high-energy phosphates in striated muscle, brain, retina, inner ear, spermatozoa, and, to a lesser degree, smooth muscle, which are tissues that can consume ATP rapidly. Logically, CK is highly abundant in these tissues, particularly heart and skeletal muscle, and is used as the most sensitive marker for skeletal muscle damage, or rhabdomyolysis. In cases of acute rhabdomyolysis, CK levels as high as 1000-fold normal can be seen. Table 10.2 lists causes of rhabdomyolysis and other conditions associated with elevations of CK in plasma, including pre-analytical variables.

Various forms of CK exist, including the mitochondrial enzyme, which predominantly catalyzes the transfer of phosphate from ATP to creatine, and the "cytosolic" form, which catalyzes the reverse reaction, regenerating ATP from phosphocreatine. Three isoenzymes of cytosolic CK occur, with different tissue expression, each composed of a dimer of two subunits—either B (brain type), coded by the *CKB* gene, or M (muscle type), coded by *CKM*. The CK-BB homodimer is abundant in brain and at lower levels in a variety of tissues; the CK-MM homodimer is abundant (98% of CK activity) in skeletal muscle; and CK-MB heterodimers

TABLE 10.2 Causes of CK elevation.

Cause	Specific example
Rhabdomyolysis	• Crush syndrome, blunt trauma, extensive surgery, intramuscular injections, bee stings • Seizures, tetanus, exercise, childbirth, status asthmaticus, intense coughing • Malignant hyperthermia, prolonged hypothermia • Ischemic injury (compression as a result of a coma, sickle cell disease, intoxication) • Metabolic, inflammatory, and dystrophic myopathies • Toxins: quails, snake and insect venous, buffalo fish, tetanus, toluene, gasoline, carbon monoxide, halothane • Drugs: alcohol, cocaine, heroin, amphetamine, phencyclidine, colchicine, corticosteroids, fibrates, isoniazid, statins, neuroleptics, opioids, zidovudine, etc.
Cardiac damage	• Myocardial infarction • Other causes (see Table 10.3)
Brain damage	• Extensive brain infarction • Acute psychotic reaction • Head injury, subarachnoid hemorrhage
Other tissue damage	• Hypoxic shock • Cancer of prostate, bladder, gastrointestinal tract, lung, breast, uterus, testis • Pulmonary embolism, gastrointestinal ischemic necrosis
Other causes of CK elevation	• Hypothyroidism • Reye's syndrome • Sepsis, typhoid fever • Smoking • CK levels correlate with muscle mass: higher in males and African Americans, lower in females and elderly individuals • Last trimester of pregnancy • Neonates and infants during first year of life
Pre-analytical factors	• Prolonged tourniquet application (~10% increase after 6 min) • Hemolysis • Repeated freeze and thaw • Exposure to 56 °C

For details, see References [31], [38], [117].

are most abundant in heart, in which they comprise approximately 25−30% of CK activity. In the past, elevations of CK-MB were used in the diagnosis of myocardial infarction, but it has been essentially superseded with the more sensitive and specific cardiac troponin measurements.

Pre-analytical variables affecting CK measurements are listed in Table 9.2. Note that most of these cause relatively minor changes in CK and that acute rhabdomyolysis is associated with large increases in plasma CK. However, they may become relevant when following the activity of chronic muscle disease such as polymyositis or Duchenne dystrophy.

Methods for CK analysis are based on either the forward reaction (creatine → phosphocreatine) or the reverse reaction (phosphocreatine → creatine). The cytosolic enzyme favors the reverse reaction and therefore it proceeds approximately sixfold faster. The ATP generated can be measured by coupling to a pair of reactions, first using hexokinase to phosphorylate glucose to glucose-6-phosphate and then using glucose-6-phosphate dehydrogenase to generate NADPH and 6-phosphoglutonate. Alternatively, in the Vitros dry chemistry method, ATP produced by CK can be measured by a series of reactions involving glycerol kinase catalyzed production of L-α-glycerophosphate, which is then converted to dihydroxyacetone phosphate by glycerophosphate oxidase, with production of H_2O_2. Subsequently, the H_2O_2 is measured by oxidation of a leuco dye. Because adenylate kinase, which is present in large amounts in erythrocytes, can convert ADP to ATP in the absence of CK, modern assays include AMP and diadenosine pentaphosphate as adenylate kinase inhibitors. Because CK is reversibly inactivated by oxidation of cysteinyl residues, *N*-acetylcysteine is added to reverse any oxidized sulfhydryl groups. In addition to the reversible oxidation of sulfhydryl groups, progressive irreversible inactivation of CK can slowly occur, and samples stored at room temperature for more than 2 days, or refrigerated for more than 7 days, show significant decreases [31,68]. Although small degrees of hemolysis (<500 mg/dL) can be tolerated, because the red cells contain no CK and adenylate kinase is inactivated, severe hemolysis releases high enough concentrations of ADP, phosphates, glucose-6-phosphate, and other enzymes that act on the reagent components to cause significant interferences. Therapeutic concentrations of hydroxocobalamin and doxycycline can artificially increase CK measured by the Roche assay [64,69].

CARDIAC TROPONIN ANALYSIS

Troponins (I and T) are key components of striated muscle sarcomeres, where they regulate muscle contraction in response to calcium levels. Troponin exists as a trimeric complex: Troponin C senses calcium levels and induces a conformation change in troponin I, which in turn induces a change in tropomyosin to allow actin to interact with myosin for muscle contraction; troponin T anchors the complex to tropomyosin. Three genes code for the three isoforms of troponin I: slow twitch skeletal muscle *TNNI1*, fast twitch skeletal muscle *TNNI2*, and cardiac *TNNI3*. Similarly, three genes code for troponin T: the skeletal muscle *TNNT1* and *TNNT2* and the cardiac *TNNT3*. Sufficient differences exist in primary amino acid sequence at the N-terminus of cardiac troponin I and troponin T to be able to separate the cardiac isoforms from skeletal troponins with immunoassays. Cardiac troponin assays were first used in clinical practice in 1992, when monoclonal antibody sandwich immunoassays for cardiac troponin T (cTnT) and cardiac troponin I (cTnI) applicable to routine clinical detection of myocardial injury were first described [70,71]. Since then, cardiac troponin assays (cTnT and cTnI) have become the standard for detection of acute myocardial infarction, and in 2007 the European Society of Cardiology, American College of Cardiology Foundation, American Heart Association, and World Heart Federation Task Force established elevation of cardiac troponins as one key defining element in the diagnosis of acute myocardial infarction (AMI) [72].

Myocardial infarction is defined as loss of cardiac myocytes (necrosis) caused by prolonged ischemia, and AMI is a type of myocardial infarction occurring between 6 h and 7 days after the ischemic event. An AMI can be diagnosed by a rise and/or fall in markers of myocardial damage in the blood, including cardiac troponins, with at least one measurement above the 99th percentile of normal population and one of the following criteria:

- Clinical: Ischemic symptoms
- Pathology: Myocardial cell death
- Electrocardiography
  - **a.** Evidence of myocardial ischemia (new ST-segment changes or new left bundle branch block (LBBB))
  - **b.** Evidence of loss of electrically functioning tissue (Q waves)
- Imaging
  - **a.** Reduction or loss of tissue perfusion
  - **b.** Cardiac wall motion abnormalities

Acute myocardial infarctions can be classified into five clinical categories [73]. The criteria listed previously pertain to the first two clinical types of AMI, most commonly seen in emergency departments:

Type 1: Spontaneous AMI resulting from ischemia due to atherosclerotic coronary artery disease

Type 2: AMI secondary to ischemia due to increased oxygen demand (e.g., arrhythmias and hypertension) or decreased oxygen supply (e.g., anemia, coronary spasm, embolism, and hypotension)

In addition, AMI diagnoses can be made in the following clinical situations:

Type 3: Sudden, unexpected cardiac death, without available cardiac biochemical markers; often with history of symptoms of ischemia, accompanied by electrocardiographic evidence of ischemia or evidence of fresh thrombus by angiography or autopsy
Type 4: AMI post percutaneous coronary intervention. 4a: increase in cardiactroponin greater than fivefold the 99th percentile of the reference population in a patient with normal baseline troponin levels or a rise of more than 20% to the levels fivefold of the 99th percentile if a patient had stably elevated troponin; 4b: AMI associated with stent or scaffold thrombosis, using imaging or autopsy evidence.
Type 5: Coronary artery bypass grafting with an increase in biomarker greater than ten times the 99th percentile of the reference population. If the patient has stably elevated baseline troponin, the same tenfold criteria is required, provided the elevation is $>$ 20% of the baseline. Additionally, of the following is required: (1) new Q waves or new LBBB, (2) angiographic evidence of coronary occlusion, or (3) imaging evidence of new loss of viable myocardium

The broader category of acute coronary syndrome (ACS; Fig. 10.1) is defined as any constellation of clinical symptoms that are compatible with AMI, and it includes (A) unstable angina, without elevation of cardiac biomarkers, and (B) AMI, characterized by elevation of biomarkers, further classified by the electrocardiographic presentation as follows:

1. ST-elevation AMI (STEMI)
2. Non-ST-elevation AMI (NSTEMI)
3. Q-wave AMI, which can be diagnosed only after 24 h

In STEMI, rapid electrocardiogram-based diagnosis is critical for timely percutaneous intervention, and troponin measurements are not essential for rapid diagnosis. In NSTEMI, the diagnosis relies on troponin measurements. In cases in which rapid intervention may be considered, fast turnaround time for reporting troponin results is essential. In addition to cardiac ischemia and AMI, any condition resulting in myocardial injury can potentially elevate cardiac troponin levels. A comprehensive list of causes of myocardial damage and troponin elevation is presented in Table 10.3.

A common misconception is that renal failure causes troponin elevation in the absence of myocardial injury, for example, by decreasing elimination of troponin or troponin fragments. This does not appear to be the case, and it is well demonstrated that patients with renal insufficiency and troponin elevation do have myocardial damage and increased risk for adverse cardiovascular events [74–76]. However, decrease elimination of cTnT in heart failure patients with renal insufficiency may play a role in the degree of troponin elevation originating from myocardial damage [77].

Another misconception is that re-expressed fetal skeletal troponin T will cause elevations in cardiac troponin

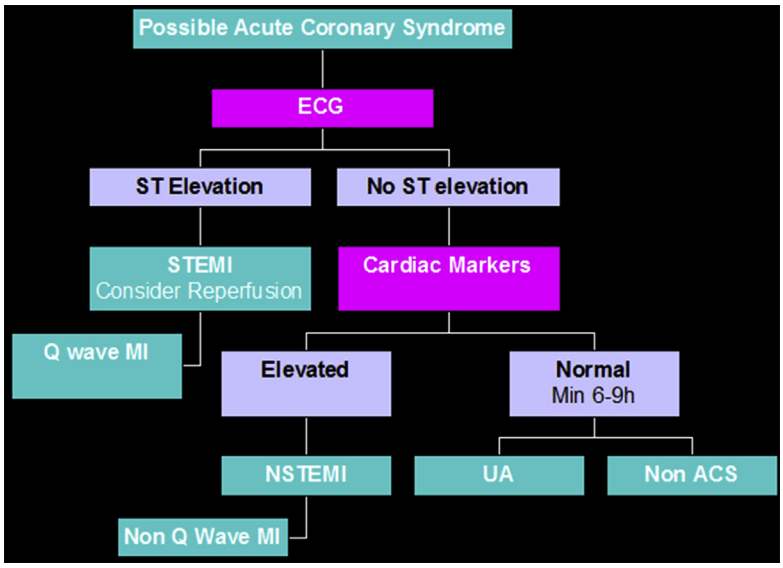


FIG. 10.1 Classification of acute coronary syndromes (ACSs). *MI*, myocardial infarction; *NSTEMI*, non-ST elevation MI; *STEMI*, ST elevation MI; *UA*, unstable angina.

TABLE 10.3 Causes of cardiac troponin elevation.

Cause	Specific example
Acute myocardial infarction	A specific cause of elevated troponin
Direct trauma to the heart	Ablation, angioplasty, cardiac surgery, implantable device discharges, pacemakers, cardioversion
Myocardial toxins	Adriamycin, 5-fluorouracil, cocaine, amphetamines, Herceptin, snake venoms
Wall stress and subendocardial hypoperfusion	Pulmonary embolism, severe systemic or pulmonary hypertension, aortic valve disease, aortic dissection, hypertrophic cardiomyopathy, acute or chronic heart failure, tachy- or bradyarrhythmias, strenuous exercise, shock and severe hypotension
Systemic disease with myocardial injury	Severe renal failure, respiratory failure, sepsis, acute central nervous system damage (stroke, hemorrhage), rhabdomyolysis, extensive burns (total body surface area >30%); other critically ill patients, especially with diabetes, hypothyroidism, transplant vasculopathy
Other causes of cardiac injury	Cardiac inflammation (pericarditis, myocarditis) Infiltrative cardiomyopathy (amyloidosis, hemochromatosis, sarcoidosis, scleroderma) Coronary vasospasm, including Takotsubo ballooning stress cardiomyopathy

T measurements in skeletal muscle disease. Although this may have been the case due to non-specificity of the antibodies against cTnT, assays have been optimized to exclude cross-reactivity against all skeletal troponins, and expression of cTnT has been excluded in a variety of muscle diseases [78]. It is possible that rarely in certain muscle diseases, true re-expression of cTnT may occur and cause elevation even with high-sensitivity fourth-generation cTnT assays [79]. However, high-sensitivity cTnI levels remain normal in these patients.

In general, troponin elevations originating from myocardial damage not associated with acute ischemia do not follow a typical rise and fall observed during an AMI: With high-sensitivity assays, troponin elevations can be seen as soon as 2 or 3 h after initiation of the ischemic event, peak at approximately 24 h, and remain elevated for 7–14 days. The troponin levels at 24–72 h correlate with infarct size, although this is better evaluated with imaging studies. Re-infarction can be detected even in the presence of troponin elevation by noting an increase in serial troponin levels.

A key concept in measuring troponins is functional sensitivity, or the minimum concentration of troponin that can be detected by an assay with imprecision of 10% or less. The consensus definition of AMI requires that troponin assays have a functional sensitivity below the upper limit of the reference range, which is defined as the 99th percentile of a healthy population [72,73]. It is important to note that the latest generation cardiac troponin immunoassays are able to reliably detect plasma levels of troponins in a majority of normal individuals—that is, the functional sensitivity is significantly below the 99th percentile ("high-sensitivity" assays). It is also important to note that excluding apparently healthy individuals with cardiovascular risk factors, cardiac imaging, or increased B-natriuretic peptide in order to determine the 99th percentile will improve the sensitivity for cardiovascular risk detection in patients with ACS [80].

It is well-established that patients with troponin elevations above the reference range are at short- and long-term risk for cardiovascular events and may benefit from therapeutic intervention [72]. It is also possible that even in apparently healthy individuals, detectable levels of troponin may indicate minor cardiac damage and increased risk of adverse cardiovascular events in the future [81]. This use of cardiac troponins for risk assessment should be distinguished from its diagnostic value for detection of AMI.

The following are helpful criteria to interpret troponin results and distinguish evolving AMI from non-ischemic causes of troponin elevation:

1. The pretest probability of ACS and coronary artery disease (CAD) should be taken into consideration: In a patient with nonspecific symptoms and low pretest probability of cardiovascular disease, a cardiac troponin level in the reference range is reassuring and may allow the patient to be discharged from the emergency department.
2. In a patient with a high probability of ACS (typical chest pain, history of CAD, etc.), if an initial cardiac troponin is negative or very mildly elevated, an increase greater than 30% in cTnI levels measured 6 h later is consistent with AMI [82]. Other criteria based on delta changes have been proposed for higher sensitivity and timeliness of diagnosis; for example, an absolute change of at least 9.2 ng/L (0.0092 ng/mL) with a high-sensitivity cTnI assay had the highest area under the receiver operator curve for diagnosis of NSTEMI [83] and a rise of >20% has been adopted by the Fourth Universal Definition of AMI [73]. With newer higher sensitivity cardiac troponin assays, a repeat test can be performed within 1–2 h and a repeatedly negative result used to exclude AMI with high sensitivity in patients presenting >2 h after

symptom initiation [73]. Importantly, these delta criteria must be optimized for each assay, time window, and patient population [84].

3. Most non-ischemic causes of myocardial damage result in relatively stable, mild elevations of troponins, whereas rapidly increasing, significant elevations of cardiac troponin (e.g., cTnI > 0.5 ng/mL) are virtually diagnostic of AMI.
4. Increments in troponins may not be seen if patients present with AMI more than 24 h after development of symptoms. In some cases, a decrease in serial troponin results may indicate that the patient suffered an AMI one or more days ago, but the decrease may be much slower than the antecedent rise.

Reversible ischemia induced by exercise does not appear to result in significant troponin release, although there is some controversy regarding whether minor amounts of intact troponins or degradation products can be released with reversible ischemia or even tachycardia-induced cardiac myocyte stretching [85]. After heavy exercise, such as in marathon runners, troponin levels are typically elevated, in some cases above 0.5 ng/mL, but it is unclear whether they indicate some degree of cardiac damage [86] and may instead reflect cardiomyocyte membrane leakage associated with mild cardiac inflammation [87]. Rapid normalization of troponin levels 24 h after a race is a good indicator that the troponin elevation represents a benign, reversible process [88].

Analytical issues

Cardiac troponins are released in the circulation in various forms; including holo-complex of cTnI/cTnT/cTnC, free intact molecules, and a variety of degradation products and post-translationally modified forms, including phosphorylated molecules [89,90]. This heterogeneity complicates the development of standardized reference materials and comparability of the troponin assays because there are differences in the ability of the various antibodies to recognize the multiple forms [89]. When the classification of patients according to elevations above the 99th percentile was compared between four different assays, 3 or 4% were differently classified between cTnI assays and 15–17% between cTnI and cTnT assays [91]. Because of the lack of standardization, it is important to compare results from the same assay methodology when interpreting serial elevations or decreases of cardiac troponin levels.

Potential pitfalls and interferences in troponin measurements are common with other immunoassays. See Chapters 5–7 for an in-depth discussion of immunoassay design and interferences observed in immunoassays. For example, heterophilic antibodies, rheumatoid factors, and anti-animal antibodies in the serum of certain individuals can cause persistent falsely elevated or depressed troponin results, which can vary depending on the specific immunoassay used [92]. Similarly, autoantibodies directed against cardiac troponins can cause falsely decreased (up to 20-fold) measurements [93]. Cardiac troponin I macrocomplexes with immunoglobulins causing persistent elevation in the absence of cardiac pathology have been described [94]. High levels of biotin ingestion (e.g. 10 mg/day) can interfere with cardiac troponin assays, including fifth generation high-sensitive methods [95,96].

Severe hemolysis typically interferes with immunoassays, particularly those using fluorometric or electrochemiluminescence detection. Some assays are more susceptible to particles such as fibrin strands or bubbles in the sample. Because measurements in serum and plasma are not always comparable, the same sample type should be used consistently in serial measurements. Samples should be promptly analyzed or refrigerated because troponin is relatively unstable at room temperature and susceptible to *in vitro* degradation by proteases. Because the proteolytic fragments may or may not be detected by a particular assay, the manufacturer's instructions for specimen processing should be followed.

B-TYPE NATRIURETIC PEPTIDE ANALYSIS

The B-type natriuretic peptide (BNP) is an important regulator of renal sodium excretion, as well as cardiac function, through the action of BNP-receptors (NPRA and NPRB) coupled to GMP cyclase. Injection of recombinant BNP (Nesiritide) can be used clinically to improve cardiac function in patients with heart failure (HF). In normal hearts, BNP is produced in atrial but not ventricular myocytes as pro-$BNP_{1–108}$ (intact pro-BNP), which is stored in atrial secretory granules. In situations of atrial dilation or increased wall tension, intact pro-BNP is cleaved by proteases into an inactive N-terminal fragment containing amino acid residues 1–76 (NT-pro-$BNP_{1–76}$) and the bioactive peptide BNP_{32}, which contains the C-terminal 32 amino acids. Once secreted in circulation, BNP has a very short half-life of 13–20 min due to cellular uptake, renal excretion, and inactivation by endothelial-bound neutral endopeptidases. In contrast, NT-pro-BNP has a half-life of 25–70 min, mostly determined by renal excretion; therefore, it is measured at higher levels in plasma. In the case of renal insufficiency, the levels of BNP and more extensively NT-pro-BNP are elevated.

BNP can be produced by the ventricular myocytes in situations of chronic myocardial stress (stretch and

wall tension) and myocardial remodeling associated with AMI and cardiac failure. Therefore, measurements of BNP or NT-pro-BNP have been used in the evaluation of patients with AMI and cardiac insufficiency. A good correlation between the levels of natriuretic peptides and the severity of HF (American Heart Association classes I–IV) can be observed, and natriuretic peptides can be in the diagnosis and follow-up of acute and chronic HF. However, these assays should be complementary and not replace other clinical diagnostic tools for HF diagnosis and therapy. In general, BNP and NT-pro-BNP are best used when the cause of acute dyspnea is clinically unclear because in patients with obvious HF or very low probability of HF, the predictive values of natriuretic peptide measurements are poor [97]. When clinical uncertainty exists natriuretic peptide values are most helpful if levels are markedly elevated (suggesting HF), for example, BNP > 400 pg/mL, or low (unlikely to be acute HF), e.g., <100 pg/mL in the acute setting and <35 pg/mL in the ambulatory or chronic setting. Corresponding diagnostic values for NT-pro-BNP are >450 (in those younger than 50 years), >900 (in those 50–75 years old), or >1800 pg/mL (in those older than 75 years) for ruling in HF versus <300 pg/mL in the acute setting and <125 pg/mL in the ambulatory setting, for exclusion of HF [98]. Intermediate levels have the lowest predictive values, and the patient may be a candidate for further evaluation with specialist assessment and echocardiography depending on the clinical assessment.

Because BNP responds to ventricular wall stress, it can be increased in many other conditions other than cardiac failure, including the following [99]:

1. Other cardiac diseases
   - **a.** Acute coronary syndrome
   - **b.** Valvular heart disease
   - **c.** Left ventricular hypertrophy with or without arterial hypertension
   - **d.** Atrial fibrillation
   - **e.** Inflammatory cardiac disease
   - **f.** Pericardial disease
   - **g.** Cardiac surgery, cardioversion, trauma
   - **h.** Toxic-metabolic injury to the myocardium, including chemotherapy
2. High-output stress (such as tachycardia, hypoxemia, anemia, sepsis, severe burns, hyperthyroidism, hyperaldosteronism, Cushing's syndrome, and advanced liver cirrhosis with ascites)
3. Pulmonary disease and pulmonary vascular conditions (such as embolism, severe pulmonary hypertension, chronic obstructive pulmonary disease, and cor pulmonale)
4. Acute or chronic renal failure (eGFR < 60 mL/min/1.73 m^2)
5. Severe neurological disease, such as subarachnoid hemorrhage, stroke, and trauma
6. Critical disease
7. Advancing age

The 2017 American College of Cardiology/American Heart Association and International Society for Heart and Lung Transplantation updated guidelines for the management of heart failure now recommend measurement of natriuretic peptides to (a) screen for HF in patients at risk, (b) diagnose HF in the setting of acute dyspnea, and (c) establish the prognosis of hospitalized patients with known HF with an admission and discharge measurement [99]. Other useful guidelines for the use of BNP or NT-pro-BNP include the following [100]:

1. Given high physiologic intra-individual variation (~30–50%) and analytical imprecision (up to 15%), only changes greater than 40–100% compared to the previous result are clinically significant [101–103].
2. The test should not be ordered more than once per week because real changes related to pathophysiology or therapy require time to occur.
3. Patients with chronic HF may have elevated natriuretic peptide levels and acute dyspnea of noncardiac origin (see above).
4. The levels of natriuretic peptide elevations do not differentiate between HF due to left ventricular systolic dysfunction and HF with preserved ejection fraction, although values tend to be lower in patients with diastolic HF.
5. Cutoffs for the use of NT-pro-BNP have been published by the International Collaborative for NT-proBNP Study (ICON) [98]:
   - **a.** For monitoring therapy and risk stratification in HF, it is reasonable to collect a baseline sample and a second prior to discharge for risk assessment and monitor therapeutic effectiveness. Patients are at lower risk with reductions greater than 50%. These measurements identify those in need of more aggressive management. For example, the threshold with the best balance of sensitivity and specificity for 1-year mortality is approximately 1000 pg/mL for NT-pro-BNP. An admission NT-pro-BNP concentration greater than 5180 pg/mL is strongly predictive of death by 76 days.
   - **b.** If estimated glomerular filtration rate is less than 60 mL/min/1.73 m^2, a higher threshold (e.g., NT-pro-BNP > 1200 pg/mL) is best for exclusion of HF.
   - **c.** For risk stratification in pulmonary embolism, values below 1000 pg/mL indicate good prognosis. Persistent elevations of NT-pro-BNP greater than 7500 pg/mL after 24 h or less than 50% decrease indicate right ventricular dysfunction and a poor prognosis.

d. For risk stratification in ACS, values less than 1115 pg/mL indicate a high probability for recovery of left ventricular function. Values greater than 1170 pg/mL for men and greater than 2150 pg/mL for women identify high-risk patients.

False-negative results—that is, normal natriuretic peptide levels despite the presence of HF—can be observed in the following situations:

1. Obesity, diuretics, ACE inhibitors, β-blockers, aldosterone receptor blockers, and aldosterone antagonists can reduce levels and decrease sensitivity for HF.
2. Patients with end-stage HF can have low levels of circulating natriuretic peptides.
3. Lack of elevation despite acute hemodynamic compromise and/or severe acute pulmonary congestion can occur in patients who present within the first hour of the onset of pulmonary edema caused by acute mitral regurgitation, in patients with preserved ejection fractions, and in those with constrictive pericarditis without intrinsic heart disease.

Pre-analytical considerations

Due to poor stability in serum, BNP assays should be performed in EDTA anticoagulated plasma and samples should not be stored for more than 4 h at room temperature. NT-pro-BNP measurements can be performed in either serum or plasma, and the sample is stable for at least 72 h at room temperature. Serum samples show approximately 10% higher levels of NT-pro-BNP compared to EDTA-anticoagulated samples [104].

Analytical issues

Like troponin, natriuretic peptide measurements are immunoassays and suffer from the same limitations discussed in Chapters 5–7. For example, a macro-BNP complex resulted in elevation of BNP but not NT-Pro-BNP [105] and high biotin levels can falsely reduce BNP and some NT-pro-BNP results [96]. There is considerable heterogeneity in the exact peptides that are measured by the various BNP and NT-pro-BNP assays [106]. "BNP" assays typically measure intact pro-BNP_{1-108} in addition to BNP_{32}, although it is unclear if this affects the diagnostic ability of BNP assays. In plasma, BNP_{32} quickly loses the N-terminal three amino acids to yield BNP_{3-32}, which may not be fully measured with some BNP assays. No other proteolytic fragments appear to circulate in patients with HF [106,107].

All "NT-pro-BNP" assays measure NT-pro-BNP_{1-76} and pro-BNP_{1-108}. However, intact NT-pro-BNP_{1-76} has not been detected in circulation; instead, several other N-proteolytic fragments originating from cleavage at both the N-terminus and the C-terminus of NT-pro-BNP_{1-108} can be present in plasma and more extensively in serum. For this reason, the best assays for pro-BNP should be directed at the central amino acids. Current U.S. Food and Drug Administration-licensed assays use a sandwich immunoassay approach, with the capture antibody directed against amino acids 1–21 and the detection antibody directed against amino acids 39–50.

Glycosylation of the central portion of pro-BNP has been described, which may affect interaction with antibodies directed against amino acids 39–50 and significantly increase renal excretion. Oligomerization of pro-BNP peptides can occur through leucine zipper motifs at the N-terminus of pro-BNP [108], potentially hiding or exposing epitopes and changing the half-life and therefore the levels of measured BNP or NT-pro-BNP.

IRON STUDIES

In this section we will discuss several assays typically used to diagnose and monitor diseases of iron metabolism, leading to either functional iron-deficiency (iron deficiency anemia and anemia of inflammation) or iron accumulation (hemochromatosis). These assays include measurement of total serum iron levels and total iron binding capacity (TIBC), as well as proteins that are established (transferrin and ferritin) and emerging (soluble transferrin receptor, hepcidin) markers of iron metabolism.

Iron metabolism is complex and a full description is beyond the scope of this work, but it can be summarized in the following steps:

(1) Ingestion of iron heme containing food such as meat, shellfish, fish, and poultry, and non-heme iron in plants such as spinach, beans, lentils, and in iron-fortified foods. Normally about 10–15 mg a day of iron are ingested.
(2) Intestinal absorption involving a transporter for heme iron, the heme transporter *SLC46A1,* and a transporter for non-heme iron, a complex of the divalent metal transporter 1 (*DMT1*) and duodenal cytochrome b reductase 1 (*CYBRD1*), which reduces dietary non-heme iron from ferric (Fe^{3+}) to ferrous (Fe^{2+}). Dietary heme iron is better absorbed (30%) than non-heme iron (10%). Normally about 1–2 mg/day of iron is absorbed and added to the circulation.
(3) Transport of iron from enterocytes to transferrin in circulation, using ferroportin 1 (*SLC40A1*) and hephaestin (*HEPH*) transporters. Apotransferrin

synthesis by the liver can be increased in iron deficiency to promote increased absorption. Intestinal iron transport to the circulation can be decreased by hepcidin (*HAMP*) in iron overload. Excess iron is stored in intestinal cells in complexes with ferritin and eventually eliminated by shedding of the enterocytes. The total amount of iron transported by transferrin in circulation is about 3−7 mg.

(4) Capture of circulating transferrin by target cells, e.g., erythroblasts in the bone marrow, myocytes, or placental cells, using their membrane-bound transferrin receptor. Iron is released into acidified vacuoles while apotransferrin is recycled to the circulation. In iron deficiency, the transferrin receptor is proteolysed and released into circulation. About 1−2 mg/day of iron is consumed by tissues, about 80% in the bone marrow. Total iron content in bone marrow and muscles is about 300 mg and in circulating RBCs is about 1800−2500 mg.

(5) Transferrin iron can be also transferred to hepatocytes in liver parenchyma for storage in ferritin granules. The homeostatic iron regulator (*HFE*) gene binds to the transferrin receptor and regulates the uptake of transferrin iron by the liver and the production of hepcidin. Mutations of HFE in hereditary hemochromatosis result in decreased hepcidin production and increased iron absorption and storage. About 300−1000 mg of iron are normally stored in the liver parenchyma and about 600 mg in reticuloendothelial macrophages.

(6) Iron released by dying cells (e.g., RBC) is captured in reticuloendothelial macrophages, predominantly in the spleen and the liver, and complexed with ferritin and hemosiderin. This release of iron from intracellular stores can be blocked by hepcidin in iron overload conditions. About 20−25 mg/day of iron is recycled by the reticuloendothelial system.

(7) About 1−2 mg/day of iron is lost under normal conditions, predominantly through exfoliation of enterocytes containing ferritin iron stores. Additionally menstruating women lose 0.5−1 mg per day of menses. Loss of iron is not specifically regulated.

Iron studies are predominantly used to identify and monitor disorders of iron metabolism, particularly anemias and iron overload. Table 10.4 shows the changes in iron studies in the various disorders of iron metabolism and some other conditions that may affect iron studies.

The major causes of anemia worldwide involve iron metabolism, namely iron deficiency anemia and anemia of chronic disease. Causes of iron deficiency anemia can be grouped as lower iron intake (malnutrition, malabsorption) and increased iron consumption (chronic blood loss, hemolysis, pregnancy). Anemia of chronic disease is due to the decreased red blood cell (RBC) production (erythropoiesis) related to unavailability of iron for use by RBC precursors. Anemia of chronic disease is associated with both acute and chronic inflammation and therefore is better named as anemia of inflammation. Acute and chronic inflammation states include infection, auto-immune disorders, immunizations, transplant rejection, necrosis (liver, myocardial, trauma, surgery), chronic kidney disease, chronic obstructive pulmonary disease, diabetes, metabolic syndrome, and malignancies.

Iron overload diseases (hemochromatosis and hemosiderosis) can be congenital (due to mutations in the *HFE* and other genes, including *SLC40A1*, transferrin receptor 2, ferroportin, ceruloplasmin, hemojuvelin, and ferritin) or acquired (increased ingestion, injections of hemin, chronic hemolysis, ineffective erythropoiesis, polycythemia, chronic transfusions, chronic liver disease). Treatment of hemochromatosis often includes therapeutic phlebotomy (each unit of blood corresponding to about 250 mg of iron) and requires close monitoring of iron and erythrocyte indices.

Iron deficiency anemia is characterized by low hemoglobin, and it is usually microcytic and hypochromic. Total iron and ferritin are decreased and transferrin, TIBC, and transferrin saturation (TSat) are increased. There is no consensus on the best algorithm for iron deficiency diagnosis. The most reliable method for diagnosis of Fe deficiency, in expert hands, is staining for bone marrow iron content, but this requires an invasive procedure. Ferritin <100 ng/mL has low sensitivity (25−48%) and moderate specificity (75−78%). Transferrin saturation $<16\%$ has low sensitivity (20%) but high specificity (90%) whereas a TSat $< 20\%$ has 88% sensitivity and 63% specificity for iron deficiency. A TSat $<20\%$ may be preferred for screening for iron deficiency [109].

The reticulocyte hemoglobin content is probably the most sensitive and specific non-invasive marker for iron deficiency, performing better than total hemoglobin, medium corpuscular volume, TIBC, and ferritin, and it is becoming a convenient marker for screening for iron deficiency. Reticulocytes represent 1-2 day-old RBCs and therefore their hemoglobin content reflects the adequacy of iron availability of erythropoiesis in the last 3−4 days.

It i important to note that detection of iron deficiency without anemia is controversial; may be useful in infants and pregnant women to treat subclinical iron deficiency to prevent poor outcomes; however, anemia occurs in only about 10% of iron deficient patients.

TABLE 10.4 Iron related issues in clinical chemistry.

Condition	Hgb	Serum iron	TIBC/ transferrin	UIBC	Transferrin saturation	Ferritin	Additional markers
Iron deficiency	↓N	↓N	↑N(↓)[a]	↑	↓N	↓	sTrR ↑
Acute iron ingestion	N	↑	N	↓	↑	N	
Excess parenteral iron	N	↑	N(↑)[b]	↓	N↑	N	
Chronic iron overload	N ↓ ↑	↑	N↓	↓	↑	↑	Liver MRI/biopsy, HFE mutation sTrR N↓, ZnPP↓
Sideroblastic anemia	↓N	N↑	N↓	N↓	↑	↑	CBC, Protoporphyrin↑
Chronic inflammation	N ↓	↓ N	↓N	↓N	↓N	↑	CRP ↑,
Acute inflammation	N ↓	N ↓	N↓	↓	↑	↑	CRP ↑
Inflammation + iron deficiency	↓N	↓	↓	↓	↓	↓N	sTrR N↑, ZnPP↑
Acute liver necrosis/ hepatitis	N ↓	↑	N↓ (↑)[c]	N↓	Variable	↑	Hepatic pPanel, viral markers
Nephrotic syndrome	N ↓	N ↓	↓	↓	↓N↑	N↑	Albumin ↓
Menstruation	N ↓	↓	N	N↑	↓	N	
Premenstrual interval	N ↓	N↑	N	N↓	N	N	
Contraceptives, mid term pregnancy[d]	N ↓	↑	↑	N↑	N	N↑[e]	
Exercise (athletes)	N ↓	N ↓	N	N↑	N ↓	N ↓	Hepcidin↑
Hemodialysis with ESA[f]	N ↓	N↓	N	N↑	↓	N↑	Reticulocyte CH
Megaloblastic anemia:	↓	↑	N↓	↓ N	↑	↑	Vitamin B12, Folate
• Untreated • Early Treatment	N ↓	↓	N↓	N↓	↓N	N↓	

N, Normal; ↓ decreased; ↑ increased, in order of frequency for each entry.

[a]*Transferrin/TIBC can be decreased in protein malnutrition, impaired liver synthesis and cutaneous, intestinal or renal protein loss.*

[b]*Parenteral administration of iron dextran will increase total iron and directly measured TIBC. The latter may be artificially increased because of interference from the iron dextran, whereas UIBC and transferrin levels are normal.*

[c]*TIBC but not transferrin can be artificially increased due to excessive release of ferritin-iron complexes in circulation interfering with the assay.*

[d]*Pregnancy with iron supplementation. Iron stores normally increase during pregnancy and peak at 12–16 weeks of gestation. In late pregnancy without iron supplementation iron deficiency can occur, usually without anemia, and even with iron supplementation a small proportion of women will have iron deficiency at term. On the other hand, elevated iron stores and ferritin may be associated with unfavorable pregnancy outcomes.*

[e]*Chronic contraceptive use has been associated with higher average iron stores over time, probably due to reduced menstrual blood loss.*

[f]*Chronic treatment with erythropoiesis-stimulating agents such as erythropoietin creates a state of functional iron deficiency, as increased demand for iron for erythropoiesis is not matched by available iron stores.*

Anemia of inflammation is usually mild (10–11 g/dL of hemoglobin) although it can be severe (<8 g/dL) in 20% of the cases. It is usually a normochromic, normocytic anemia, although low mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) are seen in about a quarter. Iron studies usually show low iron and low transferrin with increase iron saturation, and increased ferritin, but in 20% or so transferrin and TSat may be normal.

Iron overload is usually associated with normal or increased hemoglobin levels, unless caused by chronic hemolysis, ineffective erythropoiesis or chronic transfusions. Persistently elevated transferrin saturation is the earliest and most sensitive marker of iron overload, but ferritin is more reliable to diagnose and monitor iron excess, e.g. when performing phlebotomies for treatment of hemochromatosis, due to high intra-individual biologic variability of TSat measurements. However, it is important to note that ferritin can be increased in situations other than iron overload: acute and chronic inflammation states (see above) and liver injury (hepatitis, toxic, alcohol abuse, steatohepatitis associated with obesity), although the magnitude of the increase rarely exceeds two or three times the upper limit of normal. Measurement of C-reactive protein is useful to rule out inflammatory conditions and liver injury is unlikely with normal transaminase and bilirubin.

Iron levels have high intra-individual day-to-day and intra-day variation, with highest levels in the morning and decreasing progressively by 10–20 μg/dL near midnight. However, this circadian variation is not consistently observed and is of limited clinical significance [110].

Patients should be fasting as ingestion of products containing high iron content such as red meat will affect results. However, fasting did not seem to improve iron overload screening programs [111]. Iron measurements can be easily affected by iron contamination of sample tubes and reagents, and by hemolysis. It is recommended that serum samples be analyzed the same day of collection [110].

Total iron methods are usually based on release of iron from transferrin by acid pH followed by reduction from ferric to ferrous ions and complexing with a chromogen, such as tripyridyl triazine (TPTZ), sulphonated bathophenanthroline, ferene, terozite, or ferrozine, to produce a colored product.

To directly measure TIBC, excess iron is added to the serum sample at alkaline pH, unbound iron is removed (e.g., with magnesium carbonate + centrifugation, alumina or ion-exchange columns, or magnetic aluminum particles), and total iron is measured by the method above. Because of the manual steps involved in removing excess iron, most automated methods measure unbound iron-binding capacity (UIBC) by adding a fixed amount of iron to serum. Since only unbound iron forms a complex with the chromogen the UIBC is calculated from the difference between added iron and measured excess unbound iron. UIBC is usually not reported and TIBC is calculated by the sum of total iron and UIBC.

Some UIBC assays can be interfered by very high levels of albumin (>7 g/dL), iron-binding drugs such as deferoxamine, tetracycline drugs, very high (>1200 μg/L) ferritin levels, and rarely certain IgM monoclonal gammopathies. UIBC can be less accurate than direct TIBC measurements in cases of iron overload with high total iron and low transferrin [110].

Transferrin saturation equals total iron divided by the transferrin iron binding capacity, calculated by one of three different methods:

(1) Directly measured TIBC
(2) Total serum iron + directly measured UIBC
(3) TIBC (ug/dL) = directly measured transferrin (mg/dL) × conversion factor.

The factor for converting transferrin levels to TIBC is 1.1–1.5 and varies between different assays and in some pathologic conditions.

Transferrin is measured by immunoassays assays, which are more reproducible and accurate but can be more expensive than TIBC assays. In populations with large genetic variation in transferrin, particularly some African and indigenous Oceanic populations, the TIBC may be more reliable than transferrin to assess iron status. In healthy individuals from these populations, ferritin tends to be elevated and therefore should be interpreted with caution when screening for iron overload [111]. In extremely rare cases of congenital atransferrinemia or with pronounced protein losses as in the nephrotic syndrome, neither transferrin nor TIBC are accurate for assessing iron status.

As with any immunoassay, transferrin and ferritin measurements may be subject to interferences with antigen-antibody binding, including heterophilic antibodies and immune complexes, or with the detection system, e.g. when streptavidin is used in sera with high biotin concentrations (see Chapter 8), or with hemolysis and lipemia. A transferrin-immune complex disease with markedly elevated serum iron and transferrin and variably elevated iron saturation and liver iron stores has been described in association with monoclonal gammopathy of unknown significance and autoantibodies against transferrin [112].

Causes of decreased transferrin and TIBC include decreased protein synthesis, increased protein loss (nephrotic syndrome and other glomerulopathies, enteropathies, extensive burns), and inflammation (since transferrin is a negative acute phase reactant). Conversely, transferrin and TIBC can be increased in iron-deficiency and hormonal excess (progesterone, estrogens, oral contraceptives, anabolic steroids, late pregnancy).

Emerging markers in iron metabolism

The erythroblast transferrin receptors are induced by iron deficiency to increase capture of iron by red cell precursors and therefore the measurement of plasma levels of soluble transferrin receptor (sTfR) can be an earlier marker of iron deficiency, particularly in patients with chronic disease, as sTfR is not usually affected by inflammatory states.

However, transferrin receptors are more abundant in early erythroid precursors therefore sTfR also reflects the extent of erythropoiesis. In situations with bone marrow stimulation, such as hemolysis, thalassemias, sickle cell disease, acute blood loss, myeloproliferative syndromes, and erythropoiesis-stimulating therapy, sTfR can be elevated in the absence of iron deficiency and conversely, in conditions with suppression of erythropoiesis, such as chronic renal failure, aplastic and advanced megaloblastic or iron-deficiency anemias, the sTfR may be falsely low in the presence of iron deficiency.

The ratio of sTfR to ferritin may be particularly useful to distinguish anemia of chronic disease from iron

deficiency anemia, as these markers change in opposite directions in these two conditions: sTfR increases and ferritin decreases in iron deficiency and vice-versa in anemia of chronic disease. In one study, a ratio of sTfR (in mg/L) to the log ferritin levels of greater than 2 suggested iron deficiency and a ratio of less than 1 suggested chronic inflammation [113,114]. This study used the R&D Systems sTfR ELISA assay (subsequently automated in the Beckman Coulter Access analyzer) and a ferritin radioimmunoassay. However, since equivalence to assays from different manufacturers has not been established, caution should be used when interpreting cutoff values for diagnosis [110].

Hepcidin is a component of the innate immune response to infection and serum levels of hepcidin rise in patients with acute and chronic inflammation. Hepcidin inhibits iron absorption in the intestine and iron release from hemosiderin containing macrophages. On the other hand, suppression of erythropoiesis reduces hepcidin levels. Elevation of serum hepcidin levels is a potential marker for anemia of chronic disease and iron overload, however, hepcidin immunoassays have not been widely available in clinical practice, mainly due to the lack of a gold standard calibrator [115] and high intra-individual variability [116].

An additional marker that may be useful in the diagnosis of anemias is the red blood cell zinc protoporphyrin content (ZnPP), which accumulates when the RBC precursors are unable to complete the synthesis of heme due to lack of iron or inhibition of heme synthesis, e.g., with lead poisoning. ZnPP elevation is highly sensitive (up to 94%) for iron deficiency and can be measured by simple fluorimetry, but has low specificity as it is affected by lead poisoning, chronic disease, hemolysis, administration of gadolinium and iodine contrast agents, and various hemoglobinopathies.

CONCLUSIONS

Proteins and enzymes are useful biomarkers, and only the most commonly assayed proteins in clinical laboratory medicine were discussed in this chapter. Enzyme assays are subject to a variety of interferences depending on the particular approach used for product detection, whereas immunoassays for biomarkers suffer from common pitfalls, including interference by heterophilic antibodies, rheumatoid factors, autoantibodies, hemolysis, lipemia, and fibrin particles and bubbles. Occasionally, falsely elevated levels of protein biomarkers or enzymes occur due to complexes with immunoglobulins, the most common examples being macroamylase and macro-CK. As with all other tests, particular attention to each manufacturer's specifications for preanalytical and analytical test characteristics is essential to avoid errors and explain discrepancies with other clinical parameters.

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CHAPTER

11

Challenges in endocrinology testing

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INTRODUCTION

The proper interpretation of endocrine hormone measurements requires consideration of the hormone concentration in the plasma, the sensitivity of the responding cells and tissues, and the clinical symptoms of the patient [1]. For example, in states of thyroid hormone resistance, the concentrations of the thyroid hormones and thyroid stimulating hormone (TSH) are typically elevated but the affected patients are usually euthyroid or even possibly hypothyroid [2]. In another example, in type 2 diabetes the absolute insulin concentration may be elevated because of end-organ resistance. In such a case, hyperinsulinism reflects a lack of insulin responsiveness and not a primary excess of insulin secretion [3]. Fortunately, neither insulin nor C-peptide concentrations are recommended for use in the diagnosis of diabetes mellitus [4].

A variety of interferences can perturb the results of endocrine testing [5]. In this chapter we will highlight pre-analytic considerations, sample collection and processing, assay specificity, high-dose hook effect, macro-analyte complexes, human anti-mouse antibodies, rheumatoid factor, heterophile antibodies, and biotin-ingestion interferences.

PRE-ANALYTICAL CONSIDERATIONS

Similar to any analytical determination, the pre-analytical status of the sample is crucial [6]. The sample must be drawn on the right patient, at the right time, in the correct volume, properly identified, placed into the correct tube and properly processed. Some hormones vary considerably in concentration over the course of 24 h yet a single reference interval is applied (e.g., thyrotropin, which is also known as TSH, and testosterone). For other hormones with a more clearly evident diurnal, or circadian, rhythm (e.g., cortisol), reference intervals apply to the time of the day of sampling (e.g., a.m. vs. p.m.). However even the definition of "PM" can be controversial: "Does p.m. mean 4 p.m., 8 p.m. or midnight?" It is best to specify a specific time if there is a difference between 4, 8 or 12 p.m. Some hormones exhibit ultradian variation within a 24-h day (e.g., luteinizing hormone and follicle stimulating hormone) or infradian variation that occurs over periods longer than a day (e.g., estradiol and progesterone in menstruating women). Such variations must be taken into consideration when the laboratorian or clinician interprets the significance of a hormone concentration in an individual.

Even the location of the phlebotomy can affect the measurement. For example, parathyroid hormone (PTH) measurements taken downstream of (i.e., proximal to) a forearm-implanted parathyroid gland can display higher PTH measurements than when the PTH is drawn from the contralateral arm [7]. For certain immunoassays or mass spectroscopy methods, sample preparation (e.g., extraction) is also a key factor for proper analysis [8–11].

Sample collection and processing

Proper selection of the collection tube and post-phlebotomy sample handling is very important for many hormones. For example, glucagon and adrenocorticotropic hormone (ACTH) degrade quickly. To avoid this, plasma for glucagon measurement is prepared using an EDTA (ethylenediamine tetraacetic acid) tube containing a protease inhibitor (e.g., 0.04 mL of aprotinin added to

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each 1 mL of whole blood in a pink-peach color top tube or special tubes containing PPACK: Phe-Pro-Arg-chloromethylketone) followed by centrifugation (within 2 h) and freezing of the plasma. Aprotinin is a bovine pancreatic trypsin inhibitor that is typically purified from bovine lung. Vasoactive intestinal polypeptide (VIP) is similarly collected. For ACTH, blood is drawn into a chilled EDTA tube that must be plastic or siliconized glass, transported on ice, and then separated and frozen within 4 h. For antidiuretic hormone (ADH; e.g., arginine vasopressin), the EDTA sample should be centrifuged in a refrigerated centrifuge, separated and frozen within 2 h of collection. Regarding TSH, in one study ~2% of TSH measurements were elevated in lithium-heparin plasma separator tubes compared to rapid serum tubes [12].

The clinical laboratory must inform the medical and nursing staff of the pre-analytical requirements of sample acquisition. Currently many facilities provide this information on-line.

ASSAYS FOR HORMONAL ANALYSIS

Usually immunoassays are used in clinical laboratories for analysis of hormones. Many factors affect the accuracy of results obtained using these immunoassays including assay specificity and various other factors that may interfere with assay technology. These factors are discussed in this section.

Assay specificity

In selecting the proper analytical assay, the laboratorian should advise the clinician concerning the specificity of the assay performance. For example, many steroid immunoassays are not specific for a single steroid but may identify several members of a class of related steroids [13]. Estradiol assays detect a variety of estrogens [14] and cortisol immunoassays detect a variety of glucocorticoids [15]. For example, in the Advia Centaur cortisol immunoassay [16] the following selected percent cross reactivities are reported: corticosterone: 5.3%, cortisone: 31%, 11-deosxycortisol: 23%, 6 beta-hydroxycortisol: 6.8%, prednisolone: 109%, 6-methyl-prednisolone: 26% and prednisone: 34%. Other assay platforms demonstrate this cortisol interference though the percentages of cross reactivity vary. This lack of specificity results from the highly similar general structure of the compound of interest to other glucocorticoids [17]. There may also be negative interferences in immunoassays as reported for unconjugated estriol lowering the measured estradiol concentration [18]. In addition to specificity problems, immunoassays may lack low end analytical sensitivity [19]. This is problematic for testosterone immunoassays. Low testosterone concentrations (e.g., less than ~200 ng/dL) should be measured by mass spectrometry.

High-dose hook effect

Laboratorians must be aware of the possibility of the high-dose hook effect in immunoassays where samples with very high concentrations of an analyte produce a falsely low result [20]. In competitive immunoassays, one hypothesis explaining the high-dose hook effect is that the analyte self-aggregates and the analyte is thus under-detected. In double antibody assays, high-dose hook effect might result from such a great excess of analyte that there is insufficient capture antibody and detection antibody to measure the analyte.

The high-dose hook effect is probably of greatest importance for prolactin (Prl) [21,22]. Prolactinomas can produce Prl levels (in ng/mL) in the hundreds to tens of thousands. However, damage to the hypothalamus or hypothalamic-pituitary portal system can also raise Prl at least into the hundreds and less dramatic rises can be seen with multiple medications (Table 11.1) [23]. The difference between these clinical situations is critically important: prolactinomas can be treated with dopamine agonists whereas other large anterior pituitary adenomas are generally treated surgically.

TABLE 11.1 The differential diagnosis of hyperprolactinemia.

Central causes including PRIH[a] (dopamine) deficiency:
- Hypothalamic disease (tumors, granulomatous/vascular/autoimmune disease, irradiation)
- Interruption in the hypothalamic-pituitary portal system
- Prolactinoma or other pituitary lesions; Cushing disease
Medications:
- Dopamine antagonists
- Cholinergic antagonists
- Serotonergic antagonists
- Others: Tricyclic antidepressants, MAO-I, prokinetics (metoclopramide, domperidone), antihypertensives (alpha-methyldopa, verapamil), opiates (morphine), H_2 antagonists (cimetidine, randitidne)
Endocrine causes:
- Estrogen use
- Pregnancy, postpartum
- Hypothyroidism (pathologically elevated TRH can release prolactin)
Neurogenic
- Nursing (nipple stimulation)
- Chest wall disease
- Spinal cord injury
Other disorders:
- Cirrhosis
- Chronic renal disease

[a]PRIH, *prolactin release inhibiting hormone.*

Therefore, dilution and re-assay may be required in certain clinical situations to exclude the high-dose hook effect where the Prl result is falsely low and incongruent with the clinical picture. The lower analyte concentration in the diluted sample would allow less Prl aggregation in competitive immunoassays and reduce the amount of Prl captured by the capture antibody.

Macro-complexes

Autoantibodies against hormones can raise the circulating concentration of the hormone without producing clinical symptomatology of hormone excess because the free (unbound) hormone remains within the reference interval. The phenomenon of macro-complexes is most widely observed for amylase [24]. For hormones, this is best appreciated for Prl (23 kDa) where Prl can be bound by a spontaneously-developing anti-Prl antibody producing a complex of 150–160 kDa. Antibody-bound Prl is termed big-big Prl, or more commonly, macroprolactin. Another form of non-antibody-bound Prl is big Prl that is ~50 kDa. The biologic nature of big Prl is little understood. Big Prl and macroprolactin are thought to be biologically inactive and unable to reach target tissues (though they may be a source of circulating "little" Prl) [25]. Big Prl and macroprolactin elevate total Prl concentrations. When there is an unexplained Prl elevation, macroprolactinemia should be excluded. Treatment of the patient's serum or plasma with polyethylene glycol (PEG) will precipitate such antigen-antibody complexes and the resulting supernatant is then assayed. If the decline in the measured analyte (Prl in this case) is 50% or more, macroprolactin is present. However, the astute laboratorian (and clinician) will recognize that macroprolactinemia does not exclude true hyperprolactinemia of any cause. Other causes of hyperprolactinemia were discussed above and are reviewed in Table 11.1.

Anti-hormone autoantibodies that raise a hormone's concentration have also been reported for TSH [26–28], estradiol [29], T4 and T3 [30–32], insulin [33], ACTH [34], and non-hormone assays such as cardiac troponin I [35].

Human anti-mouse antibodies, rheumatoid factor and heterophile antibodies

Human anti-mouse antibodies (HAMAs), rheumatoid factor (RF) and heterophile antibodies can cause false positive immunoassay results. Heterophile antibodies represent naturally-occurring antibodies and autoantibodies directed against heterogeneous and ill-described antigens [36]. For more details see Chapter 7 and also the excellent review provided by Tate and Ward [37].

Rheumatoid factor is an IgM antibody that binds to the Fc portion of an IgG molecule. For example, in a recent report from France, heterophile antibodies increased the measured TSH concentration leading to overtreatment of a patient with suspected hypothyroidism [38]. Surprisingly, heterophile autoantibodies have even been reported to lower hormone concentrations [39].

Many non-competitive immunoassays use capture and detection monoclonal antibodies that are made in mice. Even without outright exposure to mice (e.g., somebody that works in a mouse colony or handles mice), the general population is exposed to mouse proteins and immunoglobulins (e.g., exposure of grain products to mouse urine). Individuals treated with therapeutic mouse monoclonal antibodies can certainly develop HAMA (Table 11.2) [40]. Responses to mouse immunoglobulins can therefore produce HAMAs. Manufacturers attempt to minimize the interference from HAMA by adding excess mouse immunoglobulin to their assay buffers. Thus, HAMA would bind to the free mouse immunoglobulin and would not bind to the mouse monoclonal immunoglobulins used in the assay. Some manufacturers have replaced mouse antibodies with antibodies from another species such as rabbit. For a discussion of the effect of HAMA on immunoassays see the excellent earlier paper published by Kricka et al. [41].

The best-known false positive results from HAMA involve false positive results in human chorionic

TABLE 11.2 Examples of mouse monoclonal autoantibodies in therapeutic use.

Monoclonal antibody	Activity
Abciximab	anti-GPIIbIIIa
Adalimumab	anti-tumor necrosis factor-alpha
Alemtuzumab	anti-CD52
Basiliximab	anti-IL-2
Bevacizumab	anti-vascular endothelial growth factor (VEGF)
Cetuximab	anti-epidermal growth factor receptor
Infliximab	anti-tumor necrosis factor-alpha
Muromonab-CD3	anti-CD3 (Orthoclone)
Ofatumumab	anti-CD20 cytolytic
Omalizumab	anti-IgE
Palivizumab	anti-respiratory syncytial virus (RSV)
Rituximab	anti-CD20
Situximab	anti-IL-6
Trastuzumab	anti-human epidermal growth factor receptor-2 [HER2]

gonadotropin (hCG) testing. This can lead to the errant diagnosis of choriocarcinoma. In at least one case, a woman with a false positive hCG result was treated with chemotherapy for choriocarcinoma followed by total bilateral oophorectomy and hysterosalpingectomy. Similarly, beta-hCG positivity was falsely seen in 30% of an IgA-deficient population due to heterophile antibodies [42]. HAMA interferences have also been reported in the measurement of TSH [43–46]. Heterophile antibodies, confirmed after incubation in heterophile antibody blocking tubes, have also been reported for FSH, intact PTH, ACTH, IGF-1, and calcitonin [47]. HAMA should also be considered in non-hormone immunoassays [48]. For example, a HAMA interference in digoxin measurements has been reported [49]. Other analytes with reported HAMA interferences include: B-type natriuretic peptide, CA-125 [50–52], HBsAg (hepatitis B surface antigen) [53], and cardiac-specific troponins I and T [54].

When HAMA interferences are suspected, the assay should be rerun on an immunoassay platform of another manufacturer. Serial dilution linearity should also be sought. With serial dilution, if the analyte recovery is greater than expected, HAMA is likely present. However serial dilutions that yield the expected recoveries do not exclude HAMA. As IgG antibodies, HAMA do not enter the urine and thus serum or plasma immunoassays can be falsely positive yet the urine immunoassays are not interfered with and are correctly negative which can be used to clarify the hCG-status of the patient (e.g., hCG is or is not present). Assays for HAMA are available in the evaluation of suspected HAMA interferences [48].

Although the release of hormones from plasma binding proteins is not an issue of interference, total hormone methods should release all protein-bound hormones for proper analysis. For example, this is important in measuring IGF-I. As well, significant variations in binding protein concentrations are not interferences; however, such aberrations can produce clinically important changes in the total concentrations of hormones in the blood stream. For example, total T4 is affected by concentration changes in thyroxine-binding globulin (TBG) and thyroxine-binding prealbumin (TBPA, also known as transthyretin) [55]. Total T3 is also affected by concentration changes in TBG. Abnormal affinity for T4 raises total T4 in dysalbuminemic hyperthyroxinemia and familial euthyroid thyroxine excess [56,57]. Sex hormone binding globulin (SHBG) has high affinity for testosterone and estradiol (an estrogen) and changes in SHBG levels can cause aberrant results. The calculated amount of free testosterone is still dependent on the concentration of SHBG. Even assays designed to measure free hormones are not immune from the effects of different binding protein concentrations [58].

The recognition and prevention of errors is a major goal of medicine in the present day [59]. Some interference can develop that are rare, and/or are unexplained [60]. The laboratorian must be able to assist the clinician when the laboratory findings and the patient's history and physical examination findings do not match. Under such circumstances, analytical errors or interferences may be present, the clinical diagnosis may be incorrect, or the blood sample may have been misidentified. As laboratorians, we likely spend much of our time concerned with the analyte's measurement; however, analytical errors may only account for 10% of all laboratory "mistakes" [61].

Biotin ingestion-associated interference

The type and frequency of immunoassay interferences can vary with changing trends in health and health care. A new type of interference to arise in the last decade involves the oral ingestion of excess biotin, a supplement, and its interactions with immunoassays that take advantage of the strong non-covalent binding between biotin and streptavidin (a large protein often coated onto magnetic particles) to allow quantification of the analyte in question. Biotin, a B vitamin, is a co-factor for multiple carboxylases involved in glucose, amino acid and fatty acid metabolism; it is found naturally in almonds, cauliflower, banana, yogurt, tomatoes, eggs and other foods. The recent popularity of biotin supplementation has arisen from reports of high dose biotin boosting nail and hair strength despite a lack of large, controlled studies supporting any true benefit [62]. Recent studies, including a randomized, double-blind, placebo-controlled trial support very high dose biotin therapy (100 mg three times a day) in progressive multiple sclerosis where improvements in clinical activity scores were seen in a small number of patients [63]. Other less well-studied, proposed health benefits of biotin include the treatment of lipid disorders, diabetes, and diabetic peripheral neuropathy [64]. High doses of biotin, on the order of 10–15 mg per day from over-the-counter supplementation, exceed the NIH Office of Supplements recommended daily intake (up to 30 mcg a day) by 330-fold. As part of a multivitamin, only 30–300 mcg of biotin are included which is minimal compared to the degree of supplementation seen in individuals in the community. Comparatively, biotinidase deficiency, a clinical indication for biotin supplementation, is rare (worldwide incidence less than 1:~61,000 if partial deficiencies are included) and require 5–10 mg/day of biotin replacement [65].

Biotin-streptavidin-dependent hormone assays run the risk of interference with supra-physiologic doses of biotin in the serum (Fig. 11.1). Excess biotin ingestion

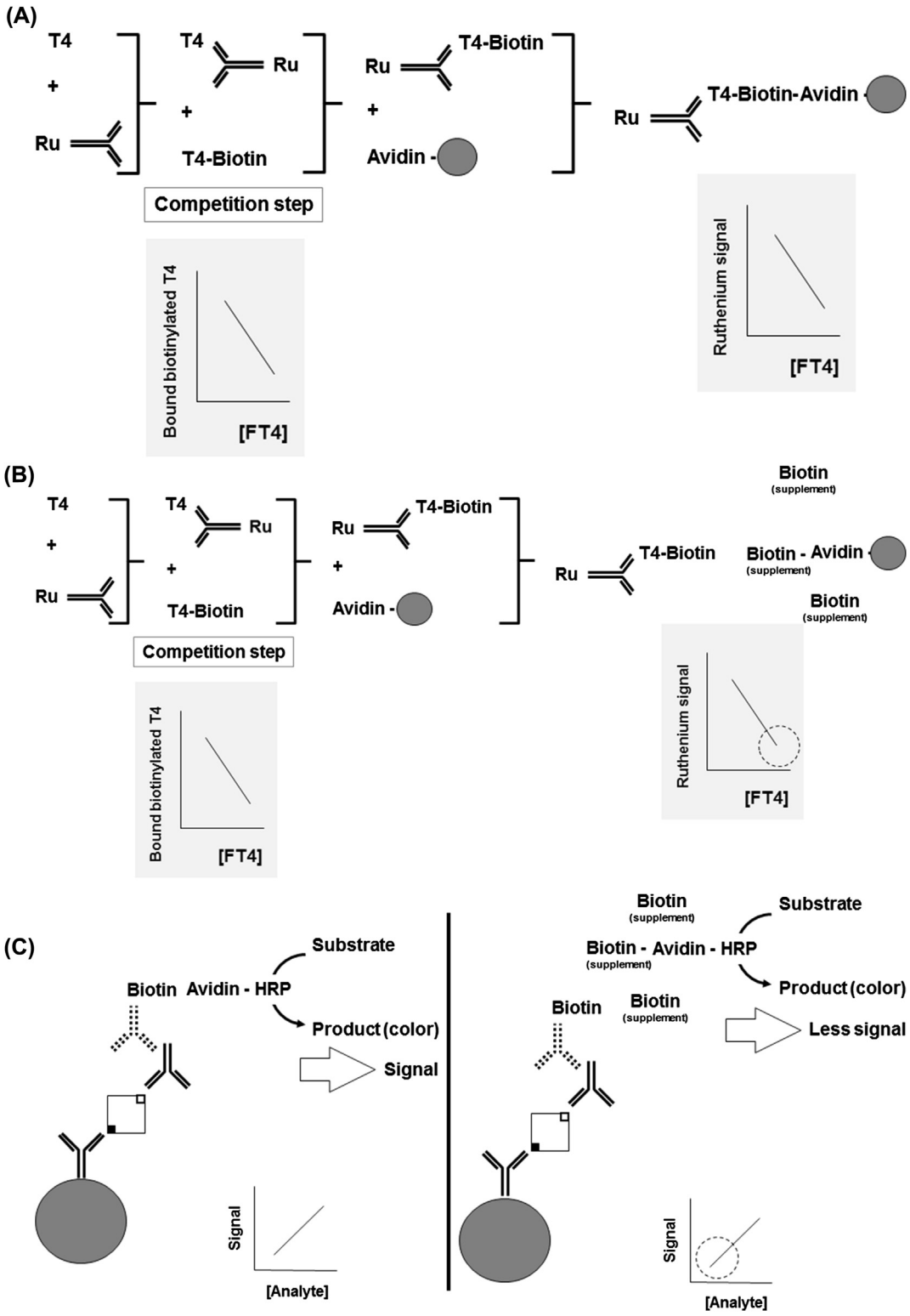


FIG. 11.1 (A) This figure depicts a typical measurement of unbound (free) T4 (FT4) in a competitive immunoassay format using a labeled antibody. An anti-T4 antibody labeled with ruthenium is added to the patient's serum. The unbound T4 (FT4) binds to the anti-T4 antibody labeled with ruthenium. The open binding sites are then occupied by added biotinylated T4 (T4-biotin). The amount of biotinylated T4 that binds to the antibody is inversely related to the FT4 concentration in the patient's serum. Next avidin-coated beads are added and a 3-part complex is formed: anti-T4 antibody labeled with ruthenium − T4-biotin − avidin-coated bead. The ruthenium signal is inversely proportional to the FT4 concentration. (B) This figure depicts the consequences of high serum concentrations of biotin where such "free" biotin interferes with the capture of the 2-part species of the "anti-T4 antibody labeled with ruthenium − T4-biotin" by the avidin-coated bead. This decreases the ruthenium signal falsely raising the apparent FT4 concentration (i.e., the dashed-line circle on the graph of the ruthenium signal vs. the FT4 concentration ("[FT4]"). (C) The figure on the left depicts a possible double antibody format for the measurement of an analyte of sufficient size that it contains two separate epitopes that are not sterically hindered. Initially the analyte is captured by the antibody attached to the bead. The analyte is then bound by a second antibody (*solid lines*; the detection antibody). The detection antibody is then bound by a third antibody (*dashed lines*). This later antibody is labeled with biotin. Next an avidin − horse radish peroxidase (HRP) conjugate is added. The amount of substrate produced is proportional to the analyte concentration. On right is depicted the consequences of exogenous biotin which lowers the signal reducing the measured analyte concentration (i.e., the dashed-line circle on the graph on the signal vs. analyte concentration).

can result in falsely low readings for large molecules such as TSH and PTH analyzed by some sandwich, or double antibody, immunoassays that employ biotin-streptavidin binding. This occurs via decreased streptavidin binding to biotinylated TSH antibody where a labeled anti-TSH "detection" antibody would complete the reaction. Excess unbound biotin binds streptavidin and the TSH-TSH biotinylated antibody sandwich complexes are washed away without being bound to the solid state with streptavidin lowering the measured TSH concentration. The same mechanism is true for PTH and this has been confirmed with multiple enzyme-linked immunosorbent assay (ELISA) results [66]. In contrast in the measurement of small molecules, such as thyroxine, triiodothyronine, and 25-hydroxyvitamin D, which are detected through competitive immunoassays that utilize biotin-streptavidin interactions, hormone levels are detected as falsely elevated. Again there is a decreased ability of streptavidin to capture the biotin-labeled analyte because of exogenous biotin; however, here a biotinylated T4 (not the patient's T4) is binding to streptavidin and the excess (true T4) is washed away. Biotin supplementation competes with the biotinylated T4. We must remember that in competitive immunoassays the label or output (e.g., with ruthenium) produces an inverse response to the amount of analyte present so when a lower signal is detected due to excess biotin binding then a higher value is reported.

The complicated clinical picture that can arise from, for example, a decrease in TSH and an increase in free T4, can lead to improper diagnosis and treatment of hyperthyroidism [67,68]. This does not occur with every streptavidin-biotin-based immunoassay but Li et al., and others have compiled lists of immunoassays and analyzers frequently affected [64,69]. Asking about supplements such as biotin must be considered prior to ordering such tests or upon receipt of abnormal laboratory studies that do not fit the clinical picture. Evaluation on a different platform that does not use a biotin-streptavidin interaction is the first step when this interference is considered followed by discontinuation of the supplement and retesting. Pharmacokinetic studies in healthy adults following one dose of biotin (5–20 mg) demonstrate a spike of biotin concentration within 1 h that wanes 12 h later (biotin concentrations fell below 50 ng/mL) [70]. However, other studies show a slower return to baseline for the analyte being tested of up to 24–48 h after biotin discontinuation for TSH and free T4 or longer, and up to 7 days for anti-thyrotropin receptor antibodies [71,72]. However, specific recommendations for patients on much higher biotin doses treated for longer periods of time are not available. Additionally, individual laboratories have developed protocols to retest or treat the blood samples such as with streptavidin agarose to deplete excess biotin. Equipment manufacturers may provide information about biotin thresholds in the serum above which interference is possible. Another example, 25-hydroxyvitamin D is labeled with biotin to form a complex in the solid phase with streptavidin and laboratory-specific testing determined that concentrations of biotin in the serum up to 70 ng/mL have not shown interferences [73]. Lastly some immunoassays use biotin and an anti-biotin antibody for binding assay reagents; therefore biotin can be used in an immunoassay in the absence of streptavidin, yet exogenous biotin can still interfere.

CHALLENGES IN TESTING OF HORMONES SECRETED BY PITUITARY

The pituitary gland in the human brain is responsible for secretion of many hormones and accurate measurement of these hormones is crucial for diagnosis of many illnesses. In this section, assays for these hormones are discussed with an emphasis on sources of errors in their measurement and steps that can be taken to avoid such errors.

Growth hormone

The measurement of growth hormone (GH) can be helpful in the diagnosis of disorders involving either growth hormone excess or deficiency. Due to the pulsatile nature of GH release, measurements are typically performed during dynamic testing in an attempt to assist in the interpretation of the results. Aside from the pulsatile release of GH, a number of other interferences, both related to the patient's lifestyle as well as the laboratory's ability to measure the analyte, can alter the measurement.

The release of GH from the pituitary is controlled and stimulated by a number of processes. GH release is normally stimulated by growth hormone-releasing hormone (GHRH) and inhibited by somatotropin release-inhibitory hormone (SRIH), both of which are released from the hypothalamus under the control of a number of different stimuli. SRIH is also known as somatostatin, which is also secreted by the delta cells of the islets of Langerhans. It has been well documented that following the onset of exercise, GH levels will rise in the plasma [74,75]. GH increases can also be seen due to stress, hypoglycemia, sleep, and various pharmacologic agents such as glucagon, clonidine and L-dopa; likewise, GH suppression is seen with somatostatin, cortisol, obesity and hyperglycemia.

TABLE 11.3 Circulating forms of growth hormone (GH).

22 kDa GH
20 kDa GH
GH-GH (dimmer; a.k.a., "big" GH)
GH-GH binding protein (GHBP; "big-big" GH)

There are a number of variables that can influence the measurement of GH in the laboratory. Much of the difficulty in measuring GH lies in the inherent heterogeneity of the analyte (Table 11.3). GH is released primarily from 2 different sites, the somatotroph cells of the pituitary gland and the placenta. Placental GH appears between 15 and 20 weeks gestation and is present until term, completely replacing the pituitary GH which becomes undetectable [75]. While this form of GH only appears during pregnancy, to what degree it cross-reacts with GH immunoassays is uncertain and interpretation should be performed with caution. It would be an extremely unusual clinical case if GH measurements were required during pregnancy. Due to alternative splicing of the gene encoding GH, two isoforms exist: the 22 kDa (191 amino acids) and 20 kDa (176 amino acids) forms. The 22 kDa isoform that represents 85%−90% of all GH is known to be the more biologically active molecule and it is typically detected in all GH immunoassays. Efforts have been made to specifically detect the 20 kDa form through the development of a "non-22K assay" where monoclonal antibodies against the 22 kDa isoform are used to complex and remove this isoform and the remaining GH is measured using a polyclonal immunoassay. An alternative method involves raising monoclonal antibodies specifically against the 20 kDa isoform [76]. Not only are there 2 GH isoforms, but GH can also exist as homodimers ("big GH"), heterodimers (GH plus its binding protein) and multimers [77].

Clinical measurement of GH is typically achieved by the use of an immunoassay and because of the heterogeneity of GH, commutability of the assays is problematic. If monoclonal antibodies are used for capture, then only a subset of the GH molecules will be measured. When comparing assays that employ monoclonal versus polyclonal antibodies, the latter can show higher measured concentrations of GH due to an increase in the forms of GH detected. When immunoassays were first introduced for the detection of GH, commutability was less of a problem; however, as the assays increasingly utilize specific monoclonal antibodies, the differences among assays have become more pronounced. Another problem with commutability of the assays is the lack of a reference standard with which to standardize the assays. Various circulating growth hormones are listed in Table 11.3.

Indirect interferences in the assay also exist, adding more layers to the difficulty of GH measurement. In the body, up to 50% of GH is bound to GH binding protein. Depending on the epitope that a monoclonal antibody binds to on GH, steric hindrance (the inability of the epitope to be accessed by the antibody) can occur, thereby falsely decreasing the results. Another cause of falsely altered results can be a consequence of the use of therapeutic GH analogs. This was reported in the use of Pegvisomant, a modified GH analog that is used as a GH receptor antagonist [78]. Patients taking this drug had falsely low GH concentrations in most of the assays tested. Because the concentration of Pegvisomant was 1000-fold that of GH, the authors hypothesized that the drug was binding the capture antibody in the assay while blocking the formation of the sandwich with the second antibody, resulting in decreased levels.

In athletics, detection of GH abuse could be afforded by specifically measuring the 20 and 22 kDa GH isoforms separately. Both the 20 and 22 kDa forms should be found in the plasma of normal individuals. However, the detection of exclusively the 22 kDa form would suggest the administration of exogenous GH because recombinant DNA-derived GH consists of only this isoform and lacks the 20 kDa isoform.

Adrenocorticotropic hormone

The measurement of ACTH can be helpful in determining the etiology of pathologically decreased cortisol (e.g., Addison disease) or increased cortisol (e.g., Cushing disease or ectopic ACTH). ACTH is secreted from corticotrophic cells of the anterior pituitary in pulsatile bursts creating fluctuations in ACTH blood levels at time scales ranging from minutes to days. Hypothalamic corticotrophin-releasing hormone (CRH) mediates control of ACTH secretion based on cortisol levels. A negative feedback system operates between cortisol and CRH—as cortisol levels decrease, CRH is increased, and vice versa. CRH directly stimulates ACTH and ACTH directly stimulates cortisol release. Aside from the feedback loop mentioned above, the neurotransmitters serotonin and acetylcholine are also known to stimulate ACTH, while GABA inhibits its secretion. There are a number of other endogenous factors (fever, hypoglycemia, cytokines, anti-diuretic hormone, etc.) that can affect the release of ACTH.

Aside from the endogenous regulation and variation, ACTH measurement is very sensitive to pre-analytical variables. It has been shown that as little as 0.1% hemolysis can cause significant loss of ACTH immunoreactivity [79]. Storage temperature and time to centrifugation also play important roles in the stability of the ACTH prior to measurement. In the evaluation of blood

samples taken from 10 healthy volunteers, the storage temperature of the blood prior to centrifugation was found to affect stability [80]. A more recent study highlighted that the more critical factor affecting stability is the amount of time expired prior to centrifugation [81]. For samples that were kept at room temperature and stored at variable time periods prior to centrifugation, the authors saw no relevant change at 4 h and a mean decrease of 10% in the ACTH concentration by 24 h as measured with an automated chemiluminescence assay (confirming earlier reports from a manual radioactive version of the same assay). Based on their study, the authors recommended that centrifugation be performed within 4 h of the sample being drawn.

The methodology used to measure ACTH in the clinical laboratory has evolved from highly complex bioassays [82] and two-site immunoradiometric assays to automated immunoassays offering high through-put and fast turn-around times. There are a number of different assays available, and similar to other antibody-based assays, standardization and agreeability are problematic. A 2011 publication compared 25 fresh-frozen plasma samples assayed by 7 different ACTH assays in 35 different laboratories [83]. The authors reported a wide range of inter and intra-assay coefficients of variation across these assays, ranging from <10% to >20%. Not only did these assays show substantial differences with respect to imprecision, they also demonstrated variable agreement among the methods. While the assays were able to correctly classify most patients with high or normal ACTH values, only 60% of measurements of samples with low ACTH concentrations were correctly classified. The conclusion from this paper is one found widely across multiple analytes within the endocrine field: ACTH assays should be standardized.

Thyroid stimulating hormone

With the number of cases of thyroid disease on the rise, the measurement of TSH continues to be an important part of laboratory medicine. TSH measurements are frequently ordered by primary care physicians and endocrinologists. In addition, TSH measurements may be requested on a stat basis by emergency department physicians for suspected cases of thyroid storm, myxedema coma or myxedematous heart failure. TSH concentrations in the serum can vary based on the time of day with maximum levels achieved at 2–4 a.m. and minimum levels achieved between 6 and 10 p.m. The minimum level can be decreased by as much as 50% compared to the maximum level, which can make interpretation difficult although in euthyroid subjects, the TSH maximum and minimum will remain within the reference interval. Even though TSH is a routinely measured analyte, it is still prone to many of the interferences that plague all immunoassays. A number of endogenous interferences have been reported, such as rheumatoid factor, heterophilic antibodies [84] and paraproteins [85]. Another confounding factor is that when exogenous T4 is ingested, the T4 (or free T4, FT4) measurements can become discordant from the TSH measurements. For example, suppose a patient has primary hypothyroidism with a low FT4 level and an elevated TSH level. With oral administration of thyroxine, the FT4 can return to the reference interval far more quickly than the TSH where the TSH may not return to the reference interval for several weeks.

TSH is typically measured by a two-site immunoassay and interference from other endogenous antibodies is an ongoing problem. There have been reports of macro-TSH, where TSH has complexed with an immunoglobulin, which is believed to have a decreased clearance resulting in the accumulation of these molecules, falsely increasing the TSH level. This is typically identified due to an unusually increased TSH level with normal free T3 and free T4 levels and no symptoms. Similar laboratory findings are also seen in obesity where mild TSH elevations (but normal T4 and T3) are produced due to excess leptin-induced stimulation of thyrotropin-releasing hormone [86].

Identification of macro-TSH can be through the use of protein A beads to remove IgG-complexed TSH molecules or it can be a diagnosis of exclusion due to thyroid studies within the reference intervals and a clinically euthyroid state with a persistently elevated TSH level [87]. There was an interesting case of macro-TSH in a neonate who was identified with increased TSH levels, but normal T4 and T3, on neonatal screening. Both newborn and mother had elevated TSH levels that were due to macro-TSH, as shown by gel filtration chromatography. In the case of the newborn, the macro-TSH slowly decreased only to completely disappear after 8 months of life, consistent with the rate of elimination of maternal IgG. TSH is unique in that the identification of an elevated TSH level in an otherwise euthyroid patient does not immediately identify a macro-TSH. A persistently elevated TSH in a patient with free T4 and T3 levels within the reference intervals may indicate subclinical hypothyroidism [88], which is likely more common than the existence of macro-TSH. In addition, TSH with impaired biologic activity may have a similar presentation to macro-TSH: an elevated TSH with a normal free T3 and T4 along with the presence of a higher molecular weight TSH by chromatography [89,90]. Mutations in the TSH beta subunit have been reported in patients with impaired TSH activity [91]. A distinguishing characteristic for patients whose TSH is inactive is the reduction in TSH after the

administration of exogenous free T3; if the TSH was elevated purely because of binding with IgG, exogenous T3 should not be able to influence the rate of clearance of TSH (and TSH would not decline in such a case).

Luteinizing hormone/follicle stimulating hormone

Luteinizing hormone (LH) and follicle stimulating hormone (FSH) are both gonadotropins that control the functional activity of the gonads. The release of LH and FSH from the anterior pituitary is stimulated by gonadotropin releasing hormone (GnRH). The release of LH/FSH is episodic. The levels of LH and FSH vary during the course of the menstrual cycle. Post-menopausal women have persistent elevations in LH, and particularly, FSH.

Initial LH measurements were performed using radioimmunoassay (RIA) which included the use of polyclonal antibodies. One limitation of the use of polyclonal antibodies in the RIA was cross-reactivity with hCG and an increased non-specific background when compared to the use of some monoclonal antibodies [92]. As this methodology was replaced by more easily automated immunoassays, monoclonal antibodies were incorporated into these assays. While the performance of these assays was considered to be acceptable, the results obtained across platforms were not identical. Studies were initiated in an effort to better understand the differences among assays and there were multiple conclusions. For 11 different commercial companies manufacturing LH immunoassays, there were a total of 55 different monoclonal antibodies used [93]. These antibodies further differed in the LH epitopes that they recognized, including antibodies specific for the alpha subunit, the beta subunit and the holomolecule (anti-alpha beta). The variety of antibodies used across differing vendors contributes not only to a lack of standardization, but can lead to very different concentrations measured by an assay, based on the characteristics of the antibodies used. Studies with these kits were undertaken using an international standard for LH (IS 80/552) in which the authors were able to determine that the calibration curve differed among kits, adding to the discrepancy between the kits [94]. Similarly, these same problems were encountered when the RIA for FSH was converted to an immunometric assay. The immunometric assay gave results that were an average of 17% higher than the RIA results, while the LH results were 33% lower for the assays evaluated in this study. This presented a unique problem in that LH:FSH ratios were used when evaluating a patient for polycystic ovarian syndrome (PCOS). In a study of 78 women presenting with polycystic ovaries on pelvic ultrasound and 59 controls with normal ovaries by ultrasound, by comparison of both the RIA and two different monoclonal immunoassays, the cut-off ratio for PCOS used would need to be re-established using the new monoclonal assays [95]. Having mentioned PCOS, current draft PCOS medical guidelines do not use the LH to FSH ratio as a diagnostic marker of PCOS [96]. Evaluation of different vendor assays in different patient populations have also further confirmed the variation seen between assays [97].

Similar to other hormone assay interferences, a macro-FSH has also been reported in the literature [98]. In this case, the presence of this macrocomplex resulted in a false positive interference in a sandwich immunoassay. As for LH, a patient with a macrocomplex was also identified for this hormone in 2003 [99]. In this case report, the patient showed a high LH value while other hormone studies were normal except for high concentrations of thyroperoxidase antibodies. The authors identified a macrocomplex through gel filtration and chromatography.

Prolactin

Prolactin (Prl) is a hormone released from the anterior pituitary. Its physiologic role is in lactation where suckling increases Prl concentrations. Levels of this hormone are at their lowest at midday and peak at night just after the onset of deep sleep and can be stimulated in a number of events such as stress. Increases in estrogen can also stimulate secretion and gene transcription of prolactin [100]. Multiple drug classes can have effects on prolactin release. Antipsychotics are the most common drugs to cause increased prolactin levels. Increases can also be seen with morphine and morphine analog use, while lithium can decrease Prl levels by 40% [101,102]. Very high levels of Prl can generally fall into two categories, either a prolactinoma or macroprolactin. Prolactinoma describes a tumor in the anterior pituitary that secretes large amounts of Prl. Due to the high levels of Prl that are produced by this tumor, laboratory measurement of Prl in these patients can be challenging due to the high-dose hook effect. In a case report by Frieze et al., they describe a male patient who initially presented with headaches, personality changes and bulging eyes [21]. His initial Prl levels were elevated at 164.5 ng/mL (reference interval, 1.6–18.8 ng/mL). A biopsy from a 10-cm mass in his anterior skull base and cranial fossa confirmed the presence of a prolactinoma. Due to this discrepancy in lab values with the clinical diagnosis of a large tumor, the original serum was retested with serial dilutions eventually revealing a Prl of 26,000 ng/mL.

Macroprolactin, also known as big-big Prl, has a molecular weight greater than 100 kDa typically due to complex formation with an immunoglobulin.

The characteristics of macroprolactin are similar to other macrocomplexes that result from immunoglobulins, typically IgG, binding to Prl contributing to the larger molecular mass and increasing its persistence in the circulation. Classically, the presence of a macroprolactin does not contribute any diagnostic information because Prl in this form is not biologically active; however, macroprolactin can pose a serious problem when interpreting laboratory results. The high Prl results typically seen in patients with prolactinomas can also be encountered in patients with macroprolactin, potentially leading to the misdiagnosis of a patient. In an effort to correctly classify patients, routine screening of hyperprolactinemic samples (females $>$ 500 mU/L and male $>$ 290 mU/L) for macroprolactin has been recommended [103].

According to the published literature, macroprolactin can account for up to 26% of hyperprolactinemia cases reported depending upon the commercial immunoassay used [104,105]. In a report by Smith et al., they identified 10 patient samples with elevated Prl levels due to macroprolactin. They then analyzed these samples at 18 different clinical laboratories using a total of 9 different assay systems [106]. Across the range of assay systems evaluated, they reported differences in Prl ranging from 2.3 to 7.8-fold, with the Roche Elecsys users reporting the highest Prl levels. These authors utilized the technique of gel chromatography to establish the presence of macroprolactin; however, the more frequently used technique was immune complex precipitation using polyethylene glycol (PEG). This method is not without problems, as there are reported interferences of PEG with the Prl immunoassay on a variety of analyzers [107]. In an effort to circumvent this issue, reference intervals have been determined for Prl values of PEG-treated samples. Evaluating samples from 146 healthy volunteers, reference intervals were established for the Abbott Architect, ADVIA Centaur, Siemens Immulite, Beckman Coulter Access, Roche Elecsys and Tosoh A1A [106]. The intervals were evaluated using samples with known macroprolactins and non-macroprolactin, as established by gel filtration chromatography. Another interference with PEG precipitation is increased concentrations of serum globulins, as seen in cases of hypergammaglobulinemia from IgG myeloma and HIV infection [107]. In both cases, it was concluded that monomeric Prl was being precipitated along with the immunoglobulins, leading to a falsely low level of Prl after PEG treatment.

CHALLENGES IN MEASURING HUMAN CHORIONIC GONADOTROPIN

The measurement of human chorionic gonadotropin (hCG) is clinically used in the determination of pregnancy. Depending on the assay, the specimen may be either urine or blood and the measurement either qualitative (as in point-of-care) or quantitative. One of the biggest challenges in the measurement of hCG is the presence of different biologic forms of hCG. There exist at least six hCG variants including intact hCG (nicked and non-nicked), the free beta subunit (hCGβ; nicked and non-nicked) and the free hCG alpha subunit (regular and hyperglycosylated). These different forms can be released in varied ratios based on disease state and assays measuring this hormone can vary widely with respect to the different forms that are detected. Of the six forms listed above, most assays focus either on measuring intact hCG alone or hCGβ plus intact hCG.

Urine pregnancy tests play an important role in both emergency departments (EDs) and radiology suites to detect the pregnancy status of patients where it may be unknown, or to rule out pregnancy prior to radiation. The high-dose hook effect can have a serious effect on these tests, producing false negatives and potentially leading the physician in an incorrect direction. There are reports in the literature where the hook effect has contributed to false negative results in the patients with molar pregnancies [108,109]. The presence of a hydatidiform mole can result in hCG concentrations of greater than 1,000,000 mIU/mL and those cases are at high risk of false negatives due to the hook effect [109]. In these cases, dilutions of samples and reanalysis for hCG can help determine if the hook effect is contributing to a false negative result. The sensitivity of a particular assay to the hook effect varies greatly dependent on the immunoassay method used. To better understand the extent of this variation, a study evaluated 6 different commercial assays for hCG measurement [110]. Using a sample with a total hCG concentration of approximately 3,600,000 mIU/mL, the authors measured this sample on the six different platforms. Out of the 6 analyzers evaluated, four demonstrated the hook effect while the remaining 2 analyzers did not.

In addition to the well-known high dose hook effect, another cause of a false negative result could be from a "hook-like effect" due to an increase in hCGβ core fragment (hCG-βcf). This fragment is formed by proteolytic degradation of hCG in the kidneys and is then excreted. The amount of hCG-βcf in the urine is first detected in the later stages of early pregnancy (5–8 weeks of gestation) until term and it is hypothesized to bind and saturate the primary antibody in a sandwich immunoassay, essentially blocking any binding for other forms of hCG [111,112]. The second antibody in these methods does not recognize hCG-βcf as they were optimized for early pregnancy detection, and the result is a false negative [113]. This theory was proven by Gronowski et al., through the evaluation of three urine samples with apparent false negative results using a

point-of-care test [114]. In this study, the authors used specific hCG antibody assays to measure the different forms of hCG present in the urine samples. They found that in these false negative samples, the hCG-βcf was in molar excess of the intact hCG (64%–78% of all hCG immunoreactivity). They further showed that this was the fragment causing the false negative result by adding 1,000,000 pmol/L hCG-βcf to urine that had been previously determined to have a concentration of 17,800 IU/L hCG. The point-of-care urine hCG result changed from positive prior to the addition, to negative after the hCG-βcf was added in 2 out of 3 of the POC assays evaluated. This work was expanded to look at a fourth urine point-of-care pregnancy device, and they found that false negatives due to the addition of hCG-βcf to urine varied with device lot number [115]. The interference from hCG-βcf also translated to the serum assay, where investigators found that in 2 out of the 9 assays evaluated, values with greater than a 50% reduction were observed in the presence of elevated hCG-βcf [116]. While the issues concerning measurement of hCG-βcf are fairly well documented, the hCG hormone can exist in a number of different forms which can also make interpretation of hCG assays difficult. A published case describes a discrepancy between results obtained using urine and plasma samples from the same patient [117]. A 41-year-old woman presented with vaginal blood loss and was hospitalized. Both qualitative and quantitative assays were used to test 2 successive urine samples for hCG, with all results positive, while the corresponding plasma samples resulted in no hCG present. Through hCG isoform analysis, the authors were able to identify that the majority of hCG being produced was a degraded form of the β-subunit that lacked the C-terminal peptide. This variant was thought to have a fast clearance, as a possible explanation as to why it was undetectable in the plasma, yet measured repeatedly in the urine.

While the occurrence of false positive results is less frequent than false negatives, false positive results are still encountered in the measurement of hCG. Specific to the hCG assay, false positives can exist in two different categories: a falsely increased hCG level or a positive pregnancy test when the patient is not pregnant. Analytically, the serum/plasma hCG assay is susceptible to many of the same interferences as any traditional immunoassay, such as heterophile antibodies [118]. There is even a report of macro-hCG involving IgM [119]. However, because these molecules are too large to be filtered by the glomeruli, the urine hCG test typically does not encounter these interferences. There have been cases of a true analytical interference causing a false positive urine test [120], although these appear be less frequent than false positive serum hCG tests. In this particular case, the patient had a tubo-ovarian abscess from an intrauterine contraception device with a positive enzyme-linked immunosorbent assay (ELISA)-based urine pregnancy kit. Pathologic evaluation showed a bacterial colony consistent with *Actinomyces* with no notation regarding the presence of trophoblastic tissue and a negative serum hCG result. The actual mechanism of interference in this case was unresolved.

CHALLENGES IN THYROID FUNCTION TESTS

Numerous interferences in thyroid immunoassays have been identified as noted in the introduction including macro-TSH, and T4 and T3 autoantibodies [121,122]. More recently, increased intake of the supplement biotin has shed light on certain thyroid testing immunoassays that are disturbed by such supplements. These interferences were discussed previously. However, interferences in thyroglobulin (Tg) and calcitonin immunoassays were not mentioned previously in this chapter and will be examined here [123].

Thyroglobulin

Papillary and follicular carcinomas are differentiated thyroid cancers. Tg can be measured as a tumor marker of such thyroid cancers following surgical thyroidectomy [124–127]. Pre-operative Tg measurements have no role in the diagnosis of thyroid cancer. However, pre-operative confirmation that a differentiated thyroid cancer produces Tg would allow Tg use as a tumor marker in such cases post-operatively. Uncommonly, differentiated thyroid cancers will not produce Tg and therefore Tg production must be confirmed before it is used as a tumor marker [128]. Tg measurements do not have a role in the management of anaplastic thyroid carcinoma or medullary thyroid carcinoma (where calcitonin is a tumor marker; see below).

If a patient has had a complete thyroidectomy and there are no metastases, Tg should not be detectable several weeks post-operatively [129]. In order to ensure that any remaining functional thyroid tissue is maximally stimulated for detection post-operatively, thyroxine replacement is discontinued until the TSH is clearly elevated (which takes ~2 weeks) or recombinant-DNA-produced TSH (Thyrogen-alpha) is injected (two doses are administered 24 h apart with Tg measured 72 h after the second dose). If the Tg remains undetectable, the prognosis is good and the patient should be retested for Tg periodically in the future [130]. Many experts believe that such Tg measurements have replaced the need for repeated thyroid scans for the detection of thyroid tissue and metastases [131].

Heterophile antibody interference is seen in Tg assays and can lead to false elevation and potentially unnecessary therapy such as radioactive iodine ablation in differentiated thyroid carcinoma patients following thyroidectomy [132]. Another important, and common, interference in Tg assays is the presence of thyroglobulin autoantibodies (TGAs) [133]. Whereas the general population frequency of TGA is ~5%, for unclear reasons, TGA can be found in ~10% of patients with differentiated thyroid carcinomas. TGA interferences can be more significant for double antibody (sandwich) assays than competitive immunoassays but TGA can interfere in either assay format.

While it might be expected that TGA would lower Tg measurements (if the affinity of TGA for Tg is very high), there is no way to predict if the TGA interference will be a positive interference or a negative interference. Therefore, the best practice is to not report Tg measurements if TGA are present. This implies that every Tg measurement requires that TGA also be measured (which is a best practice) [134]. Recovery studies are not reliable to determine the "true" Tg level when TGA are present. Methods for mass spectrometry (LC-MS/MS) measurement of Tg have been reported in the literature in persons with TGA [135].

Although Tg should not be used to assess post-operative status in the presence of TGA, absolute TGA levels may substitute as a tumor marker. Here the hypothesis is as follows: if TGA are detected in a post-operative patient with a history of differentiated thyroid cancer, the TGA would only be present if there were thyroid tissue remaining (either normal thyroid or thyroid cancer). Therefore higher TGA levels would correlate with a higher post-operative tumor burden [135,136].

Calcitonin

The tumor marker used in cases of medullary thyroid carcinoma (MTC) is calcitonin (CT). Like Tg, the CT value does not distinguish benign from malignant tumors. CT is followed post-operatively to monitor the completeness of surgical removal of the MTC and the possibility of local tumor recurrence or metastases. Because basal CT levels may not be detectable in MTC, CT should be measured after stimulation with intravenous calcium [137,138]. Elevated calcitonin levels due to the presence of heterophile antibodies have been reported in ~1% of patients with thyroid nodules [138].

ADRENAL FUNCTION TESTS

Cortisol is probably the most commonly measured analyte for evaluating adrenal function although aldosterone and renin are also ordered frequently. In this section pitfalls in testing these analytes are addressed.

Cortisol

The levels of endogenous cortisol can be very sensitive to drugs that the patient may be taking. Such drugs can include various glucocorticoids or drugs that impair the synthesis of cortisol. Cortisol levels can be either suppressed or elevated dependent on the specific drug. A fairly comprehensive review has been written by Ambrogio et al., covering the effects of drugs on the hypothalamic-pituitary-adrenal axis [139]. In addition, there have been reports of assay interference in the patient population taking the drug metyrapone, which is used in the management of Cushing syndrome but with decreasing frequency. For patients taking this drug, the current guidelines recommend determining the dose of metyrapone based on the serum cortisol concentration. The mechanism of action for this drug is inhibition of the enzyme 11β-hydroxylase, which can lead to the accumulation of cortisol precursor steroids such as 11-deoxycortisol. In one study, the authors were able to identify that the increased cortisol precursor concentrations caused a positive interference in the cortisol immunoassay in two patients taking the drug [140]. They were able to identify the increased results from immunoassay by simultaneously measuring these samples by liquid chromatography mass spectrometry (LC/MS). Further study compared cortisol measurements made in 3 different groups: patients receiving metyrapone, patients diagnosed with Cushing syndrome not receiving any treatment, and patients with no adrenal pathology and no medication known to interfere with the cortisol immunoassay [141]. In these 3 different patient populations, they measured cortisol by both immunoassay and liquid chromatography combined with mass spectrometry (LC/MS). The two control groups not receiving metyrapone showed good agreement between immunoassay and LC/MS; in contrast, the metyrapone group showed a positive bias of 23% and the difference between the 2 assays positively correlated with drug dose and 11-deoxycortisol concentration.

The immunoassay used to measure cortisol can also be affected by heterophile antibodies [142]. In one case, the interference led to a falsely low cortisol result and the patient was given cortisol replacement for suspected adrenal insufficiency [143]. Due to the clinical doubt surrounding the patient's diagnosis, further work-up with the samples was performed, which included measurement of cortisol with a different immunoassay and incubation with a heterophile antibody blocking tube. The authors were able to identify the source of the

problem and stopped the patient's glucocorticoid replacement therapy.

Heterophile antibodies are just one contributing factor to the controversy surrounding the use of immunoassays [144]. There has been investigation into other ways to measure cortisol, aside from the use of immunoassays. The use of LC/MS has the potential to avoid some of the traditional pitfalls of using immunoassays for analyte measurements, such as interferences caused by endogenous immunoglobulins or the high-dose hook effect [145]. The success of an LC/MS assay relies heavily on the development and validation of the method. The mechanisms of interference when using mass spectrometry are very different from immunoassays. A source of potential interference in LC/MS is from other analytes. These can be isobaric compounds which have the same molecular mass, such as cortisol and tetrahydro prednisolone, or the A + 2 isotopes of their unsaturated analogs, such as cortisone and cortisol [146]. There also was a report where fenofibrate, which is not chemically or structurally related to cortisol, resulted in a falsely elevated urine free cortisol result by an LC/MS method [147]. This interference overlapped with the major transition of cortisol; however, a second mass transition was free from the interference and allowed for accurate quantitation. Another potential source of interference specific to LC/MS methods includes false elevations/depressions due to ion enhancements/suppression [148]. Ion suppression affects the amount of analyte that is introduced into the mass spectrometer. This can happen due to the presence of nonvolatile or less volatile solutes in the sample matrix, such as salts, ion pairing agents, endogenous compounds, etc. [149]. This can also happen if other molecules being ionized have a higher mass or are more polar, which may decrease the ability of the analyte of interest to be ionized [150,151]. The use of liquid chromatography combined with tandem mass spectrometry (LC/MS/MS) and isotopically-labelled analogous internal standards can minimize such interferences.

Measurement of urinary free cortisol (UFC) is a screening test used in cases of suspected Cushing syndrome. While the use of LC/MS is increasing, traditional measurement techniques have often used immunoassays. The urine provides a different matrix with different potential interferences when compared to the serum assay. One problem specific to urine is that the hydrophobic conjugate derivatives of cortisol are present in large excess concentrations when compared to free cortisol, providing an opportunity for cross-reactivity [152]. A study was performed comparing cortisol measurements by gas chromatography-mass spectrometry (GC-MS), LC/MS and two commercial immunoassays [11]. The two immunoassays that were evaluated both overestimated the UFC concentrations by approximately 1.6–1.9-fold when compared with GC-MS. Both ring-A dihydro- and tetrahydro-metabolites contributed to this overestimation and the positive interference was not seen in the GC-MS or LC/MS methods. Quantitation of UFC by immunoassay typically requires an extraction step with a solvent to release free cortisol from conjugated steroids, but interference from unconjugated steroids can still be a potential source of error [153]. A recent study evaluated a different sample preparation technique, i.e., solid phase extraction, to remove the excess steroids. The authors were able to show that solid phase extraction was effective in removal of 6-β-hydroxycortisol from urine samples thereby eliminating this as a possible interference in treated urine samples.

Aldosterone & renin

Primary aldosteronism is being increasingly investigated as a possible secondary cause of hypertension in adults. From the laboratory perspective, this usually involves measuring both aldosterone and renin and calculating an aldosterone-to-renin ratio (ARR). Aldosterone is typically measured using either a radioimmunoassay or chemiluminescence immunoassay; however, LC/MS applications have also been developed for the measurement of this analyte [154]. The renin value in the ARR can be generated either by measuring the plasma renin activity (PRA) or the direct renin concentration (DRC). The actual method used to measure the effect of renin in the ARR can also impact the results, as it has been reported that PRA is preferable to DRC when calculating the ARR [130]. The PRA is measured by allowing renin in plasma to convert endogenous angiotensinogen to angiotensin I and then measuring angiotensin I by immunoassay. DRC assays are measured usually through antibody-based testing, such a radioimmunoassay and chemiluminescent immunoassays. The cutoffs used to define primary hyperaldosteronism must be separately defined when using the aldosterone to PRA ratio versus the aldosterone to DRA ratio.

As for endogenous variables affecting this assay, a number of factors can affect the ARR, such as posture, time of day, age, gender, sodium intake, potassium level and renal dysfunction [155]. A woman's menstrual cycle can also have an effect, and has led to a study investigating whether women are more at risk for false positives in primary aldosteronism screening [155,156]. The authors concluded that ARRs generated using DRC in normal women during the luteal phase can lead to a higher incidence of false positive ARRs in women than in men.

Multiple drugs can alter the endogenous levels of aldosterone and plasma renin activity. Angiotensin

converting enzyme inhibitors, angiotensin receptor antagonists, and diuretics can all contribute to potential false elevations in PRA and should be interpreted with caution in these individuals [157]. Mineralocorticoid receptor antagonists and high-dose amiloride must be discontinued at least 6 weeks prior to testing, due to interference in the interpretation of the ARR. In the case of the other drugs, a detectable low PRA level does not exclude primary aldosteronism and an undetectably low PRA in patients taking these drugs should be considered highly suspicious. Adrenergic inhibitors also suppress renin secretion which can then lead to suppressed aldosterone secretion in normal patients [158]. A 2002 study evaluated the bias introduced by a number of antihypertensive drugs on the ARR [159]. Measurements were made in samples from patients taking the drugs and the percent changes from the control group were as follows: amlodipine -17% ± 32, atenolol 62% ± 82, doxazosin −5% ± 26, fosinopril −30% ± 24 and irbesartan −43% ± 27. The measurements for PRA and aldosterone concentrations in this work utilized radioimmunoassays.

While drugs typically act to change endogenous levels of renin and/or aldosterone, steroids and other metabolites can cause false results due to cross reactions with the antibodies used in the assays. It has been hypothesized that increases in 21-deoxyaldosterone and other steroids in patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency could be responsible for false increases in aldosterone measurements when compared with LC/MS [154]. Another study compared an automated chemiluminescence immunoassay with a radioimmunoassay both with and without extraction steps in samples obtained after saline infusion [160]. While the vendors of the assays used in this study do provide a list of steroids that interfere with their assays, the authors speculate that other interfering substances may be playing a role in their discrepant results.

TESTING OF PARATHYROID FUNCTION

Usually parathyroid hormone and 25-hydroxyvitamin D are measured during the evaluation of parathyroid function. In this section challenges in measuring these analytes are addressed.

Parathyroid hormone

Parathyroid hormone (PTH) plays an important role in mineral and bone metabolism. PTH is secreted from the four parathyroid glands in response to decreased ionized calcium levels. The PTH secreted is often referred to as "intact PTH," and it refers to the form of the hormone which is 1-84 amino acids (a.k.a. − "full length PTH"). With secretion, 1-84 amino acid PTH can be rapidly degraded to 7-84 amino acid PTH, which lacks biologic activity. Due to the existence of various forms of PTH with varying biological activity, the measurement of PTH by sandwich immunoassay techniques is not straight forward. The majority of assays in use today are either second or third generation assays. Both utilize antibodies directed at the C-terminal end of the hormone, but the second generation assay has increased interference from the N-terminal truncated fragment (7-84 amino acids) [161] due to the location of binding of the C-terminal directed antibody. The ratio of intact PTH (1-84) and the N-terminal truncated fragment (7-84) is roughly 1:1 in a healthy population but the ratio can vary based on the presence of disease [162]. In an effort to circumvent this interference, third generation assays were developed so that the N-terminal antibody recognizes the first ~4 N-terminal amino acids, which are absent in the truncated 7-84 amino acid fragment. "True-intact," "whole," "bio-intact" and "cyclase activating" PTH are terms applied to the 1-84 amino acid PTH assays. There was debate as to whether such third generation assays offer any improvement in sensitivity or specificity over the second generation assays [163,164]. Presently there is little belief that 1-84 amino acid PTH assays are clinically necessary.

Similar to many other hormones measured by immunoassay, heterophile antibodies and rheumatoid factor interference have been a problem for PTH assays [165,166]. Tumors can be a source of excess PTH production, increasing the PTH level and making interpretation of results difficult. While adenomas, hyperplasia, and carcinoma of the parathyroid are all causes of hyperparathyroidism, there have also been cases of non-parathyroid tumors producing PTH [167]. Surgery is the usual treatment for parathyroid masses and intraoperative measurement of PTH can assist the surgeon in elucidating if all of the parathyroid mass has been removed.

Exogenous factors can also have an effect on PTH producing misleading interpretations of laboratory results. Recently, biotin ingestion, as discussed previously, has been found to falsely lower PTH levels measured in sandwich immunoassays. Additionally, a study examined the effect of three different anesthetic techniques on intraoperative PTH levels [168]. The intraoperative PTH levels measured at 3 min post-induction increased by 28% with monitored anesthetic care, 45% with general anesthesia with laryngeal mask airway, and 65% with general anesthesia and endotracheal intubation, compared with preinduction levels. A substance that can decrease levels of PTH is cinacalcet HCl, a calcimimetic. *In vivo*, the release of PTH is regulated by free ionized calcium in the blood, which interacts with the calcium sensing receptor on the parathyroid gland.

In the case of hyperparathyroidism, cinacalcet HCl is used to increase the sensitivity of the calcium-sensing receptor to ionized calcium, thereby reducing PTH levels [169]. A case study evaluated the dynamic changes in PTH and PTH fragments in a patient receiving cinacalcet HCl for secondary hyperparathyroidism [170]. To assess the effect of cinacalcet HCl, this study utilized 3 different immunoassays for PTH; 2 measuring intact PTH (one measuring full length PTH (1-84) and the other measuring full and non-full length fragments), and an assay using an antibody specific to the C-terminal region of PTH. The authors found that the relationships between the PTH assays did not remain linear during therapy and found that the PTH response after therapy was assay dependent.

Assays for 25-hydroxyvitamin D

The popularity of measuring 25-hydroxyvitamin D (25-OHD) has vastly increased since the introduction of the first assay in 1971. The first assay was a competitive protein binding assay using vitamin D binding protein as the binding agent [171]. Since then, efforts have been made to create new assays that were easier to incorporate into the clinical laboratory. Today, multiple different methods are employed for the measurement of 25-OHD including immunoassays, liquid chromatography, and liquid chromatography tandem mass spectrometry (LC/MS/MS) approaches. Immunoassays are currently the dominant choice among clinical laboratories for the measurement of 25-OHD, most likely due to their compatibility with the high volume demands of the clinical laboratory. However, these assays are prone to the same interferences as all immunoassays, such as heterophile antibodies [172], and come with their own unique issues as well, including false elevations from biotin-ingestion interferences. In a recent report, investigators evaluated their 25-OHD assay by retesting stored samples that had been previously tested for 25-OHD [173]. Using the DiaSorin Liaison for both the initial and repeat testing, they found that 5% of their results were discrepant from the original results. They further investigated the source of the discrepancy by using heterophile blocking tubes to assess the presence of heterophile antibodies and found that the samples did not differ significantly after treatment.

One of the largest interferences reported for immunoassay measurement of 25-OHD is vitamin D binding protein (VDBP). 25-OHD is hydrophobic and therefore tightly bound to VDBP in the circulation. Releasing 25-OHD from VDBP has been done traditionally by using organic extraction protocols yet these solvents are not compatible with the automated immunoassays. One study compared 5 automated immunoassays, a radioimmunoassay and 2 independent LC/MS/MS methods [174]. They found excellent concordance across the LC/MS/MS assays and outstanding performance by the DiaSorin radioimmunoassay. The automated immunoassays did not fare nearly as well and it is speculated that the extraction step without aggressive solvents may be a contributing factor to this lack of comparability. It has also been discovered that inaccuracies found in immunoassays are VDBP concentration dependent [175]. The inability to effectively and reproducibly remove 25-OHD from VDBP often contributes to a negative bias thereby falsely decreasing the total 25-OHD. This is thought to be a larger problem for 25-OHD_2 which is often under measured in total 25-OHD immunoassays. This is particularly a problem for countries like the U.S., where vitamin D_2 is prescribed for supplementation. Underestimating 25-OHD_2 can lead to false conclusions regarding compliance with therapy and mislabeling patients as vitamin D deficient, when in fact, it could be the result of falsely decreased values.

One way to avoid the problems encountered when immunoassays are used to measure 25-OHD is to use a more direct measurement approach such as LC/MS. The major analytes contributing to interference using this technique are from the 25-OHD_3 epimer and other isobaric compounds. The epimer, 3-epi-25-OHD_3, is the most abundant vitamin D metabolite and it has the same molecular weight as vitamin D_3, and it also forms the same mass to charge parent and product ions when ionized. There is an inverse relationship between patient age and epimer percentage, and in 172 children tested, the epimers contributed 8.7−61.1% of the total measured 25-OHD [176]. A novel method reports a LC/MS method that is able to separate the epimers and other isobaric compounds, such as 3-epi-25-OHD_3, 1-α-OHD_3, and the bile precursor 7-α-hydroxy-4-cholesten-3-one from 25-OHD_2 and 25-OHD_3 [177]. Interferences have also been noted using LC/MS when samples are collected in serum separator tubes [178]. Further investigation identified that only 25-OHD_2 was falsely increased upon storage of the samples in the serum separator tubes and the authors were able to circumvent this problem by using an alternative transition for monitoring 25-OHD_2.

GONADAL AND REPRODUCTIVE MEDICINE

The most important endocrine immunoassay interferences in the field of reproductive medicine are false positive hCG results as discussed in the introduction [179]. HAMA, rheumatoid factor and heterophile antibodies can also affect LH and FSH measurements [180]. Assay specificity is an important issue for sex steroid measurements. Many experts in this field are

utilizing tandem mass spectroscopy as the definitive method for steroid analysis [181].

Prior to immunoassays of the last ~20 years, some older LH assays cross-reacted with TSH. For example, in cases of severe primary hypothyroidism with a greatly elevated TSH level, there could be a false elevation in the measured LH. In a woman of reproductive age with untreated or undertreated primary hypothyroidism, if she had menstrual irregularity, primary hypogonadism might be mistakenly diagnosed on the basis of an elevated LH that really resulted from cross-reactivity with a massively elevated TSH.

Specificity of sex steroid measurements is very important in patient care [182–186]. As well, sex steroid analysis is extremely important in the field of competitive athletics [187–189]. The laboratorian must be aware of all such possibilities and investigate possible interferences when an inquiry is made by a clinician [190,191].

Although not interferences, proper interpretation of sex steroid and gonadotropin results requires that the proper reference intervals be used. For women of reproductive age, this can be challenging because the LH, FSH, estradiol and progesterone levels are changing throughout the menstrual cycle. There is diurnal variation in testosterone although in a healthy male, these variations do not fall outside the reference interval [192]. Of interest, testosterone appears to rise with stress [193].

Possibly the tumor marker with the most widespread clinical use is prostate specific antigen (PSA) [194]. Total and free PSA can be measured [195]. Nevertheless, the use of PSA testing remains controversial [196].

PSA is measured by immunoassay [197]. Rarely (in 0.3% of samples) heterophile antibodies falsely raise PSA, although in the study reporting this finding, there were no adverse clinical consequences identified [198]. In another report, heterophile antibodies raised the PSA concentration leading to inappropriate therapy [199]. In post-prostatectomy patients treated for prostate cancer, if PSA rises without any clinical evidence of metastases or worsening metastases, heterophile antibodies should be considered and tested for [200,201]. PSA assays may be improved using F(ab′)2 antibody fragments [202]. PSA assays are further discussed in Chapter 12.

TESTING FOR INSULIN LIKE GROWTH FACTOR-I

Insulin-like growth factor I (IGF-I) is measured in cases of growth hormone disorders. IGF-I measurements are often preferred over growth hormone measurements due to the stability of IGF-I and IGF-I has been shown to be useful in screening for growth hormone deficiency. IGF-I is a small molecule and is carried through the circulation attached to larger proteins, known as IGF-I binding proteins, specifically, IGF binding protein-3 (IGFBP-3) together with the acid-labile subunit (ALS). Releasing of IGF-I from the binding proteins is necessary for its measurement and incomplete liberation can cause discrepancies among methods. Acid gel filtration chromatography is the best method available for accomplishing separation of IGF-I from its binding proteins; however, this approach is not compatible with the typical high-volume clinical laboratory setting. An alternative method has been developed which includes dissociation by low pH, precipitation of the binding proteins by ethanol and the separation of free IGF-I with centrifugation. This method encountered two main problems, the first is that not all the binding proteins were precipitated with ethanol and the second is precipitation of some of the IGF-I, falsely decreasing the IGF-I values [203–205]. To circumvent these problems, excess IGF-II is introduced into the sample after the acid/ethanol precipitation to occupy the binding protein sites that would still remain [206]. Similar procedures have been employed when measuring IGF-II, with the exception of IGF-I addition to saturate the binding protein [207]. Sandwich immunoassays have also been developed for the measurement of IGF-I [208]. Heterophilic antibodies have been shown to interfere and falsely decrease IGF-I levels measured by the Immulite 2000. In this case, the low IGF-I values suggested growth hormone deficiency but neither the clinical signs nor the growth hormone stimulation test results supported this diagnosis. After treatment of the sample with a heterophile blocking tube, the IGF-I results were within the reference interval.

While IGF-I is not as variable as growth hormone, there are still multiple factors that can impact circulating IGF-I levels. Age and sex are both related to IGF-I levels to the extent that reference intervals change based on these demographics. A multicenter study evaluated 3,961 healthy subjects aged 1 month to 88 years to describe age and sex specific reference intervals [209]. Using an automated chemiluminescence immunoassay system, researchers found that in people under age 20, females had higher serum IGF-I levels with a mean peak of 410 μg/L at age 14 while males had a mean peak of 382 μg/L at age 16. The age dependence slowed and after 25 years males had higher IGF-I levels than females. Diet, lifestyle and ethnicity have been shown to correlate with IGF-I levels [210,211]. While growth hormone levels can change significantly in the circulation over short periods of time, IGF-I levels are relatively stable during the day and the timing of sample collection generally does not impact IGF-I results.

MEASUREMENT OF OTHER HORMONES INCLUDING INSULIN

The normal endocrine products of the islets of Langerhans include insulin, glucagon, somatostatin and pancreatic polypeptide [212]. The only situation in clinical medicine where glucagon might be measured is in cases of suspected glucagonoma [213]. Glucagonoma can present with secretory diarrhea, glucose intolerance or frank diabetes and rash (necrolytic migratory erythema). In the absence of long-standing diabetes, glucagon deficiency is rare [214]. The pre-analytical concerns in measuring glucagon were discussed previously. A macro-glucagon (IgG bound to glucagon) has been reported to cause hyperglucagonemia [215]. Somatostatinomas may also present with diabetes and secretory diarrhea as well as cholelithiasis [216]. Samples for somatostatin should be drawn into a pre-chilled EDTA tube with separation of the plasma from the cells within 2 h of collection. The sample is shipped frozen. Under pathologic circumstances, vasoactive intestinal polypeptide (VIP)-secreting islet tumors (i.e., VIPomas) develop that produce secretory diarrhea and flushing [217]. The collection of blood for VIP has been discussed. Pancreatic polypeptidomas are not endocrinologically active as they do not secrete pancreatic polypeptide nor do they cause endocrine symptoms [218]. Such tumors do present as mass lesions [219].

An important clinical interference in insulin assays is the common development of insulin antibodies in insulin-treated patients [220,221]. These antibodies may raise total insulin concentrations making such measurements not interpretable in assessing endogenous insulin secretion in insulin-treated patients. Attempts have been made to measure "free insulin" after polyethylene glycol precipitation of the antibody-bound insulin complexes; however, "free insulin" has not proven to be a reliable measure of the biologically active insulin and such measurements are not recommended [222,223]. Lack of standardization contributes to this assay variability as well.

For each insulin molecule released by the pancreas into the splenic vein, one molecule of connecting peptide (C-peptide) will also be released. Because injected insulin preparations lack C-peptide, C-peptide antibodies do not develop. Therefore C-peptide can be measured as an index of endogenous insulin production in insulin-treated patients. An important caveat is that approximately 50% of the insulin secreted from the pancreas is cleared by the liver on its first pass. This reflects the fact that hepatocytes are a major target of insulin action. Because insulin clearance by the liver is greater than C-peptide clearance by the liver, in peripheral blood the molar ratio of C-peptide to insulin is greater than 1. Nevertheless, assuming that the hepatic clearances of insulin and C-peptide are comparable among individuals, C-peptide concentrations can be used as a proxy for endogenous insulin secretion.

Insulin autoantibodies can be found in individuals at increased risk for the development of type 1 diabetes as well as at the onset of type 1 diabetes [224]. Insulin autoantibodies arise spontaneously and are not the consequence of exposure to exogenous insulin injections. These autoantibodies are usually of very low titer compared to the insulin antibodies that develop in response to exogenous insulin administration. Within 14 days of the initiation of insulin treatment of an insulin-naive individual, insulin antibodies can appear. Autoantibodies to proinsulin have also been described [225]. The insulin antibody assays do not discriminate autoantibodies from antibodies induced by insulin exposure. It should be noted that insulin antibodies develop in response to both animal and human insulins.

The analytical specificity of insulin and C-peptide assays is an important consideration. The insulin immunoassay should not detect C-peptide, proinsulin or partially cleaved proinsulin. Unfortunately in both type 1 and type 2 diabetes, relative elevations in the secretion of proinsulin occur that likely reflect the failing nature of the beta cell and/or beta cells that are extremely stimulated [226] Similarly, the C-peptide assay should not detect insulin, proinsulin or partially cleaved proinsulin. Proinsulin-specific immunoassays have been reported [227,228]. Hyper-proinsulinopathies are rare inborn errors that result from incomplete cleavage of the proinsulin to insulin plus C-peptide [229]. It is uncommon that diabetes would result from a hyper-proinsulinopathy alone.

Still another issue affecting insulin immunoassays is the detection of recombinant DNA-derived insulins whose sequences have been altered compared to human insulin. Laboratorians need to educate clinicians about the specificity of their insulin immunoassay. For example, the Roche Elecsys E170 analyzer does detect glargine [230]. Additionally, the Centaur and Immulite 2000 analyzers do detect glargine, aspart, and lispro. A 1999 publication reported on the development of an immunoassay specific for lispro insulin [231].

As with other hormone assays, standardization of insulin measurements across platforms has not been achieved but is an important goal for laboratory scientists [232,233]. Such work is in process [234–237]. Likewise, C-peptide assays are not standardized across platforms [238–240].

PRENATAL TESTING

A variety of hormones and proteins are measured in second trimester maternal serum in screening for neural

tube defects (e.g., anencephaly and spina bifida), Down syndrome (trisomy 21) and Edward syndrome (trisomy 18). Alpha fetoprotein (AFP), hCG and unconjugated estriol constitute the triple screen whereas the quadruple screen is constituted with the addition of dimeric inhibin A measurements [241,242]. The quadruple screen has a sensitivity of 80% and specificity of 95% for the detection of Down syndrome [243,244]. First trimester prenatal screening for Down syndrome and Edward syndrome includes ultrasound-detected nuchal translucency and measurements of pregnancy-associated polypeptide-A (PAPP-A) and hCG [245].

A PubMed search (on 10-13-2017) revealed no reported analytical interferences for PAPP-A or alpha fetoprotein. Estriol measurements should suffer from similar specificity problems as estradiol measurements and other steroids [246]. False positive hCG measurements have been discussed due to HAMA. Not in the setting of pregnancy, heterophile antibodies have been reported to raise measured hCG levels [247]. Heterophile antibodies producing a false elevation in inhibin A have been described [248]. In an ELISA format assay to measure inhibin A, a catalase inhibitor was recommended to prevent a decline in the measured inhibin A level in cases of delayed separation of serum from the whole blood [249].

CONCLUSIONS

As discussed throughout this chapter, measuring hormones by immunoassay in the clinical laboratory can be technically demanding. Proper pre-analytical management of the sample is critical especially for hormones that are particularly labile. The collection tubes, position of patient during phlebotomy (e.g., renin measurements), patient preparation and the time of the day can all alter the results. Sample processing can also impact the results, as seen with ACTH where the timing of centrifugation and storage of these samples is critical.

The analytical challenges in hormone immunoassays are multiple: hormones are typically present in the plasma in low concentrations; the biologically active hormone may only be the free (or unbound) fraction; hormones often circulate attached to binding proteins; hormones may exist in a variety of forms (e.g., the 22 and 20 kDa forms of GH); hormone metabolites may cross react with the parent compound; and cross reactivity between hormones, especially steroid hormones, may exist. Added to these challenges are the analytical interferences discussed above: even if the measurement of the hormone by immunoassay is theoretically straight-forward, the opportunity exists for interference from HAMAs, heterophile antibodies, biotin, and more.

Even the best validated assays may encounter some of the interferences discussed in this chapter. As laboratorians, we may not be able to prevent these interferences, but we can be prepared to recognize the possibility of such interferences when there is discordance between the laboratory result and clinical findings in the patient. In this way, the laboratorian can assist the clinician in the proper interpretation of the result. Further studies may be indicated, for example, to prove or disprove the possibility of HAMA interference. A number of tools for identifying the presence of interference, such as heterophile blocking tubes or PEG precipitation, are available. In the field of immunoassays, it is wise for the clinician and the laboratorian to both remember the Latin phrase “Caveat emptor” which translates as “Buyer beware.”

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CHAPTER

12

Pitfalls in testing for common tumor markers

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INTRODUCTION

Tumor markers are molecules that are produced by cancer cells or by other cells of the body in response to cancer or under certain benign (noncancerous) conditions. Most tumor markers are produced by normal cells as well as by cancer cells but in the process of developing cancer, concentrations of these markers are elevated many fold compared to very low concentrations of these markers observed in healthy people [1]. In addition to blood, some these markers are also found in urine, stool, or bodily fluids of patients with cancer. So far, more than 20 different tumor markers have been characterized and are used clinically for diagnosis and monitoring of treatment. Some tumor markers are elevated with only one type of cancer, whereas others are associated with two or more cancer types. There is no "universal" tumor marker for detection of a particular type of cancer. Commonly monitored tumor markers are summarized in Table 12.1.

Although theoretically any type of biological molecule can act as tumor marker, in practice, most markers are either proteins or glycoproteins. However, low-molecular-weight substances, for example, vanillylmandelic acid and homovanillic acid in neuroblastoma are also used and nucleic acids (both DNA and RNA) are currently being evaluated as possible tumor markers. More recently, patterns of gene expression and changes to DNA are also under intense investigation be used as tumor markers. These types of markers are measured specifically in tumor tissues [1]. Most of the traditionally used markers are probably not involved in tumorigenesis but are likely to be byproducts of malignant transformation. In the future, however, molecules involved in carcinogenesis or cancer progression are likely to find increasing use as markers [2].

CLINICAL APPLICATION OF TUMOR MARKERS

Tumor markers have five potential uses [3].

1. Screening a healthy person or a high risk person for the probable presence of cancer
2. Diagnosis of cancer or a specific type of cancer
3. Evaluating prognosis in a patient
4. Monitoring cancer therapy
5. Detecting relapses

Screening and early detection of cancer

Tumor markers were first developed to test for cancer in people without symptoms, but very few markers are effective in achieving this goal. Today, the most widely used tumor marker in the clinical setting is prostate specific antigen (PSA). In addition, only a few markers that are now available have clinically useful predictive values for cancer at an early stage, and only when patients at high risk are tested.

Diagnosis of cancer

Tumor markers are not the gold standard for diagnosis of a cancer. In most cases, a suspected cancer can only be diagnosed by a biopsy. Alpha-fetoprotein (AFP) is an example of a tumor marker that can be

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TABLE 12.1 Tumor markers commonly tested in clinical laboratories.

Marker	Application	Normal range in serum (adults)
Prostate specific antigen (PSA)	Prostate carcinoma	0–4 ng/mL
Cancer antigen 125 (CA 125)	Ovarian and fallopian carcinoma	0–35 U/mL
Cancer antigen 15-3 (CA 15-3)	Breast cancer	0–30 U/mL
Cancer antigen 19-9 (CA 19-9)	Pancreatic and ovarian cancer	0–35 U/mL
Alpha-fetoprotein	Hepatoblastoma, hepatocellular carcinoma, and germ cell tumors	<15 ng/mL
Carcinoembryonic antigen (CEA)	Colorectal, gastric, pancreatic, lung, and breast carcinomas	<2.5 ng/mL, nonsmokers <5 ng/mL, smokers
Beta-2-microglobin (B2M)	Multiple myeloma and lymphoma	Up to 3 μg/mL
Human chorionic gonadotropin (hCG)	Choriocarcinoma and testicular carcinoma	Men: <5 mIU/mL Non-pregnant women: <5 mIU/mL Post-menopausal woman: <10 mIU/L

used to aid in diagnosis of cancer, especially hepatocellular carcinoma (HCC). However, the level of AFP can also be increased in certain liver diseases, although when it reaches a certain threshold it is usually indicative of hepatocellular carcinoma.

Evaluating prognosis

Some types of cancer grow and spread faster than others while some cancers also respond well to various therapies. Sometimes the level of a tumor marker can be useful in predicting the behavior and outcome for certain cancers. For example, in testicular cancer, very high levels of a tumor marker such as human chorionic gonadotropin (hCG) or alpha-fetoprotein (AFP) may indicate an aggressive cancer with poor survival outcome. Patients with these high levels may require very aggressive therapy even at the initiation of cancer therapy.

Monitoring therapy

Certain markers found in cancer cells can be used to predict if a particular treatment is likely to produce a favorable outcome or not. For example, in breast and stomach cancer, if the cells have too much of a protein termed human epidermal growth factor receptor 2 (HER2), drugs such as trastuzumab (Herceptin) can be helpful if used during chemotherapy. If there is normal expression of HER2, these drugs may not produce expected therapeutic benefits. Therefore expression of HER2 is ordered prior to chemotherapy.

Detecting relapses

Tumor markers are also used to identify recurrence of certain tumors after successful therapy. Certain tumor markers may be useful for further evaluation of a patient after completion of the treatment when there is no obvious sign of cancer in the body. Commonly measured tumor markers in clinical laboratories include:

- Prostate specific antigen (PSA) for prostate cancer
- Human chorionic gonadotropin (hCG) for gestational trophoblastic tumors and some germ cell tumors but the most common application of hCG is diagnosis of pregnancy
- Alpha-fetoprotein (AFP) for certain germ cell tumors and hepatocellular carcinoma
- CA-125 (carbohydrate antigen or cancer antigen 125) for ovarian cancer
- CA 19-9 (carbohydrate antigen 19-9) for pancreatic and gastro-intestinal cancers
- Carcinoembryonic antigen (CEA) for colon and rectal cancers
- CA-15-3 (cancer antigen 15-3), marker for breast cancer

Less commonly monitored tumor markers include:

- CA 72-4 (cancer antigen 72-4), marker for colorectal cancer
- CYFRA 21-1 (cytokeratin fragment), marker for lung cancer
- Human epididymis protein (HE4), marker for ovarian cancer
- Human epidermal growth factor receptor 2 (HER2/neu), marker for breast cancer
- Squamous cell carcinoma antigen, maker for various cancer
- Chromogranin A, marker for neuroendocrine tumor

In this chapter major emphasis is placed on discussion of common tumor markers, including pitfalls in measuring these markers.

PROSTATE SPECIFIC ANTIGEN (PSA)

Prostate specific antigen (PSA), a serine protease belonging to the kallikrein family is a single chain glycoprotein containing 237 amino acid and four carbohydrate side chains (molecular weight: 28,430 Da). PSA is expressed by both normal and neoplastic prostate tissue.

PSA expression and processing

Under normal conditions, PSA is produced as a proantigen (proPSA) by the secretory cells that line the prostate glands and then secreted into the lumen, where the propeptide moiety is removed to generate active PSA. The active PSA can then undergo proteolysis to generate inactive PSA, of which a small portion enters the bloodstream and circulates in unbound state (free PSA). Alternatively, active PSA can diffuse directly into the circulation where it is rapidly bound to protease inhibitors, including alpha-1-antichymotrypsin (ACT) and alpha-2-macroglobulin [4].

Although generating less PSA per cell than normal tissue, prostate cancer tissues lack basal cells, resulting in the disruption of the basement membrane and normal lumen architecture. As a result, the secreted proPSA and several truncated forms can be released into circulation directly resulting in more PSA "leaked" into the blood. In addition, a larger fraction of the PSA produced by malignant tissue escapes proteolytic processing. The end result is that more PSA is found in blood in patients with prostate cancer compared to patients with no cancer. Prostate cancer is a commonly encountered cancer in men.

In men with a normal prostate, the majority of free PSA in the serum reflects the mature protein that has been inactivated by internal proteolytic cleavage. In contrast, this cleaved fraction is relatively decreased in patients with prostate cancer. Thus, the percentage of free or unbound PSA is lower in the serum of men with prostate cancer and conversely, the amount of complexed PSA "PSA complexed to α1-antichymotrypsin" is higher compared with those who have a normal prostate or benign prostatic hyperplasia (BPH) [5,6]. This finding has contributed to the use of the ratio of free to total PSA and complexed PSA (cPSA) as a means of distinguishing between prostate cancer and BPH as a cause of an elevated PSA. Causes of elevated PSA include:

- Benign prostatic hyperplasia (BPH)
- Prostate cancer
- Prostatic inflammation/infection
- Perineal trauma

Benign prostatic hyperplasia (BPH)

The most common explanation for an elevated serum PSA is BPH due to the high prevalence of this condition in men over the age of 50. Serum PSA levels overlap considerably in men with BPH and those with prostate cancer. As an example, one published report retrospectively examined preoperative serum PSA in 187 men with a histologic diagnosis of BPH on a transurethral resection of the prostate (TURP) specimen and 198 men with organ-confined prostate cancer as determined by step-section analysis of a radical prostatectomy specimen. The median serum PSA concentrations were 3.9 ng/mL (range 0.2–55 ng/mL) and 5.9 ng/mL (range 0.4–58 ng/mL) respectively. Although this difference was statistically significant, the distribution of serum PSA values in both groups overlapped considerably with a clustering of PSA values below 10.0 ng/mL. A potentially confounding problem is that medical treatment for BPH can also reduce serum PSA concentrations [7].

Elevated PSA in prostate cancer and other conditions

Studies in the 1980s confirmed that serum total PSA could be used as a screening tool to identify men with prostate cancer because elevated serum PSA is clearly a more sensitive marker that digital rectal examination. Although the majority of prostate cancers express PSA, between 20 and 50% of men with newly diagnosed prostate cancers in the United States have serum PSA values below 4.0 ng/mL (upper end of normal is usually considered as 4.0 ng/mL) indicating that PSA lacks specificity as a tumor marker. In general patients with PSA below 4.0 ng/mL are more likely to have prostate cancer that is confined to the organ. These patients have a better prognosis than patients with prostate cancer who showed levels above 4.0 ng/mL [8].

Prostatitis with or without active infection is an important cause of an elevated PSA and levels as high as 75 ng/mL have been reported in the literature. Thus, many physicians often initially treat a man with an isolated elevated serum PSA with antibiotics for a presumed diagnosis of prostatitis and then obtain a repeat serum PSA for further clinical evaluation. The percent free PSA may be less affected by the presence of inflammation, particularly when the total serum PSA is less than 10 ng/mL. However, at least one study suggested that the free to total ratio of serum PSA is unable to distinguish chronic inflammation from prostate cancer, as both conditions may lower the percentage of free PSA. This would be expected because inflammation leads to elevated serum PSA in a similar fashion as

prostate cancer through disruption of the basal membrane and increased leakage of "immature" PSA into the blood stream [9].

Any perineal trauma can also increase the serum PSA. Prostate massage and digital rectal examination may cause minor transient elevations which may be clinically insignificant. For example in one study, 2750 healthy men and patients over the age of 40 undergoing digital rectal examination were divided into four groups based upon their initial serum PSA. The two groups with the lowest initial serum PSA values (0.1−4 and 4.1−10 ng/mL) had statistically insignificant changes in the serum PSA after digital rectal examination, although PSA increased in the group with an initial serum PSA 10.1−20 ng/mL, indicating a trend toward statistical significance. In contrast, patients with an initial serum PSA greater than 20 ng/mL had statistically significant increases in PSA after digital rectal examination. Thus, it is reasonable to perform PSA testing without regard to whether a patient has had a recent digital rectal examination [10].

Mechanical manipulation of the prostate by cystoscopy, prostate biopsy, or transrectal ultrasound can more significantly affect the serum PSA. Vigorous bicycle riding has been reported to cause substantial elevations in the serum PSA, but this is not a consistent finding. Luboldt et al. based on a study of 33 men aged 50−74 years reported that after a 13 mile bicycle ride, no statistically significant change was observed in total, free and percent free PSA values compared to pre bicycle ride values [11]. Sexual activity can minimally elevate the PSA (usually in the 0.4−0.5 ng/mL range) for approximately 48−72 h after ejaculation.

PSA testing

Emerging concepts regarding PSA testing that may help refine the interpretation of an elevated concentration include:

- PSA density
- PSA velocity
- Free versus complexed or bound PSA

These modifications would presumably be most useful for prostate cancer screening when the total PSA is between 2.5 and 10.0 ng/mL, the range in which decisions regarding further diagnostic testing are most difficult. To more directly compensate for BPH and prostate size, transrectal ultrasound (TRUS) has been used to measure prostate volume. Serum PSA is then divided by prostate volume to obtain PSA density, with higher PSA density values (greater than 0.15 ng/mL/cc) being more suggestive of prostate cancer while lower values are more suggestive of BPH. While an early study suggested that PSA density was a promising method for distinguishing patients with benign and malignant prostate disease, subsequent reports have found considerable overlap in PSA densities between these groups. One multicenter study that compared PSA density versus PSA for the early detection of prostate cancer found that almost one-half of the cancers would have been missed using 0.15 ng/mL/cc as a cutoff for biopsy. There are also inherent difficulties in measuring PSA density, which include errors of prostatic volume measurement with TRUS and an intra-patient variation of up to 15% in PSA density with repeated measurements [12]. Furthermore, although PSA density can reliably differentiate between large groups of patients with BPH and prostate cancer, the ability to extrapolate these data to the individual patient is quite limited. Most importantly, the requirement for TRUS substantially increases cost and discomfort, and is impractical for widespread screening purposes.

Another approach has been to assess the rate of PSA change over time (the PSA velocity). An elevated serum PSA that continues to rise over time is more likely to reflect prostate cancer than one that is consistently stable. For practical purposes, the clinical usefulness of PSA velocity is in part limited by intra-patient variability in the serum PSA; at least three consecutive measurements should be performed. A longer time over which values are continuously measured can be useful in reducing the general variation in the PSA measurements. Depending upon the magnitude of the abnormal serum PSA, it is likely that a patient with an initially abnormal level would undergo prostate biopsy before waiting for a second measurement to be performed one year later. Therefore, a rapid PSA velocity should quickly result in an abnormal PSA level, which would be further evaluated due the PSA elevation alone even in the absence of a rapid velocity. Furthermore, men with cancer often have a PSA velocity less than 0.75 ng/mL per year, especially those with lower PSA levels. Multivariate and receiver operator characteristic (ROC) analyses as well as a systematic review suggest that calculation of PSA velocity and PSA doubling time (PSA doubling time is the number of months for a certain level of PSA to increase by a factor of two) are of limited value in screening, though some studies do suggest they may be useful in detecting the most aggressive cancers [13].

Serum free and bound PSA

Prostate cancer is associated with a lower percentage of free PSA in the serum compared to PSA values observed in benign conditions. The percentage of free PSA has been used to improve the sensitivity of cancer detection when total PSA is in the normal range (<4 ng/mL) and also to increase the specificity of cancer

detection when total PSA in the "gray zone" (4.1−10 ng/mL). In this latter group (PSA between 4.1 and 10 ng/mL) the lower the value of free PSA, the greater the likelihood that an elevated PSA representing cancer rather than BPH. For example, in one study involving men with PSA values in the range of 4.1−10 ng/mL the probability of cancer with a free to total PSA ration below 10% was 56%, compared with only 8% when the ratio was greater than 25% [14]. As with PSA, there is no absolute free/total cutoff that can completely differentiate prostate cancer from BPH. The optimal cutoff value for free PSA is unclear and depends upon whether optimal sensitivity or specificity is sought. The higher the cutoff value the greater is the sensitivity but the lower is the specificity.

Free PSA could be useful for risk stratification in men with prostate cancer. A lower percentage of free to total PSA may be associated with a more aggressive form of prostate cancer. This was illustrated in a study that examined banked frozen serum from 20 men with prostate cancer. Ten years before the diagnosis of cancer, at a time when the total PSA was not different between those who ultimately developed aggressive (aggressive defined by clinical stage T3 disease, nodal or bone metastases, pathologically positive margins, or Gleason score of 7 or greater) versus nonaggressive tumors, there was a statistically significant difference in the percentage of free PSA between the two groups. All eight men with aggressive cancers had a free PSA of $\leq$14%, compared with only two of six (33%) with nonaggressive cancer [15].

Complexed PSA

Assays for alpha-1-antichymotrypsin (ACT)-complexed PSA (cPSA) have been implemented that could theoretically provide a similar enhanced degree of specificity compared to free to total PSA ratio, but requiring measurement of a single analyte. Most but not all reports suggest that cPSA outperforms both total PSA and the ratio of free to total PSA, with similar sensitivity but a higher specificity [16]. According to one study, for men with total PSA in the diagnostic gray zone (4.0 and 10.0 ng/mL), the use of cPSA alone would have missed only one of the 36 men with cancer who would be diagnosed with prostate cancer using both total PSA and biopsies. Interestingly, free to total PSA alone would also have also missed one cancer, but eliminated biopsy in only 20 men compared to 34 men where biopsy could be eliminated using cPSA assay alone. The utility of cPSA in men with a lower total PSA (2−4 ng/mL) is under investigation as there are conflicting data as to whether cPSA improves specificity compared with the free to total PSA ratio [17]. Complexed PSA has been approved for the monitoring of men with prostatic carcinoma. The utility of complexed PSA for screening is uncertain and is not routinely used at this time in clinical practice.

Percent [-2]proPSA

PSA is initially produced as proPSA and this form can preferentially leak into the blood stream in men with prostate cancer. One specific isoform of proPSA is [-2]proPSA, which is unbound and potentially higher in concentration in men with prostate cancer. Based upon this observation, there has been growing interest in using the ratio of [-2]proPSA to free PSA (expressed as percent [-2]proPSA or %[-2]proPSA) for screening of prostate cancer. Authors in one multicenter study involving 566 men undergoing biopsy observed that %[-2]proPSA significantly outperformed both total PSA and percent free PSA [18]. At 80% sensitivity, %[-2]proPSA had 52% specificity, compared to 30% for PSA and 29% for percent free PSA. %[-2]proPSA is currently approved by the European Union for prostate cancer detection. The Prostate Health Index (PHI), a mathematical formula incorporating [-2]proPSA, free PSA and total PSA [PHI = -([-2]proPSA/free PSA) $\times$ $\sqrt{}$PSA] has been approved in the United States by the Food and Drug Administration.

False positive and unexpected PSA results

False positive PSA test results may be encountered causing confusion regarding the diagnosis of prostate cancer. Kilpelainen et al., based on screening of 61,604 men in Europe, observed 17.8% false positive PSA results. However, men who tested false positive with one PSA screening test were more prone to be diagnosed with prostate cancer in the future [19]. Nevertheless, the major cause of false elevation of PSA is the presence of heterophilic antibody in the serum [20]. In fact the presence of heterophilic antibody in the specimen not only may cause false elevation of PSA but also false elevation of other tumor markers. Kamiyam et al. described a case of a 63 year old man who was diagnosed with prostate cancer with a PSA level of 5.27 ng/mL. Radical prostatectomy was performed but his PSA remain elevated to 3.32, 4.78 and 5.93 ng/mL respectively at 1, 2 and 3 month after surgery. However, radiological investigation revealed no metastases. The authors measured PSA-α1-antichemotrypsin level in this patients and the value was <0.1 ng/mL. The authors concluded that PSA values in this patient were falsely elevated due to interference from heterophilic antibody [21].

Condoms are commonly submitted for testing in crime laboratories in cases of sexual assault and rape because the presence of seminal fluid in the condom can be used as an important evidence of sexual assault. Although microscopic examination of the seminal fluid and identifications of human spermatozoa is desirable, sometimes due to degradation and other reasons, direct microscopic examination of seminal fluid is not possible. In this case chemical testing of the seminal fluid can be conducted and the presence of PSA can be indicative of seminal fluid. However, false positive tests may be observed using Seratec PSA SemiQuant Cassette test or other similar PSA test mostly due to the presence of spermicide nonoxynol-9, which is a strong detergent [22].

Using assays capable of measuring very low levels of PSA in serum, it has been demonstrated that PSA may be produced in sites other than prostate glands. Very low levels of PSA may be present in breast milk and detectable PSA levels in the serum may be observed in women with breast cancer as well as breast cysts. In addition, elevated PSA may also be observed in male patients with breast cancer, which is a rare form of cancer in males [23,24]. Furthermore, both PSA and free PSA have been detected in women with pancreatitis and pancreatic cancer [25]. Moreover, low level of PSA may be detected in women because PSA synthesis can be induced by steroid hormones in several tissues of women. The menstrual cycle is regulated by the cyclic variation of estradiol and progesterone. In one study authors detected PSA in 38.2% of all serum specimens and 10% of all saliva specimens collected from women (detection limit 0.001 ng/mL). The serum PSA was highest during the mid-follicular phase of the cycle (between 4th and 8th day of cycle) [26].

Newer urine biomarkers of prostate cancer

More recently, newer biomarkers are emerging for diagnosis of prostate cancer. Prostate specific antigen 3 has been regarded as a highly specific to prostate. A urine assay is approved by the FDA. Gene fusions of the transmembrane protease serine 2 (TMPRSS2) and the E26 transformation specific ERG oncogene have been identified as a common event in human prostate cancer. Therefore, quantitative determination of the TMPRSS2-ERG fusion gene product in urine has promise as a biomarker of prostate cancer. In addition, DNA based biomarkers, such as polymorphisms in the *GSTP1* gene, may improve its predictive value. More recently, aberrant microRNA expression in prostate cancer has been reported. Preliminary results indicate utility of circulating and urinary microRNAs in the detection and prognosis of prostate cancer [27–29].

CANCER ANTIGEN 125 (CA-125)

Cancer antigen 125 or carbohydrate antigen 125 (CA-125) is also known as mucin 16 or MUC16. CA-125 is a glycoprotein which is encoded by the *MUC16* gene in human. CA-125 is used as a tumor marker because CA-125 concentrations may be elevated in the blood of some patients with specific types of cancers, such as ovarian cancer, and in several benign conditions. CA-125 levels in serum are elevated in approximately 50% of women with early stage of disease and in over 80% of women with advanced ovarian cancer [30]. Monitoring CA-125 serum levels is also useful for determining the response of a patient to ovarian cancer therapy as well as for predicting the patient's prognosis after treatment. In general, persistence of high levels of CA-125 during therapy is associated with poor survival rates. Also, an increase in CA-125 levels in a patient during remission is a strong predictor of recurrence of ovarian cancer.

However, the specificity of CA-125 is limited. CA-125 levels are elevated in approximately 1% of healthy women and fluctuate during the menstrual cycle. CA-125 is also increased in a variety of benign and malignant conditions, including:

- Endometriosis
- Uterine leiomyoma
- Cirrhosis with or without ascites
- Pelvic inflammatory disease
- Cancers of the endometrium, breast, lung, and pancreas
- Pleural or peritoneal fluid inflammation due to any cause

Monitoring of serum CA-125 levels also has value in patients with fallopian tube cancer, a relatively rare disease. The pretreatment serum CA-125 concentration appears to have prognostic value in patients with fallopian tube cancer, and following initial treatment it is a sensitive marker for monitoring of recurrence and response to chemotherapy. This was illustrated in a series of 53 patients with fallopian tube cancer, of whom 43 (81%) had an elevated pretreatment serum CA-125 concentration. In a univariate Cox regression model, preoperative serum CA-125 was independently associated with disease-free and overall survival outcome. The level of CA-125 was also correlated with response to therapy, decreasing in all 20 patients who responded to chemotherapy, and increasing in both patients who demonstrated progressive disease. Postoperatively, in 90% of patients, an increase in serum CA-125 preceded the clinical or radiologic diagnosis of recurrent disease, with a median lead-time of three months [31].

False positive and false negative CA-125

To date, CA-125 is the most commonly measured tumor marker for screening and diagnosis of epithelial ovarian tumors, which account for 85–90% of ovarian cancers [32]. Nevertheless, both false positive and false negative results have been reported with CA-125 testing. Meigs' syndrome is the association of ovarian fibroma, pleural effusion and ascites, which may also cause marked elevation of CA-125. Timmerman et al. reported two cases of Meigs' syndrome where the authors observed elevated CA-125 and commented that a pelvic neoplasm in a woman presenting with hydrothorax ascites and elevated CA-125 levels may indicate a benign condition that can be rapidly resolved after surgical correction. Neither ultrasound nor computed tomography can offer a reliable preoperative diagnosis [33].

Case Report: A 46-year-old woman with right pleural effusion and ascites caused by an ovarian tumor showed an elevated CA-125 level of 1808 U/mL. Computed tomography showed ascites and a bilobate pelvic tumor approximately 25 cm in size. The diagnosis of advanced epithelial ovarian cancer was considered and the patient was treated with chemotherapy and after three cycles of chemotherapy, the CA-125 level was reduced to 90 U/mL. After that surgery was performed on the patient in order to remove 25 cm of the left ovarian tumor with intact capsule, histopathological examination revealed only fibroma. However, her CA-125 was further reduced to 11 U/mL postoperatively. The authors concluded that although the association between ovarian tumor, pleural effusion, and ascites with marked elevation of CA-125 is highly indicative of epithelial ovarian cancer, Meigs' syndrome must also be considered in these patients and minimally invasive surgery or biopsy collection must also be considered [34].

Abnormally high values of both CA-125 and CA-19-9 have been reported in women with benign tumors. Nagata et al. reported that three patients with endometriosis also showed elevated levels of CA-125 and two patients with dermoid cyst showed elevated levels of CA-19-9. Therefore, the authors recommended that tumor marker values should be considered along with bimanual examination, ultrasound and CT scan for diagnosis of ovarian tumors [35]. Sometimes F(ab′)2 fragments of the murine monoclonal antibody (OC-125) are administered to patients with ovarian cancer because OC-125 is directed against the CA-125 antigen present on the surface of human ovarian cancers. Exposure to such antibody may lead to development of an immune response causing the presence of HAMA (human antimouse monoclonal antibody; also broadly termed as heterophilic antibody), which may interfere in an unpredictable manner with the determination of CA-125 using serum specimens in such patients [36].

Reinsberg and Nocke reported falsely low CA-125 values as well as one case of a falsely high value in patients who developed anti-idiotypic antibodies and non specific HAMA after exposure to [^{131}I] F(ab′)2 fragments of the OC-125 antibody. However, such interference can be eliminated after removal of serum immunoglobulins, particularly IgG. Although false positive results can also be corrected by adding non-specific murine IgG to the serum, false negative results cannot be corrected by such treatment. The false negative results were due to the presence of anti-idiotypic antibody while the false positive result was due to the presence of HAMA [37]. Falsely elevated CA-125 due to the presence of heterophilic antibodies in the specimen is a serious concern.

Measurable CA-125 concentrations can also be observed in patients without any cancer. CA-125 concentrations are known to rise in patients with severe congestive heart failure and such elevations correlate with the severity of disease and increased concentration of B-type natriuretic peptide (BNP), a marker for heart failure [38]. In the menstrual phase of the cycle in women, CA-125 values may be elevated causing false positive test results [39]. CA-125 levels may also increase after abdominal surgery, chronic obstructive pulmonary disease, active tuberculosis and lupus erythematous. During pregnancy, CA-125 concentrations increase 10 week after gestation and remain high throughout the pregnancy. Moreover, during the terminal phase of pregnancy, the CA-125 concentration may be as high as twice the upper limit of the reference range [40].

Emerging biomarkers in diagnosis of ovarian cancer

Serum epididymis protein-4 (HE4; molecular weight 23–27 kDa) level is a useful biomarker for the management of ovarian and endometrial cancer patients. HE4 has been shown to be expressed and secreted by ovarian carcinoma cells. This marker can be measured in both serum and urine [41]. The ROMA (the risk of ovarian malignancy algorithm) score that utilizes serum HE-4 and CA-125 level in calculating the score can also be used for improved sensitivity and specificity for diagnosis of ovarian cancer. In one study based on measuring HE-4 and CA 125 level in healthy females, the authors concluded that the reference limits for HE4, CA125 and ROMA were 65.87 pmol/L, 32.23 U/mL and 13.14 respectively for premenopausal women. The corresponding values for postmenopausal women were 90.76 pmol/L, 27.52 U/mL and 25.46 respectively [42].

Long noncoding RNAs are non-protein coding transcripts longer than 200 nucleotides that have recently been found as key regulators of many diseases including cancers. Expression pattern of several dysregulated long noncoding RNAs (HOTAIR, H19, XIST and HOST2) have potential as biomarkers for diagnosis of ovarian cancer [43].

ALPHA-FETOPROTEIN (AFP)

Alpha-fetoprotein (AFP) or alpha-fetoglobulin, a protein with approximate molecular weight of 70 kDa, is encoded in humans by the *AFP* gene, which is located on the q arm of chromosome 4 (4q25). AFP is a major plasma protein produced by the yolk sac and the liver during fetal development and is considered as the fetal form of albumin. AFP binds to copper, nickel, fatty acids and bilirubin. AFP is found in monomeric, dimeric and trimeric forms [44]. The half-life of AFP is approximately five to seven days. Following effective cancer therapy, normalization of the serum AFP concentration over 25−30 days is expected. However, it is essentially undetectable in the serum in normal men [45]. The upper limit of normal serum AFP concentration is less than 10−15 μg/L (10−15 ng/mL). Many tissues regain the ability to produce this oncofetal protein while undergoing malignant degeneration but serum AFP concentrations above 10,000 μg/L are most commonly observed in patients with nonseminomatous germ cell tumors (NSGCTs), hepatoblastoma or hepatocellular carcinoma.

In men with NSGCTs, AFP is produced by yolk sac (endodermal sinus) tumors and less frequently by embryonal carcinomas. As with β-hCG, the frequency of an elevated serum AFP increases with advancing clinical stage of the tumor, from 10 to 20% in men with stage I tumors, to 40−60% of those with disseminated NSGCTs [44]. By definition, pure seminomas do not cause an elevated serum AFP. However, molecular studies have demonstrated AFP mRNA in minute quantities in pure seminoma and several case reports have documented pure seminoma with borderline elevations in serum AFP (10.4−16 ng/mL). Higher serum AFP concentrations are considered diagnostic of a nonseminomatous component of the tumor (especially yolk sac elements) or hepatic metastases [46]. If the presence of an elevated serum AFP is confirmed, patients should be treated as if they had an NSGCT.

Serum AFP is the most commonly used marker for diagnosis of epithelial liver tumors (hepatocellular carcinoma and hepatoblastoma) because it is often elevated in these patients. Hepatoblastomas, more common in children, are usually associated with higher levels pf AFP than hepatocellular carcinoma (HCC), more common in adolescents and adult. Serum levels of AFP do not correlate well with other clinical features of HCC, such as size, stage, or prognosis. Elevated serum AFP may also be seen in patients without HCC such as acute or chronic viral hepatitis. AFP may be slightly elevated in patients with liver cirrhosis due to chronic hepatitis C infection. A significant rise in serum AFP in a patient with cirrhosis should raise concern that HCC may be developed. It is generally accepted that serum levels greater than 500 μg/L in a high-risk patient is diagnostic of HCC. However, HCC is often diagnosed at a lower AFP level in patients undergoing screening.

Not all tumors secrete AFP, and serum concentrations of AFP could be normal in up to 40% of patients with small HCCs. Furthermore, an elevated AFP may be more likely in patients with HCC due to viral hepatitis compared to alcoholic liver disease. In a study of 357 patients with hepatitis C and without HCC, 23% had an AFP >10.0 μg/L [47]. Elevated levels were associated with the presence of stage III or IV fibrosis, an elevated international normalized ratio, and an elevated serum aspartate aminotransferase level.

AFP levels are however normal in the majority of patients with fibrolamellar carcinoma, a variant of HCC [48]. The sensitivity, specificity, and predictive value for the serum AFP in the diagnosis of HCC depends upon the characteristics of the population under study, the cutoff value chosen for establishing the diagnosis, and the gold standard used to confirm the diagnosis. A number of studies have described test characteristics in different settings. The following estimates were based upon a cutoff value of >20 μg/L (>20 ng/mL) in a systematic review that included five studies [49]:

- Sensitivity 41−65%
- Specificity 80−94%
- Positive likelihood ratio 3.1−6.8
- Negative likelihood ratio 0.4−0.6

At a prevalence of HCC of 5%, a serum AFP of ≥20 μg/L had a positive and negative predictive value of 25 and 98%, respectively. At a prevalence of 20%, these numbers were 61 and 90%, respectively. The low positive predictive values indicate the limitation of using the serum AFP as a screening test for HCC [50]. Despite the issues inherent in using AFP for the diagnosis of HCC, it has emerged as an important prognostic marker, especially in patients being considered for liver transplantation. Patients with AFP levels >1000 μg/L have an extremely high risk of recurrent disease following the transplant, irrespective of the tumor size seen on imaging.

False positive AFP

Elevations of serum AFP in the absence of liver cancer can occur from tumors of the gastrointestinal tract, particularly gastric adenocarcinoma, or from liver damage (e.g., cirrhosis, hepatitis, or drug or alcohol abuse) [46]. Lysis of tumor cells during the initiation of chemotherapy may result in a transient increase in serum AFP. Elevated serum AFP occurs in pregnancy with tumors of gonadal origin (both germ cell and non-germ cell) and in a variety of other malignancies, of which gastric cancer is the most common [51]. German et al. commented that elevated alpha-fetoprotein concentrations can also be due to liver damage secondary to drugs (chemotherapy, anesthetics or antiepileptics), virus or alcoholism without any clinical evidence of malignant tumor [52]. Johnson et al. reported the case of a patient with elevated maternal serum alpha-fetoprotein where an ultrasound scan of the fetus showed a lemon sign, a banana sign, an effaced cisterna magna and splayed lumber vertebra. After pregnancy termination, no spinal abnormality was observed during autopsy although X-rays of fetal spine showed narrowing in the thoracic spine. The sonographic cranial findings and elevated alpha-fetoprotein suggestive of neural tube defect were misleading in this case although karyotype of the fetus was 69, XXY [53]. Values up to 140 times the upper reference range have been observed in cases of hereditary tyrosinemia type 1, with 71% patients showing levels twice the upper references range, indicating liver damage and the possibility of hepatomas at risk of HCC development [54].

As expected, heterophilic antibodies, if present in the specimen, can also cause falsely elevated AFP concentration. AFP level may be falsely elevated due to the presence of rheumatoid factor in the specimen [55]. Recently, Wang et al. reported negative interference of rheumatoid factor in the chemiluminescent microparticle immunoassay of AFP using ARCHITECT analyzer (Abbott Laboratories, Abbott Park, IL) [56]. Concentration of AFP may also be increased in pregnant women with systematic lupus erythematous [57].

CARCINOEMBRYONIC ANTIGEN (CEA)

Carcinoembryonic antigen (CEA) is a glycoprotein involved in cell adhesion that is normally produced during fetal development, but the production of CEA stops before birth. Therefore, it is not usually present in the blood of healthy adults, although levels are raised in heavy smokers. CEA is a glycosyl phosphatidyl inositol (GPI)-cell surface anchored glycoprotein whose specialized sialofucosylated glycoforms serve as functional colon carcinoma L-selectin and E-selectin ligands, which may be critical to the metastatic dissemination of colon carcinoma cells [58]. It is found in sera of patients with colorectal carcinoma (CRC), gastric carcinoma, pancreatic carcinoma, lung carcinoma and breast carcinoma. Patients with medullary thyroid carcinoma also have higher levels of CEA compared to healthy individuals (above 2.5 ng/mL). However, CEA blood test is not reliable for diagnosing cancer or as a screening test for early detection of cancer. Most types of cancer do not produce a high level of CEA. Elevated CEA levels should return to normal after successful surgical resection or within 6 weeks of starting treatment if cancer treatment is successful. Because it lacks both sensitivity and specificity, serum CEA is not a useful screening tool for CRC. However, in patients with established disease, the absolute level of the serum CEA correlates with disease burden and has prognostic value [59]. Furthermore, elevated preoperative levels of CEA should return to baseline after complete resection and residual disease should be suspected if they do not.

Serum CEA concentration and colorectal carcinoma

Serum levels of the tumor marker carcinoembryonic antigen (CEA) should be routinely measured preoperatively in patients undergoing potentially curative resections for CRC for two reasons:

- Elevated preoperative CEA levels that do not normalize following surgical resection imply the presence of persistent disease and need for further evaluation.
- Preoperative CEA values are of prognostic significance. CEA levels $\geq$5.0 ng/mL are associated with an adverse impact on survival that is independent of tumor stage [60].

The 2010 TNM staging criteria for malignant tumor (T describes size of tumor, N describes lymph nodes involved and M describes distant metastatics) does not include serum CEA in stage assignment, but recommends that the information be collected for prognostic value [61]. The data also support the view that decisions regarding adjuvant chemotherapy should include preoperative CEA levels into consideration. However, while a patient with node-negative cancer and a high preoperative CEA could be considered at higher than average risk for recurrence after surgery alone, and this might influence the decision to administer adjuvant chemotherapy, particularly if other risk factors (e.g., obstruction, perforation) are present, there is no data that directly support benefit for adjuvant chemotherapy in this particular setting based on CEA concentration alone. An expert panel convened by the American Society of Clinical Oncology (ASCO) in

2006 concluded that the data were insufficient to support the use of CEA to determine whether to treat a patient with adjuvant therapy or not [62]. Serial measurement of CEA can detect disease recurrence even among patients with an initially normal CEA level, although the sensitivity is lower (between 27 and 50% in four separate studies). Thus, a postoperative CEA elevation indicates recurrence with high probability, but a normal postoperative CEA level (even if it was initially elevated) is not useful for excluding a disease recurrence.

Arguments against serial CEA testing

- 30–40% of all cases of CRC recurrences are not associated with measurable elevations in serum CEA [63].
- The benefit of CEA monitoring is limited to a small number of patients with recurrent CRC and is not cost-effective.
- There are no data showing that CEA testing improves quality of life.

Frequency of testing

The optimal timing of CEA measurements is unclear. Although one study suggests better disease-free survival in patients who have a CEA level checked every one or two months compared to less frequent intervals, these results have not been confirmed by others, and no study has shown a survival benefit for any CEA testing frequency. ASCO (American Society of Clinical Oncology) guidelines suggest that postoperative serum CEA testing be performed every three months for at least three years after initial therapy in patients with stage II or III disease, if the patient is a potential candidate for surgery or systemic therapy. Guidelines from the NCCN (National Comprehensive Cancer Network) differ slightly, suggesting postoperative CEA testing every 3–6 months for 2 years, then every 6 months for a total of 5 years. These guidelines suggest that serial testing be limited to patients who have T2 or higher stage and who would be considered potential candidates for resection of isolated metastases. However, patient compliance with ASCO guidelines is poor. Sisler et al. based on a cohort of 250 patients reported that guidelines for colonoscopy, liver imaging and CEA measurement were met by 80.4%, 47.2% and 22% of patients respectively. Adherence was lower among elderly patients (>70 years) [64].

Adjuvant therapy with a 5-fluorouracil-based regimen may falsely elevate the serum CEA level possibly because of treatment-related changes in liver function. Therefore, serum levels of CEA should not be measured during 5-fluorouracil based oral chemotherapy nor within two weeks after intravenous chemotherapy administration [65].

CEA in cholangiocarcinoma

As a single analyte, serum levels of carcinoembryonic antigen (CEA) are neither sufficiently sensitive nor specific to diagnose cholangiocarcinoma. Many conditions other than cholangiocarcinoma may increase serum levels of CEA. Noncancer-related causes of an elevated CEA include gastritis, peptic ulcer disease, diverticulitis, liver disease, chronic obstructive pulmonary disease, diabetes, and any acute or chronic inflammatory state. One large study evaluated serum CEA levels in 333 patients with primary sclerosing cholangitis (PSC), of whom 44 (13%) were diagnosed with cholangiocarcinoma either by histologic confirmation or at least one year of clinical followup. A serum CEA level >5.2 ng/mL had a sensitivity and specificity of 68 (95% CI 48 to 84%) and 82% (95% CI 74 to 88%), respectively [66]. Biliary levels of CEA have also been evaluated. In one report, the biliary CEA concentration was elevated approximately five-fold in patients with cholangiocarcinoma compared to those with benign strictures; patients with PSC or choledochal cysts without malignancy had intermediate values [67].

False positive CEA

As expected false positive CEA test results can occur due to the presence of heterophilic antibodies in the specimen (see discussion on heterophilic antibodies at the end of this chapter). However, CEA concentrations can also be elevated in non-neoplastic conditions. Renal failure and fulminant hepatitis can falsely increase CEA values. CEA concentrations may be also elevated in patients receiving hemodialysis. Patients with hypothyroidism may also elevated levels of CEA, correlated with the duration of hypothyroidism. CEA levels may also be raised in some non-neoplastic conditions like ulcerative colitis, pancreatitis, cirrhosis, chronic obstructive pulmonary disease (COPD), Crohn's disease, as well as in smokers.

Litvak et al. investigated CEA levels in 728 patients who were diagnosed with CRC and underwent an R0 resection of locoregional CRC and later showed CEA levels above normal range. Of these 728 patients, 358 patients had false positive elevations of CEA values whereas 335 patients had true elevations indicative of recurrence of CRC and 35 patients had true positive elevations of CEA due to development of a new, non-CRC malignancy. Of these falsely elevated values, 111 patients showed a single isolated CEA elevation (median highest CEA level of 5.5 ng/mL) with no further falsely

elevated CEA value, whereas 247 patients showed falsely elevated CEA in two or more occasions. Only five patients showed values greater than 15 ng/mL and no confirmed elevations greater than 35 ng/mL were false positive results. The authors concluded that false positive CEA values between 5 and 15 ng/mL are common but false positive results greater than 15 ng/mL are rare and all confirmed CEA levels greater than 35 ng/mL were associated with cancer recurrence in these patients [68].

Case Report: A 66 year old woman was admitted to the hospital for treatment of uterine and rectal prolapse, pleural and pericardial effusion as well as ascites. She complained about general fatigue, constipation and anal pain. Blood testing reveled normocytic anemia (hemoglobin 9.5 g/dL) and thyroid function tests showed severe thyroid dysfunction with a thyroid stimulating hormone (TSH) concentration of 47.09 μU/mL (normal: 0.5–3.0 μU/mL), free thyroxin 0.1 ng/dL (normal: 0.8–1.5 ng/dL) and free triiodothyronine 0.6 pg/mL (normal: 2.1–3.8 pg/mL). Her thyroid microsome antibody titer was elevated and thyroid ultrasonography demonstrated diffuse swelling of the thyroid. Therefore she was diagnosed with primary hypothyroidism. Interestingly her serum CEA was elevated to 16.1 ng/mL (normal <5 ng/mL) and her CA-125 was 193 U/mL (normal <35 U/mL). These results suggested malignancy but further investigations ruled out any malignancy. Interestingly after treating the patient with oral levothyroxine, her CEA and CA-125 levels returned to normal values [69].

CA-19-9 (CARBOHYDRATE ANTIGEN 19-9)

Carbohydrate antigen (CA 19-9), also called cancer antigen 19-9 or sialylated Lewis (a) antigen, is a tumor marker used primarily in the management of pancreatic cancer [70]. Guidelines from the American Society of Clinical Oncology discourage the use of CA-19-9 as a screening test for cancer, particularly pancreatic cancer. The reason is that the test may be falsely negative in many cases, or abnormally elevated in people with no cancer at all (false positive). However, in individuals with pancreatic masses, CA-19-9 can be useful in distinguishing between cancer and other pathology of the gland [70,71]. The reported sensitivity and specificity of CA 19-9 for pancreatic cancer are 80–90%, respectively [72]. However, these values are closely related to tumor size. The accuracy of CA 19-9 to identify patients with small surgically resectable cancers is limited [72–74]. The specificity of CA 19-9 is limited because CA 19-9 is frequently elevated in patients with cancers other than pancreatic cancer, and various benign pancreaticobiliary disorders [75]. As a result of all of these issues, CA 19-9 is not recommended as a screening test for pancreatic cancer.

The degree of elevation of CA 19-9 (both at initial presentation and in the postoperative setting) is associated with long-term prognosis [76,77]. Furthermore, in patients who appear to have potentially resectable disease, the magnitude of the CA 19-9 level can also be useful in predicting the presence of radiographically occult metastatic disease [77,78]. The rates of unresectable disease among all patients with a CA 19-9 level ≥130 units/mL versus <130 units/mL were 26 and 11%, respectively. Among patients with tumors in the body/tail of the pancreas, more than one-third of those who had a CA 19-9 level ≥130 units/mL had unresectable disease. While high levels of CA 19-9 levels may help surgeons to better select patients for staging laparoscopy, an expert panel convened by the American Society of Clinical Oncology (ASCO) recommended against the use of CA 19-9 alone as an indicator of operability.

Serial monitoring of CA 19-9 levels (once every one to three months) is useful for further monitoring of patients after potentially curative surgery and for those who are receiving chemotherapy for advanced disease. Elevated CA 19-9 levels usually precede the radiographic appearance of recurrent disease, but confirmation of disease progression should be pursued with imaging studies and/or biopsy.

CA-19-9 can be elevated in many types of gastrointestinal cancer, such as colorectal cancer, esophageal cancer and hepatocellular carcinoma. Apart from cancer, elevated levels may also occur in pancreatitis, cirrhosis, and diseases of the bile ducts. It can be elevated in people with obstruction of the bile duct [71]. In patients who lack the Lewis antigen (a blood type protein on red blood cells), which is about 10% of the Caucasian population; CA-19-9 is not expressed even in those with large tumors. This is because of a deficiency of the fucosyltransferase enzyme that is needed to produce CA-19-9 as well as the Lewis antigen [75,79].

In another report, the authors observed 5–10% false negative CA-19-9 results in Lewis negative phenotype and 10–60% false positive elevation in patients with obstructive jaundice [82]. Patients with acute or chronic pancreatitis may also have elevated levels of CA-19-9. In addition, pulmonary diseases may also elevate CA-19-9 levels. Liver cirrhosis, Crohn's disease and benign gastrointestinal diseases may also increase CA-19-9 levels [40].

Combined CEA and CA 19-9

The use of a combined index of serum CA 19-9 and CEA (CA 19-9 + [CEA × 40]) has also been proposed for screening of cholangiocarcinoma. In a more recent

study of 72 patients with primary sclerosing cholangitis, the use of CA 19-9 alone (cutoff value ≥37 U/mL) was 63% sensitive for detecting cholangiocarcinoma, while the sensitivity of the combined CA 19-9/CEA index was only 33% [80]. Determination of serum levels of carbohydrate antigen (CA) 19-9 and carcinoembryonic antigen (CEA) may aid in the differentiation of invasive from noninvasive intraductal papillary mucinous neoplasm of the pancreas (IPMN). The tests had a sensitivity of 80% and a specificity of 82%.

Pitfalls in measuring CA-19-9

Although CA-19-9 is the most commonly used and best validated serum tumor marker for pancreatic cancer diagnosis in symptomatic patients, and for monitoring therapy for patients with pancreatic adenocarcinoma, CA-19-9 is also normally synthesized by healthy pancreas and biliary ductal cells and by gastric, colon, endometrial and salivary epithelia. As a result, small amounts of CA19-9 are present in serum and can be overexpressed in several benign gastrointestinal disorders. Critical aspects of the clinical usefulness of CA-19-9 measurement include false negative results in subjects with Lewis antibody and false positive elevation, occasional and transient elevations with benign diseases and its poor positive predictive value for pancreatic cancer (72.3%). Another complicated factor is the issue of false positive and false negative results in serum CA-19-9 measurement using commercially available assays. These factors do not make CA-19-9 a good cancer biomarker. Nevertheless, CA-19-9 is the only pancreatic cancer marker in clinical use [81].

Interference of heterophilic antibodies causing false positive CA-19-9 result has been documented and usually treating the specimen with heterophilic antibody blocking agents eliminates such interference. Monaghan et al. described a case where a false positive CA-19-9 result obtained by the ADVIA Centaur CA-19-9 assay (Siemens Diagnostics) was not resolved after treating the specimen with a heterophilic antibody blocking agent. By performing a gel filtration study, the authors speculated that the interfering substance was a low molecular weight (approximately 100 KDa) compound. Interestingly, when the specimen was reanalyzed using Roche Modular Analytics E170 and Brahms KRYPTOR analyzer, CA-19-9 values were within reference limit indicating that the interfering substance is only affecting the CA-19-9 assay on the ADVIA Centaur analyzer [83]. Berth et al. reported interference of rheumatoid factor in CA 19-9 assay [84].

Case Report: A 76 year old female patient with symptoms of shaking, chills, fever, abdominal pain, pruritus, fatigue and dark urine was admitted to the hospital. Her liver enzymes were elevate with aspartate aminotransferase (AST) level of 201 U/L, alanine aminotransferase (ALT) level of 228 U/L, gamma-glutamyl transpeptidase (GGT) level of 296 U/L, alkaline phosphatase level (ALP) of 866 U/L, and total bilirubin level of 10 mg/dL. Interestingly her serum CA-19-9 was highly elevated to 9586 U/mL. Ultrasonographic examination of liver and biliary system revealed cholelithiasis, dilation of common bile duct and portal hilar lymph nodes. Abdominal computerized tomography showed a stone in the common bile duct. Endoscopic examination demonstrated dilatation of common bile duct, choledocholithiasis and multiple stones in the gall bladder. However, no cancer was detected by any investigation. She was treated with ceftriaxone 2 gm/day. Sphincterotomy and extraction of biliary stones was performed and her symptoms gradually improve over 10 days. Interestingly, not only her liver enzymes returned to normal values but her CA-19-9 level was also significantly reduced to 50 U/L [85].

Case Report: A 52 year old woman had a history of epigastric pain and anorexia for two months and also lost weight. She was a non-smoker and a non-drinker but reported over consumption of black tea (1.5−2 L) every day. Her physical examination was unremarkable, her electrolytes, liver function tests, lipid profile and fasting blood glucose were all within acceptable limits but her serum C-19-9 was significantly elevated to 1432 U/mL (normal <37 U/mL). Abdominal ultrasonography and computed tomography showed mild enlargement of pancreas but further investigation with endoscopic ultrasonography showed no pancreatic malignancy or biliary abnormality. The patient was advised to stop tea consumption and four weeks later her symptoms resolved and interestingly her CA-19-9 level was almost returned to almost normal value of 42 U/mL [86].

β_2 MICROGLOBULIN

Also known as B2M, β_2-microglobulin is a component of MHC (major histocompatibility complex) class I molecules, present on all nucleated cells (excluding red blood cells). In humans, the β_2-microglobulin protein is encoded by the *B2M* gene [87,88]. For the diagnosis of multiple myeloma, the serum β_2-microglobulin level is one of the prognostic factors incorporated into the International Staging System. The serum β_2-microglobulin level is elevated (i.e., >2.7 mg/L) in 75% of patients at the time of diagnosis. Patients with high values have inferior survival [89]. The prognostic value of serum

β_2-microglobulin levels in myeloma is probably due to two factors:

- High levels are associated with greater tumor burden.
- High levels are also associated with renal failure, which carries an unfavorable prognosis.

In lymphoma, β_2-microglobulin levels usually correlate with disease stage and tumor burden in patients with CLL (chronic lymphocytic leukemia), with increasing levels associated with a poorer prognosis. B2M may be regulated, at least in part, by exogenous cytokines. The source of these elevated cytokines in CLL is unclear, although IL-6, which inhibits apoptosis in CLL cells, may be released from vascular endothelium [90].

However, β_2-microglobulin levels also rise with worsening renal dysfunction leading some investigators to suggest a measure of β_2-microglobulin adjusted for the glomerular filtration rate (GFR-adjusted B2M) [91]. This GFR-adjusted B2M requires validation in prospective confirmatory studies. The plasma β_2-microglobulin concentration is increased in dialyzed patients, with a level ranging from 30 to 50 mg/L, much higher than the normal value of 0.8–3.0 mg/L. Infection with human immunodeficiency virus, hepatitis, and active tuberculosis may also elevate level of β_2-microglobulin [40]. Ibsen et al. reported elevated β_2-microglobulin concentration in serum in children suffering from infectious mononucleosis. The diagnosis was established by demonstration of Epstein-Barr virus specific IGM antibodies. The serum concentrations of β_2-microglobulin were significantly increased in the first specimen after onset of disease, compared to children with other infectious diseases or without diseases. The serum concentrations of β_2-microglobulin were reduced toward normal range within 3 weeks to 3 months. During that time IgM antibodies of Epstein -Barr virus also decreased to unmeasurable levels [92].

HUMAN CHORIONIC GONADOTROPIN

Human chorionic gonadotropin (hCG) is a hormone composed of alpha and beta subunits and the beta subunit (beta-hCG) provides the functional specificity of this hormone. Human chorionic gonadotropin is synthesized in large amounts by placental trophoblastic tissue and in much smaller amounts by the hypophysis and other organs such as testicles, liver and colon. Therefore elevated levels of hCG are observed during pregnancy, produced by the developing placenta after conception, and later by the placental component syncytiotrophoblast [93]. Although the major application of hCG testing is in the initial diagnosis of pregnancy, it is also used as a cancer marker for testicular and ovarian cancer, but use of hCG as a cancer marker is not approved by the FDA.

Laboratory tests for hCG are essentially very sensitive and specific for diagnosis of trophoblast-related conditions including pregnancy and the gestational trophoblastic diseases. Rarely, however, very low levels of hCG are detected in the absence of one of these conditions. hCG exists in many forms in serum including intact molecule, beta-hCG, hyper-glycosylated form other forms including C-terminal peptide. Therefore, an assay capable of measuring all forms of hCG is desirable to resolve low values and ensure indeed it is a low value. Although all assays detect regular hCG, they do not necessarily detect all hCG variants [94].

The major application of hCG testing is detection of pregnancy using urine specimens and often point of care devices are used to qualitatively measure hCG (positive or negative). In pregnancy, hCG immunoreactivity in urine is due to the presence of intact hCG, as well as hyper glycosylated hemoglobin, nicked hCG, beta-subunit of hCG, the core fragment of beta-hCG and others. As expected, these forms are also present in serum. However, many over-the-counter pregnancy tests do not measure hyperglycosylated hCG, which accounts for most of the total hCG at the time of missed menses. Clinical tests for pregnancy may only detect total hCG levels $\geq$20 mIU/mL. Therefore, when following hCG levels to negative (<1 mIU/mL) in women with gestational trophoblastic disease, it is important to use a sensitive hCG test that detects both regular and other forms of hCG. False negative results using qualitative tests for hCG in urine due to increased concentration of core fragment of beta-hCG have also been reported [95].

At levels of hCG above 500,000 mIU/mL, a "hook effect" can occur resulting in an artifactually low value for hCG (i.e., 1–100 mIU/mL) [96]. This is because the sensitivity of most hCG tests is set to the pregnancy hCG range (i.e., 27,300–233,000 mIU/mL at 8–11 weeks of gestation); therefore, when an extremely high hCG concentration is present, both the capture and tracer antibodies used in assays become saturated, preventing the binding of the two to create a sandwich [97]. For this reason, a suspected diagnosis of gestational trophoblastic disease must be communicated to the laboratory so that the hCG assay could also be performed at 1:1000 dilution.

Causes and evaluation of persistent low levels of human chorionic gonadotropin

A positive test for hCG before menopause is an indication of pregnancy. However, positive hCG test in a postmenopausal woman is a diagnostic challenge. Erroneous assumption of cancer due to positive hCG may lead to costly diagnostic tests and even cancer

chemotherapy in the absence of cancer. The pituitary is capable of producing hCG. Low levels of hCG accompany the mid-cycle pre-ovulatory surge of luteinizing hormone and production of hCG by pituitary also continues after menopause. Therefore, low levels of hCG in serum are normal in postmenopausal women. Levels as high as 32 mIU/mL have been reported. Moreover, the pituitary origin of hCG can also be confirmed by the resolution of high hCG levels after administration of hormone replacement therapy for two or more weeks [98]. In addition, low levels of hCG due to pituitary hCG are also found in women who have a history of gestational trophoblastic disease. Unless tumor is evident, it is essential to exclude these possibilities before initiating chemotherapy for assumed persistence of disease.

For a positive hCG serum level, it is important to determine if the hCG represents an actual early pregnancy (intrauterine or ectopic), active gestational trophoblastic disease (complete or partial mole, invasive mole, choriocarcinoma, PSTT), quiescent gestational trophoblastic disease, a laboratory false positive (also called phantom hCG), or a physiologic artifact (pituitary hCG). De Backer et al. reported persistent low hCG levels in two nonpregnant women. In the first case, hCG levels increased during several years following a spontaneous abortion. The likelihood of a false positive result due to interference of heterophilic antibodies was ruled out and extensive clinical investigation excluded the presence of a tumor. The diagnosis was quiescent gestational trophoblastic disease. In the second case, elevated hCG was an incidental finding where the clinician questioned the laboratory regarding validity of the test. The cause was probably an increase in pituitary hCG production and chronic renal failure in the patient [99].

Phantom hCG and phantom choriocarcinoma syndrome (pseudohypergonadotropinemia) refers to persistent mildly elevated hCG levels, which may mislead physicians to treat patients with cytotoxic chemotherapy for choriocarcinoma when in reality no true hCG level or trophoblastic disease is present. In one patient with low levels of serum hCG (49–89 mIU/mL) 11 months after the patient had miscarriage, diagnosis of choriocarcinoma was made. Despite chemotherapy and a hysterectomy, low levels of hCG were still detected in serum using three different assays, but no hCG was detected in urine. When serum was diluted levels did not decrease parallel to dilution indicating that the molecule measured in her serum was a pseudo-gonadotropin or phantom hCG, an interfering substance in the test. In the 2nd case, positive hCG was observed 7 years after normal pregnancy in a woman. A pelvic ultrasound and a laparoscopy revealed no pregnancy but hCG levels stayed between 48 and 74 mIU/mL over a three month period. Although low levels of hCG, beta-hCG and beta-core fragment were detected in serum, no activity was observed in urine. Therefore, the elevated hCG test results in serum were due to phantom hCG. The authors also described a third case where false positive hCG levels in serum (51–135 mIU/mL) were due to phantom hCG [100]. Persistent low level of phantom hCG may also be due to heterophilic mouse antibody interference.

Case Report: A 23 year old woman presented to the emergency department with persistent abdominal pain and metrorrhagia one month after miscarriage. She also showed a low level of hCG (74 mIU/mL) in her serum. As choriocarcinoma was suspected, a chemotherapy trial with methotrexate was prescribed but neither pain relief nor reduction of serum hCG level was observed. In addition, she showed a skin toxicity reaction to methotrexate. Subsequently, laparoscopy ruled out a trophoblastic residue and patient was referred to endocrinologists for further investigation. Although increased hCG levels were detected in her serum no hCG was detected in urine. When the serum was treated with a heterophilic blocking tube, no hCG was detected in the serum indicating that phantom hCG in her serum was due to heterophilic mouse antibodies [101].

False positive human chorionic gonadotropin

The capture and tracer antibodies used for hCG testing may be goat, sheep, or rabbit polyclonal antibodies or mouse, goat, or sheep monoclonal antibodies. Humans extensively exposed to animals or certain animal byproducts can develop human antibodies against animal antibodies (HAAAs). Humans with recent exposure to mononucleosis are prone to develop HAAAs; those with IgA deficiency syndrome also often have heterophilic antibodies [102]. These antibodies may develop from milk and meat ingestion, environmental or occupational exposure to animals, or from injection of mouse monoclonal antibody products for diagnosis or therapeutic purposes [99]. False positive hCG tests due to presence of heterophilic antibody in the serum specimen have been well documented in the literature. Such false positive results in the absence of pregnancy have led to many men and women misdiagnosed with cancer, confusion and misunderstanding, and needless surgery and chemotherapy [103–105]. There are two main methods for identifying false positive hCG:

- The most readily available approach is to show the absence of hCG in the patient's urine, since large molecules like an immobilized capture antibody-heterophilic antibody/HAAA-tracer antibody sandwich fail to cross the glomerular basement membrane. A true hCG elevation should be present in both serum and urine [106].

- A second useful way of identifying a false positive serum hCG result is to send the serum to two laboratories using different commercial assays. If the assay results vary greatly or are negative in one or both alternative tests, then a false positive hCG can be presumed.

Patients who have false positive hCG test results are at risk for recurrent false positive hCG assay results [107]. They are also at risk for other false positive results, such as with CA-125 and thyroid antibody tests [108]. False positive results for urine hCG have been described in cases of membranoproliferative glomerulonephritis (where rheumatoid factor was the potential interfering substance), chronic renal failure, adenomyosis, tubo-ovarian abscess, and multiple myeloma. A transient false positive urine hCG test result using a point of care device in a 38 year old woman suffering from septic shock has also been reported. The authors speculated that the false positive result that was resolved after 48 h may be due to a molecule produced as a part of host inflammatory response or from bacterial synthesis of an interfering substance with hCG like antigenic structure [109].

Case Report: A 39 year old male patient underwent a unilateral orchiectomy due to a right testicular mass. He showed an elevated serum hCG level of 30 U/L (normal <5 U/L). Histological examination revealed embryonal carcinoma. He underwent three cycles of chemotherapy with bleomycin, etoposide and cisplatin. His serum hCG after three weeks of chemotherapy was undetectable. Two months later, his serum hCG was increased to 55 U/L suggesting relapse of the cancer. However, further investigations revealed no relapse of cancer, hCG was undetectable in cerebrospinal fluid and examination of lymph node did not show any malignancy. His serum hCG increased further to 280 U/L and after chemotherapy was reduced to 55 U/L. Unfortunately, serum hCG increased again to 341 U/L and after chemotherapy it was reduced to 145 U/L. Interestingly, in the following month his serum hCG returned to a normal level, which coincided with changing the serum hCG assay to Roche Modular analyzer E170. Previously, the Abbott's assay was used on the AxSYM analyzer for measurement of serum hCG. Retesting high hCG results using the Roche analyzer showed remarkably low levels indicating interference in the Abbott's serum hCG assay. However, re-analysis of these samples using Abbott's laboratories Architect analyzer and Immulite analyzer (Siemens Diagnostics) also eliminated this interference. Unfortunately, this male patient with testicular cancer received two additional unnecessary chemotherapy cycles due to falsely elevated serum hCG levels as a result of analytical interference in the assay [110].

Case Report: A 44 year old HIV positive man presented with a painless swelling of his left testicle. He underwent left radical orchiectomy for a pathological stage T1 non-seminomatous germ cell tumor (NSGCT). However, after the procedure his serum hCG was persistently elevated and he went through four cycles of chemotherapy with etoposide and cisplatin. Despite chemotherapy his serum hCG values did not return to normal level. However, further investigations revealed that the patient was cancer-free. Suspecting false positive serum hCG levels, the authors reanalyzed the sample after adding a heterophilic antibody blocking agent and the serum hCG levels became undetectable indicating the presence of a heterophilic antibody in the serum specimen causing false positive serum hCG values in this patient. Other tumor markers including alpha-fetoprotein were not elevated [111].

MARKERS OF BREAST CANCER

Breast cancer is by far the most common cancer in women worldwide where more than 1.2 million cases are detected every year affecting 10–12% of women and responsible for approximately 500,000 deaths per year. Several tumor markers are used in diagnosis and management of patients with breast cancer but the most commonly used tumor marker is CA 15-3 (cancer antigen 15-3) which is also known as MUC-1. This tumor marker, a high molecular mass glycoprotein, is a transmembrane mucin expressed by most glandular epithelial cells and was first discovered in human breast milk where this protein is shed from lactating mammary epithelial cells. CA 15-3 was identified on the surface of many types of cancer including breast, and ovarian cancer as well as lung, pancreatic and prostate cancer, but the major application is in diagnosis and management of breast cancer. The molecules detected by CA 15-3 assay are the shed or soluble forms of the MUC-1 protein and there are many commercially available immunoassays (both competitive and sandwich type) for measuring CA 15-3 in serum or plasma. Although a major application of CA 15-3 is in monitoring patients with metastatic disease, this marker should not be used alone but should be measured in conjunction with diagnostic imaging, clinical history, and physical examination. CA 15-3 may also be used in the postoperative surveillance of asymptomatic women who have undergone surgery for invasive breast cancer. The main limitation is that serum levels of CA 15-3 are rarely increased in patients with early or localized disease. Moreover, CA 15-3 levels may be elevated in other types of advanced cancer including ovarian, pancreatic, gastric, and lung cancer [112,113]. Moreover, CA 15-3 can be significantly

elevated in patients with vitamin B12 deficiency. Increases up to twice the upper reference range have been observed in some healthy women during the menstrual cycle. Pancreatitis, Crohn's disease, ulcerative colitis and benign gastrointestinal disease may also cause some elevation of CA 15-3 [40].

Human epidermal growth factor receptor 2 (HER 2) (also known as cluster of differentiation 340 or protooncogene Neu, encoded by the *ERBB2* gene) is a 185 kDa glycoprotein consisting of three domains; a 105 kDa extracellular domain (ECD), a transmembrane lipophilic segment, and an intracellular domain with tyrosine kinase activity. The ECD portion can be released by cleavage from HER2 receptor and shed into serum. Overexpression of HER2 receptor is observed in 20−30% breast cancer patients and such overexpression is associated with an aggressive tumor subtype, reduced survival, and possible treatment with monoclonal antibody trastuzumab or other types of therapy targeted against the HER 2 receptor protein [113]. HER 2 can be measured by various methods but most commonly employed methods are immunohistochemistry and FISH (Fluorescence in situ hybridization) [114]. Testing of HER 2 in addition to testing of estrogen and progesterone receptor using immunohistochemistry may have additional advantages but detail discussion on this topic is beyond the scope of this chapter.

HETROPHILIC ANTIBODY INTERFERENCE IN TUMOR MARKERS TESTING

Heterophilic antibodies are human antibodies that interact with assay antibodies causing false positive or negative results. In this section case reports showing interferences from heterophilic antibodies on various tumor marker measurements are presented Although among tumor markers, serum hCG assay is the most affected by the presence of heterophilic antibodies, false positive test results with other tumor markers including PSA, CA-125, CA-19-9, CEA, alpha-fetoprotein and even beta2-microglobulin have also been reported. In fact interference due to heterophilic antibodies is the major problem in assays of various cancer markers. Even IgM lambda antibody to *Escherichia coli* can produce false positive test results with determination of various tumor markers as well as troponin I. However, for hCG testing a positive test result in serum but negative test result (beta-hCG) in urine may indicate interference in the serum hCG assay including interference due to the presence of heterophilic antibodies.

Case Report: A 56-year-old male presented to the hospital with a history of benign prostatic hypertrophy and a urinary tract infection probably related to urethral obstruction. The patient did not improve with intravenous ampicillin/sulbactam and cefepime therapy and several days later *E. coli* was identified in the blood culture. The patient was managed by intravenous cefepime and oral ciprofloxacin therapy. On the third day of hospitalization, the patient complained about jaw pain and his cardiac troponin I was 8.7 ng/mL, which was further elevated to 12.7 ng/mL 6 h later. As a result, the patient was admitted to the coronary care unit. However, further investigation showed no evidence of myocardial obstruction or damage. The troponin I value was even further elevated to 220 ng/mL on day 7. Interestingly, his CK-MB and myoglobin were within normal limits indicating no myocardial infarction. More strikingly his CA-125, alpha-fetoprotein and thyrotropin were all elevated as measured by the Abbott assays on an AxSYM analyzer. Suspecting false positive results for all these analytes, the authors treated the specimens with Protein A-Sepharose and murine mAB-conjugated Sepharose. Treating with Protein A-Sepharose did not eliminate the interference but treatment with mAB-conjugated Sepharose eliminated the interference indicating that the interference was due to a heterophilic antibody of the IgM class. The authors speculated that IgM antibody production in this patient was induced by *E. coli* infection [115].

Case Report: A 62 year old man underwent radical prostatectomy for localized adenocarcinoma of the prostate. His preoperative PSA level was 12.2 ng/mL. Six weeks after the procedure, during a routine visit, his PSA was 7.48 ng/mL. To rule out specimen error, the test was repeated one week later using a different sample and the PSA was 7.94 ng/mL. In order to eliminate the possibility of false positive PSA levels, the serum specimen was treated with a heterophilic antibody blocking agent and the PSA value decreased below detection level indicating that the false positive postoperative PSA in this patient was due to the presence of a heterophilic antibody [116].

Case Report: A 55 year old woman underwent surgical treatment for ovarian cancer in 1995 and was free from cancer. In July 2001, a blood specimen for CA-125 showed a CA-125 level of 11 U/mL. The test was repeated a month later and the value was still slightly elevated to 14 U/mL. When aliquots of the serum samples were sent to a different laboratory CA-125 values were 156 and 187 U/mL. Suspecting interference, specimens were treated with heterophilic antibody blocking agent and values were returned to near normal indicating falsely elevated CA-125 in sera due to the presence of interfering heterophilic antibodies [117].

Case Report: A 76 year old man was referred to the hospital, complaining of pain in his right upper quadrant. He had complained about that pain previously. Ultrasound revealed hepatomegaly and biliary polyps

while computed tomography showed pancreatic poly-cysts and a cyst on his left kidney. Although CA-125, CEA and alpha-fetoprotein concentrations were within normal ranges, his CA-19-9 was elevated to 1047.4 U/mL (normal < 37 U/mL) as measured by the Abbott's assay using the AxSYM analyzer. Interestingly, when CA-19-9 concentration was measured using a different assay (Roche assay on Modular E170 analyzer), the value was 11.9 U/mL, which was within the normal limit. Suspecting high rheumatoid factor present in the patient as the cause of interference with the Abbott assay, due to previous reports of interference of rheumatoid factors in CA-19-9 assay, the authors analyzed 18 specimens with high rheumatoid factors using the Abbott assay but did not observe any false positive CA-19-9 results. Therefore, the authors ruled out rheumatoid factor as the interfering agent in this patient. Because the patient lived in a farm where mice were present the authors suspected a heterophilic antibody (HAMA: human anti-mouse antibody) as the cause of interference. After incubating the specimen with 50% mouse serum followed by treatment with polyethylene glycol 6000, the CA-19-9 value reduced from 1047.4 to 19.8 U/mL indicating that the cause of false positive CA-19-9 in this patient using the Abbott assay was a HAMA [118].

LESS FREQUENTLY MONITORED TUMOR MARKERS

CA 72-4 (cancer antigen 72-4) is a marker for colorectal cancer. In general about 5% women show CA 72-4 values over the reference range. In addition, 3–7% patients with pancreatitis show CA72-4 value over the normal range. Interestingly approximately 50% of patients suffering from Familial Mediterranean Fever demonstrate CA72-4 values above the reference range [40].

CYFRA-21, squamous cell carcinoma antigen, and CEA are the most useful markers for diagnosis of non-small cell lung cancer whereas neuron specific enolase is a marker for small cell lung cancer [119]. However, patients with pulmonary disease without cancer may show elevated levels of CYFRA 21-1 antigen. Patients receiving dialysis, patients with renal failure and liver disease may also show elevated levels of this antigen. Chromogranin A is a marker for various neuroendocrine diseases such as gastro-entero-pancreatic tumor, endocrine tumors and bronchial carcinoid. It may also be useful in diagnosis of pheochromocytoma along with urine/plasma metanephrine determination. However, the assay for chromogranin A may be affected by the presence of heterophilic antibodies in the serum [120].

Squamous cell carcinoma antigens (SCCAs) are members of the serpin family of endogenous serine protease inhibitors. The first such marker, SCCA1 was originally identified in squamous cell carcinoma of the uterine cervix. Later a similar molecule with 92% amino acid homology with SCCA1, SCCA2, was identified. High levels of SCCA are often observed with poorly differentiated and advanced metastatic squamous cell carcinoma. Elevated levels of SCCA have been reported in cancer of epithelial (cervix, lung, head and neck) and endodermal (liver) and possibly also in advanced grade as well as high grade human breast cancer. In the clinical laboratory circulating SCCA (both SCCA1 and SCCA2) can be detected by immunoassays or by using immunohistochemistry. Tissue biopsy may also be used for measuring this tumor marker [121]. Various dermatological diseases such as psoriasis, eczema, epidermitis, erythrodermia, and atopic dermatitis may cause false elevations of squamous cell carcinoma antigen levels. Patients with chronic renal failure may also show elevation of this marker [40].

More recently circulating cell free DNA assays are getting attention as viable tumor markers that can be analyzed in blood (liquid biopsy). Most cell free DNA is released from the bone marrow and white blood cells whereas cell free DNA in cancer patient is derived from necrotic and apoptotic cancer cells. In general cell free DNA contains heterogenic defects, such as single nucleotide mutations and methylation changes, identical to the primary tumor and/or metastases. The half-life varies from 16 min to 2.5 h thus providing a dynamic monitoring of disease burden. In general the overall cell free DNA level is significantly higher in cancer patients than healthy volunteers. Nevertheless, the biggest obstacle is the detection limit of the assay as tumor related cell free DNA may represent <0.1% of total cell free DNA. Moreover, several factors such as the type of matrix, storage conditions or particular handling of blood samples may affect cell free DNA concentrations and fragmentation values [122]. The epidermal growth factor receptor (EGFR) is a transmembrane tyrosine kinase receptor that plays a central role in regulating cell division and death. Mutations and copy number changes in the gene encoding EGFR that lead to overexpression of the protein have been associated with a number of different cancers. The FDA recently approved the first such liquid biopsy test for EGFR mutation in patients with non-small cell lung cancer. This non-invasive method allows the identification of specific mutants such as EGFR T790M in patients with non-small cell lung cancer being treated with gefitinib [123]. Detailed discussion on this topic is beyond the scope of this chapter.

TABLE 12.2 Common causes of elevation of level of various tumor markers in the absence of neoplasia.

Tumor marker	Common causes of elevated levels
Prostate specific antigen	Prostatitis/Benign Prostatic Hyperplasia, Breast Cyst, Heterophilic Antibody
Alpha-Fetoprotein	Hepatobiliary Disease, Pneumonia, Pregnancy, Autoimmune Disease, Heterophilic Antibody
CA-125	Hepatobiliary Disease, Pulmonary Disease, Renal Failure, Hypothyroidism, Endometriosis, Pregnancy, Autoimmune Disease, Skin Disease, Cardiovascular Disease, Heterophilic Antibody
Carcinoembryonic antigen (CEA)	Hepatobiliary Disease, Renal Failure, Hypothyroidism, Gastrointestinal Disease, Pancreatitis, Endometriosis, Autoimmune Disease, Heterophilic Antibody
CA-19-9	Hepatobiliary Disease, Renal Failure, Pulmonary Disease, Pancreatitis, Gastrointestinal Disease, Endometriosis, Heterophilic Antibody
CA-15-3	Vitamin B12 Deficiency, Effusion, Renal Failure,
Beta-2-microglobin	Renal Failure, Autoimmune Disease, Cerebral Lesion
hCG	Renal Failure, Pregnancy, Autoimmune Disease, Heterophilic Antibody
CA-72-4	Hepatobiliary Disease, Renal Failure, Effusion, Pancreatitis, Gastrointestinal Disorder
CYFRA 21-1	Hepatobiliary Disease, Renal Failure, Effusion, Pulmonary Disease
Chromogranin A	Cardiovascular Disease, Viral Infection, Prostatitis/Benign Prostatic Hyperplasia, Gastrointestinal Disease, Heterophilic Antibody

CONCLUSIONS

Tumor markers are widely used nowadays. However, there are some limitations with the interpretation of tumor marker measurements. All the markers in current use can be synthesized by normal tissues and levels in serum can be elevated in certain benign diseases. Frequently, both benign disorder and malignant transformation of the same organ can give rise to elevations in the same marker, for example, prostate specific antigen (PSA) can be increased in both benign hypertrophy of the prostate (BPH) and prostate carcinoma. In most situations, however, marker levels are higher in malignancy (especially when distant metastases are present) than in benign disease.

Markers are only rarely elevated in the serum of patients with early malignancy or malignancy confined to the primary site. For example, carcinoembryonic antigen (CEA) is present at high concentrations in less than 10% of patients with Duke's A colorectal cancer and is only frequently elevated when distant metastases are present.

Markers are generally elevated in only a proportion of patients (at most 70–80%) with a particular tumor type, even in the presence of advanced disease, for example, CA-125 in ovarian cancer, CA 19-9 in pancreatic cancer, and AFP in hepatocellular cancer. By contrast, hCG is present at high concentrations in almost all patients with trophoblastic malignancies.

Apart from PSA, which is almost prostate specific, no other marker in clinical use is organ specific. Many markers such as CEA, CA-125, CA 15-3, CA 19- 9, and tissue polypeptide antigen (TPA) can be elevated in serum with most types of advanced adenocarcinoma. Alpha-fetoprotein (AFP), however, is relatively specific for hepatic epithelial tumors and nonseminomatous germ cell tumors, while HCG is relatively specific for trophoblastic malignancies. Although heterophilic antibodies can also cause significant interference in tumor marker immunoassays, various heterophilic blocking reagents, steps to remove immunoglobulins and serial dilutions may correct the problem. There are also various non-neoplastic conditions that may elevate values of various tumor markers as discussed throughout the chapter. In Table 12.2, common non-neoplastic causes of elevation of various tumor markers are summarized.

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CHAPTER

13

Issues of interferences in therapeutic drug monitoring

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INTRODUCTION

Therapeutic drug management (TDM) is a cornerstone of personalized medicine, designed to select and optimize drug therapy for individual patients. By optimizing therapy, the desirable effects of a drug (efficacy) can be maximized, and the undesirable effects of a drug (toxicity) minimized. TDM is also employed to evaluate patient compliance, identify drug-drug interactions or other changes in pharmacokinetics, and to both characterize and manage acute intoxications or emergent overdose situations. TDM is most useful when there is a good correlation between drug concentration in blood and effect (therapeutic and toxic). It is particularly useful for drugs that have a narrow therapeutic window, prodrugs, and for drugs that are highly bound to plasma proteins. TDM is not widely available for most drugs due in part to limitations in analytical methods.

Inaccuracies in TDM results will impact patient care by contributing to unnecessary or inappropriate dose adjustments. Inappropriate increases in dose which could lead to dose-dependent adverse drug reactions (ADRs). ADRs are reported to account for 41% of all hospital admissions, due to inappropriate dose or prescription, allergic reactions, and drug-drug interactions. The consequences of ADRs are costly, and can be fatal [1]. Errors in TDM results could also cause unnecessary dose reductions and minimize the therapeutic effect. Therapeutic failure due to inappropriately low dosing could contribute to unnecessary testing and procedures, and for time-sensitive conditions such as organ transplantation, cancer, uncontrolled seizures, and cardiac disturbances, could be fatal. The laboratory has an important opportunity to promote clinically useful TDM through involvement in appropriate specimen collection, sample handling, sample processing, selection of appropriate analytical methods, and interpretation of results. As such, the laboratory must be prepared to consult regarding therapeutic ranges, toxic thresholds, as well as strengths and limitations of available analytical methods. Inaccuracies in TDM may be a result of pre-analytical factors surrounding the specimen and patient, or analytical factors surrounding selection and performance characteristics of the analytical method (see Fig. 13.1). Sources of interference may arise from endogenous or exogenous sources, and may impact specific technologies in a direct or indirect manner. However by using good laboratory practice interferences can be identified, managed, and/or prevented.

This chapter describes sources of pre-analytical and analytical interferences that have been reported to adversely affect accuracy of TDM results. Specific drug, technology, and case examples will be provided to illustrate these sources in more detail, and guide appropriate utilization and interpretation of TDM, from the clinical laboratory perspective.

SOURCES OF PRE-ANALYTICAL FACTORS AFFECTING DRUG LEVELS

Pre-analytical sources of inaccuracy in laboratory test results are covered in detail elsewhere, within this book (Chapters 1–4). However, there are many pre-analytical factors important to TDM that are unique from other types of laboratory testing, and are briefly emphasized here. In particular, the coordination of pharmacokinetic factors for the specific drug formulation(s) of interest, with the clinical status and co-medications of the patient, will dictate the most appropriate specimen to collect, at the most appropriate

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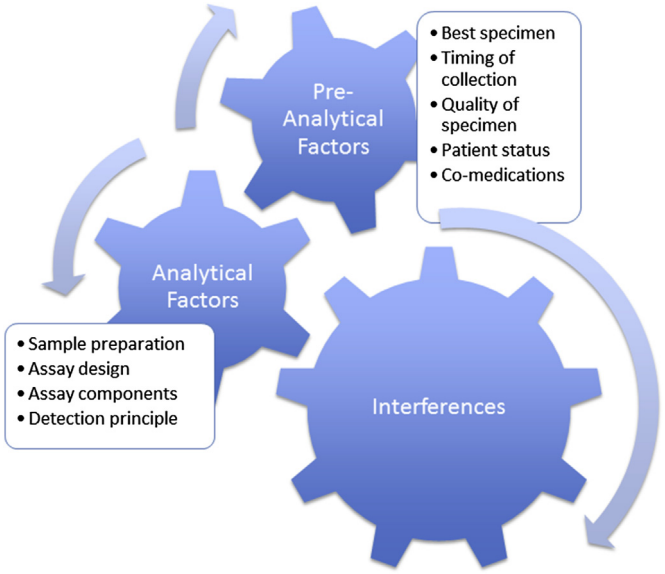


FIG. 13.1 Factors that affect accuracy of a TDM result.

time. In addition, specimen collection using the wrong anticoagulant and environmental factors such as heat and light can affect drug stability and alter the concentration of therapeutic drugs. TDM measurements are typically collected after a patient is predicted to have achieved steady state concentration, from pre-dose (trough) collections, which occur before the next scheduled dose. Steady state is achieved when the concentration of drug in the body is in equilibrium with the rate of dose administered and the rate of elimination (after initiation of therapy the time equivalent to at least five half-lives of the drug is needed for establishment of steady state). A random collection may reflect steady state for some drugs, particularly those with long elimination half-lives. Random collections are also important when evaluating therapeutic failure, toxicity, or overdose. Consideration of inaccuracies and appropriate interpretation of results depends on understanding the pre-analytical circumstances surrounding a request for TDM.

Specimen collection containers can alter TDM results by affecting drug concentration and proportional stability. Failure to separate cells promptly from serum or plasma may lead to in vitro metabolism, as has been observed with fosphenytoin and mycophenolic acid acyl glucuronide [2,3]. Serum versus plasma, variation among tube preservatives, and gel separator tubes are relevant for accurate recovery of certain drugs or fractions of drugs. For example, heparin collection tubes can increase the concentration of free (not bound to proteins) drug concentration by activating lipoprotein lipase and fatty acid concentration, which will displace protein-bound drug from albumin and alpha-1 glycoprotein [4]. Free fraction of phenobarbital, phenytoin, and valproic acid was shown to be elevated in serum versus plasma, while the free fraction of carbamazepine and theophylline was lower in serum versus plasma [5]. The proportion of free valproic acid was found to be time-sensitive, and increased significantly after 96 h storage at ambient temperature, most likely due to degradation of binding proteins. Blood specimens that are exposed to extreme temperatures can lead to plasma protein degradation as well, and increase the free fraction of drugs that are highly bound to protein (e.g., phenytoin, valproic acid). Inappropriate dose adjustments could be made when based on free drug concentrations determined with sub-optimal specimens. In addition, citrate collection tubes were associated with a decrease in the total concentration of valproic acid [6]. More dramatically, gel separator tubes have the potential to decrease total drug recovery through adsorption of the drug to the gel material. Separator tubes were reported to decrease the recovery of cardiac drugs, tricyclic antidepressants, anticonvulsants, and antipsychotics. As such, gel separator tubes should not be used as collection tubes for TDM unless validated to be appropriate [4].

Drug stability can also be influenced by sensitivity to light or heat and may lead to erroneous TDM results if specimens are stored under inappropriate conditions. Amiodarone, methotrexate, chlordiazepoxide, carbamazepine, chlorpromazine, haloperidol, clonazepam and fluoxetine are examples of light sensitive drugs. Rapid in vitro degradation is also recognized for bupropion, busulfan, carbamazepine, lithium and olanzapine. The consequences of in vitro degradation include falsely low results, which could potentially contribute to inappropriate dose adjustments. Specimens containing unstable drugs should be stored at the appropriate temperature (i.e., frozen) to preserve integrity and prevent falsely low results. In addition, the impact of repeat freeze-thaw cycles on drug integrity should be carefully evaluated [4].

TDM results can also be affected by a patient's clinical status and lifestyle factors such as diet, smoking, alcohol use, co-morbidities, pharmacogenetics, and poly-drug therapy. Food and fluid intake can alter the pharmacokinetics (absorption, distribution, metabolism, and elimination) of a drug by impacting gastric pH and emptying time which can affect drug absorption. Drugs that are administered orally are absorbed into the blood stream through the gastrointestinal tract by passive diffusion, if the drug is lipid soluble or non-ionized. Factors that affect intestinal motility, hepatic blood flow, and bile flow will also have an impact on the pharmacokinetics of a drug. For example, protein intake can affect drug-protein binding for drugs and alter drug clearance. In 1987, Fagan et al. demonstrated that

high-protein diets can increase the clearance of propranolol and theophylline [7].

Food-drug interactions will influence drug bioavailability and drug metabolism as well, by inhibiting or inducing drug metabolizing enzymes. For example, grapefruit juice can inhibit the cytochrome P450 isozyme 3A4 (CYP3A4) in the small intestine and increase bioavailability of several drugs [8]. This isozyme, part of the superfamily of CYP enzymes associated with drug metabolism, is involved in metabolism of approximately half of all drugs. Increased drug bioavailability can lead to enhanced drug activity and possible toxicity. Drug classes that are affected by this food-drug interaction include calcium channel blockers (e.g., nifedipine), antiarrhythmic drugs (e.g., amiodarone), benzodiazepines (e.g., diazepam), antiepileptic drugs (e.g., carbamazepine), antibiotics (e.g., erythromycin), antiretrovirals (e.g., indinavir), chemotherapy (e.g., docetaxel), immunosuppressants (e.g., cyclosporine A), and cholesterol lowering drugs (e.g., simvastatin) [9]. A review article by Chen et al. summarizes other juices that can cause food-drug interactions [10]. In addition, cruciferous vegetables like cabbage and cauliflower and charbroil foods can induce metabolism of drugs metabolized by the CYP1A2 isozyme. Substrates of CYP1A2 include theophylline, clozapine, and olanzapine. Alcohol consumption can also induce the CYP2E1 isozyme to increase metabolism of certain drug, in addition to the well-recognized synergistic effect with the pharmacodynamics of depressant drugs [9]. Cigarette smokers tend to require high doses of many therapeutic drugs to obtain optimal therapy, in part due to the fact that nicotine induces isozymes CYP1A1, CYP1A2, and CYP2E1.

Genetic polymorphisms in the genes that code for drug metabolizing enzymes can also affect an individual's ability to biotransform drugs for activation or elimination (pharmacogenetics). Genetic polymorphisms can, for example, cause a patient to be a poor or slow drug metabolizer. However, the overall impact of induction or inhibition of drug metabolizing enzymes, whether due to interacting substances, or genetic predispositions, depends on whether the drug substrate is activated or inactivated by the affected isozyme(s). For drugs that are inactivated by metabolism (e.g. tricyclic antidepressants, warfarin), poor metabolizers are at risk of accumulating drug and may experience toxicity if standard dosing is administered. Rapid metabolizers are at risk of therapeutic failure due to suboptimal dosing and will require higher doses or a different drug to achieve optimal therapy. The opposite is true for drugs that are activated by metabolism (e.g., clopidogrel, codeine). Accurate TDM is an important tool for optimizing dosing under conditions of unpredictable pharmacokinetics, such as pharmacogenetic variants, food-drug, and drug-drug interactions.

Another source of unpredictable pharmacokinetics that requires TDM to optimize dosing comes from patients who are critically ill. Of particular concern are patients with impaired renal, hepatic, gastrointestinal, or cardiovascular function. Renal disease will reduce the glomerular filtration rate and reduce the clearance of drugs that are eliminated via the kidney. Renal disease will also impact drug-protein binding in patients with uremia, due to uremia toxins competing for drug binding sites to albumin, and increase the concentration of non-protein-bound (free) drug and therapeutic effect. Liver toxicity or disease will decrease production of albumin and other proteins that bind to drugs. Hypoalbuminemia will increase the fraction of pharmacologically active drugs and may increase the risk of toxicity. Decreased liver function will reduce first-pass metabolism and also impact expression of CYP450 isozymes in general. Gastrointestinal disease or a history of bariatric surgery, malabsorptive disorders, or intestinal disease may impact absorption of drugs [11]. Cardiovascular disease will decrease cardiac output, tissue perfusion, drug disposition, and absorption. Reduced blood flow to the liver will decrease drug metabolism.

TDM is necessary in pregnant or nursing women as well, to accommodate changes in maternal physiology, and to protect the fetus or infant from toxicity. Pregnant women have increased body fat, total body water, and plasma volume, which will decrease plasma protein concentration and drug-protein binding. Lipophilic drugs will distribute and accumulate in fat and decrease bioavailability. Drugs that are hydrophilic will have a lower volume of distribution in the body and may have increased clearance and decreased bioavailability in patients with excessive body fat content. Cardiac output in pregnant women is increased, resulting in enhanced drug metabolism. A general recommendation is to carefully monitor pregnant or nursing women that require drug therapy to assure efficacy and safety for both mother and her unborn child [12].

SOURCES OF ANALYTICAL INTERFERENCES IN TDM

The vulnerability of an analytical method to interferences is dependent on the sample matrix, the sample preparation method, the assay design, assay components, and detection principle (Fig. 13.1). Sources of analytical interferences may be endogenous or exogenous. Laboratories must consider common sources of interferences, such as hemolysis, during development

TABLE 13.1 Example sites and sources of analytical interferences.

Analytical method	Major vulnerabilities to interference	Example sources of interference
Immunoassay	• Antibodies (species, clone, epitopes) • Assay design (homogeneous vs. heterogeneous, specific steps, timing of reactions, reagents, detection)	• Drug metabolites • Similar drugs in same drug class • Any structurally similar compounds • Heterophilic antibodies • Matrix components
Chromatography	• Sample preparation (extraction, dilution, protein precipitation) • Assay design (chemistry of phases, flow rates, temperature) • Detection technology	• Any co-eluting compounds • Compounds with similar detection characteristics • Matrix components • Carryover
Mass Spectrometry	• Sample preparation • Assay design (SIM, MRM, ions detected) • Instrument (mass resolution, ionization, voltages, data collection parameters, signal/noise)	• Isobaric or cross-talking compounds • Impurities in reagents • Matrix components • Carryover

and validation of analytical methods, and investigate suspected interferences that arise with analytical methods in routine use. Unfortunately, analytical interferences may go unrecognized by laboratories unless TDM results are questioned by clinicians who have carefully evaluated a result in the context of a specific patient scenario. Examples of analytical interference sources affecting common technologies used to support TDM are listed in Table 13.1.

Endogenous interferences (matrix components) known to affect TDM include classical biological interferences such as hemolysis, bilirubinemia, hematocrit, and lipemia. For example, hemolysis is known to compromise lithium quantitation due to a dilution effect. Both hemolysis and bilirubinemia may interfere with spectral detection methods, particularly for automated homogeneous immunoassays [13]. Also, the Tacrolimus II MEIA (microparticle enzyme immunoassays) has been shown to be affected by hematocrit, wherein a tacrolimus result is biased if the blood sample hematocrit falls outside 30−40% [14]. Electrolytes and serum protein affects ion-transfer voltammetry methods, such as in quantification of propranolol, or ion selective electrodes used for lithium quantitation [15]. Heterophilic antibodies, steroids, and other endogenous substances provide interesting case reports for isolated, patient-specific interferences with certain technologies. Examples are provided later in this chapter. It can be stated that biological samples are very complex and essentially all endogenous compounds have the potential to introduce inaccuracies through interferences, depending on the unique characteristics of an individual patient specimen, coupled with the analytical method employed.

Exogenous interferences are introduced from outside the patient, and add another level of sometimes unpredictable complexity. Such interferences may be qualified as direct or indirect. A direct exogenous interference is related to the drug of interest, including drug metabolites, or drug impurities. For example, the methotrexate immunoassay may cross-react to its inactive metabolite DAMPA, and overestimates the concentration of methotrexate in comparison to mass spectrometry results [16]. In addition, a technical note from ARK Diagnostics, Inc states that the Levetiracetam (Keppra) assay is vulnerable to interference from the drug Brivaracetam (Briviact). Patients who are undergoing a switch in drug therapy from levetiracetam to brivaracetam, should not be monitored for levetiracetam by the ARK Diagnostics assay. A mass spectrometry method should be used for drug monitoring to differentiate between the two drugs. Direct interferences can and should be carefully evaluated during assay development and validation. Compounds that interfere with a TDM technology but are unrelated to the specific drug of interest could be qualified as indirect exogenous interferences, and may be less intuitive. Examples of indirect interferences include co-medications, herbal medicines, vitamin and mineral supplements, social drugs (e.g. nicotine, ethanol), components of the diet, or components of the patient's environment (e.g., water and air sources). Indirect interferences may be predicted and characterized during assay development and validation, but are more often recognized and described after an assay is in routine use.

Analytical methods should be designed to detect, minimize or compensate for predictable interferences. Some interfering substances may be minimized through sample preparation methods such as dilution, protein precipitation, liquid/liquid or solid phase extraction. Physical separation techniques (e.g., chromatography, ultrafiltration, and ultracentrifugation) may also help minimize matrix effects. Most automated immunoassays do not incorporate any sample preparation methods and are consequently at higher risk from interferences. However, if the source of a potential interference is defined, and is measureable (e.g., hematocrit), patient specimens could be qualified for testing in advance. Those samples that that exceed established thresholds for a known interference could be

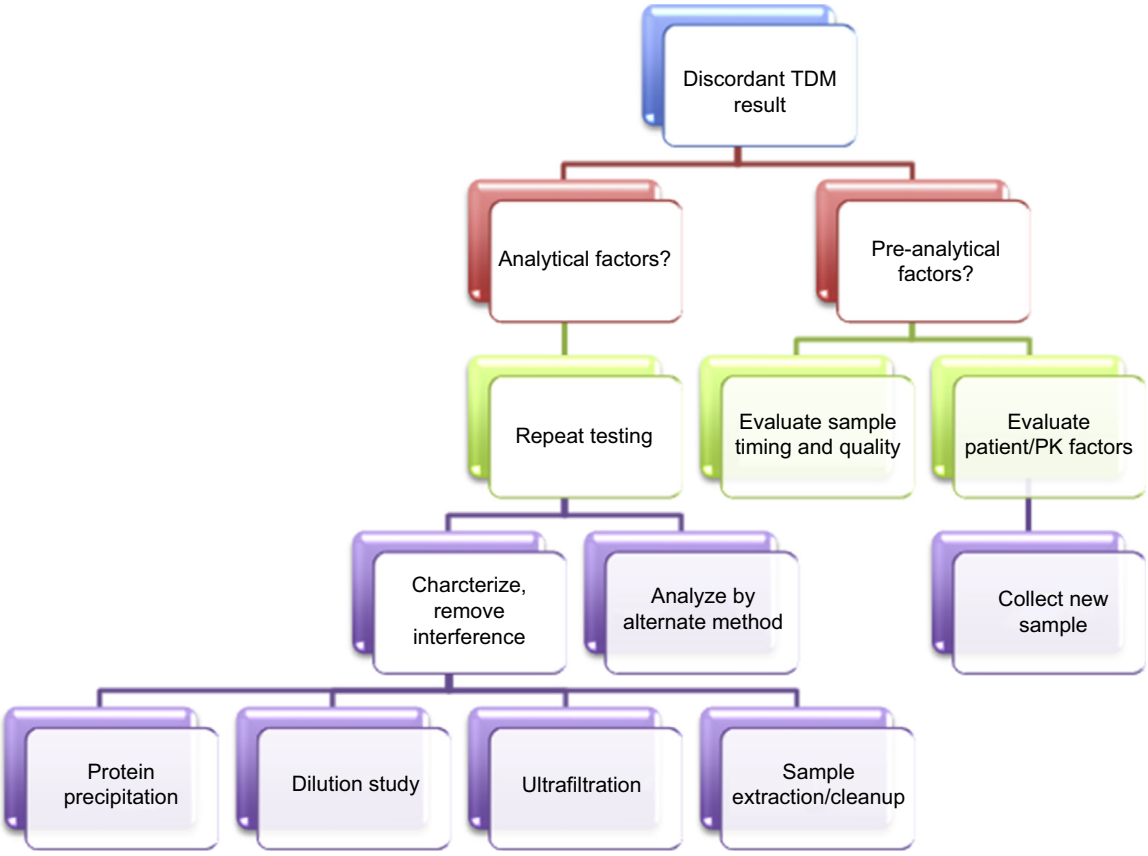


FIG. 13.2 Algorithm for evaluating a discordant TDM result.

disqualified for testing. To avoid inaccuracies in TDM results, alternate methods could be made available for disqualified specimens.

All TDM results should be interpreted within the context of the clinical, pre-analytical, and analytical scenario. Any result that is inconsistent with expectations should be investigated. Fig. 13.2 illustrates a possible algorithm for investigating a discordant TDM result, with intent to guide identification or characterization of the interference. Thus, an investigation should begin by considering pre-analytical and analytical variables. If suspicions surround the pre-analytical components of testing, a new specimen should be collected. If suspicious are aligned better with analytical components of testing, an alternate method should be sought. Repeat testing of the original sample with the original technology is also an important preliminary step for investigating the possibility of an analytical interference. Comparing results between technically-distinct analytical approaches is very informative. Additional tools that may help characterize or resolve interference include dilution studies, to potentially reduce the impact of an interference sufficiently to restore accuracy of results; removing plasma components and washing red-cells for assays based on detection of erythrocyte-bound drug; ultrafiltration or protein precipitation for physically removing large-sized or protein-based interfering substances or sample extraction methods for lipid-based interfering substances. Solid phase extraction, liquid/liquid extraction, or other sample cleanup steps should be aligned with the specimen type and the drug(s) of interest [17,18]. Many other approaches to characterizing, eliminating, and preventing interferences may be appropriate based on the actual source of interference, and the available analytical technology.

MECHANISMS OF ANALYTICAL INTERFERENCES IN TDM

Most well-described analytical interferences that affect TDM are associated with commercially available immunoassays. Among immunoassay technologies, the source of the interference may relate to the antibodies upon which the test is built, or to the assay design. Immunoassays are available in homogeneous and heterogeneous formats, which have unique vulnerabilities to interferences based on timing or various assay steps (e.g., washes, incubations), chemistry

(e.g., pH), temperatures, reactions (e.g., read times), and detection technology (e.g., spectral). All immunoassay formats employ a capture antibody, and are typically designed to detect a single representative chemical structure against which the capture antibody is raised. Sometimes immunoassays employ more than one antibody, and the antibodies may have originated from several different species (e.g., mouse, rabbit). Actual performance characteristics and vulnerabilities to specific sources of analytical interferences vary tremendously among commercial products, based in part, on the antibodies that are unique to each product. In general, immunoassays that utilize polyclonal antibodies are more likely to be associated with analytical interferences than those that utilize monoclonal antibodies, due to the fact that polyclonal antibodies have a larger number of epitopes available for binding drug antigens with similar chemical structures. And, in general, heterogeneous assay formats are less vulnerable to non-specific interferences, than homogeneous assay formats.

For drug classes that contain many structurally similar compounds, cross-reactivity across the drug class inherently compromises specificity for an individual drug [19]. For example, immunoassays designed to detect tricyclic antidepressants generally detect several common tricyclic antidepressant drugs at similar concentrations. Because an acute intoxication with a tricyclic antidepressant is likely to be managed based on the drug class and clinical presentation, rather than based on the individual drug(s) responsible, analytical interference across the class of drugs is desirable and can be useful clinically for drug detection, such as in the Emergency Department, or for a psychiatric clinic that wants to evaluate compliance with therapy. However, an immunoassay for tricyclic antidepressants is generally not useful clinically for TDM due to the fact that other tricyclic structures are known to cross-react with common immunoassays. Such assays are also not useful for supporting TDM or pharmacokinetic evaluations, or whenever concentrations of individual drugs and drug metabolites are clinically relevant. In the case of tricyclic antidepressants, the preferred technology for analysis is liquid chromatography or liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). In any case, review of the cross-reactivity profile for any immunoassay product is required, to evaluate which drugs and other compounds are likely to be detected, and with what degree of sensitivity/specificity. See discussion of tricyclic antidepressants and other specific drug examples below for more information.

Examples of specific immunoassay designs that are associated with analytical interferences known to adversely affect TDM (exemplified later in this chapter) include fluorescent polarization immunoassay (FPIA), enzyme multiplied immunoassay (EMIT), microparticle enzyme immunoassay (MEIA), and chemiluminescence immunoassay (CLIA).

Chromatography and mass spectrometry

Chromatography is long recognized to impart specificity to TDM because of the inherent ability to separate components of the matrix, and hence, to separate drugs and drug metabolites from one another. As such, chromatographic techniques physically separate the sample, and are frequently referred to as physical techniques. Chromatographic methods are typically developed by individual laboratories, and vary tremendously relative to sample preparation methods, chemistry of related solid (e.g., column) and mobile (e.g., gas or liquid) phases, flow rates, temperatures, instrument platforms and associated capabilities, detection technologies, methods of data collection, and extent of data analysis. Chromatographic methods are extremely vulnerable to interferences from co-eluting compounds that may or may not be structurally similar to the compounds of interest. The relevance of a co-eluting substance depends on whether that substance interferes with the analyte signal that is generated, which is based very much on the signal to noise ratio, and on the detection method. Common detection methods utilized include spectral (e.g., UV, fluorescent), electrochemical, and mass spectrometry. Despite possible limitations, chromatographic methods, particularly coupled with mass spectrometric detection, are recognized to resolve many of the interferences that are encountered with immunoassay methods, and are generally regarded as gold standard platforms for TDM.

Mass spectrometry is a highly selective detection technology that detects mass to charge ratios of ionized molecules and fragments. This detection technology is frequently coupled to gas or liquid chromatography. Output of data can be quantitative, qualitative for targeted masses, be compared to a library of data to identify a particular compound, or can be used to determine element composition and structure. Assays can be designed using selected ion monitoring (SIM), which is a mode that scans a limited mass to charge range, multiple reaction monitoring (MRM), which an ion mass is selected in the first mass spectrometer and the fragment ion mass is selected in the second mass spectrometer, or full mass spectrum, which scans a wide mass to charge ratio range.

Although mass spectrometric methods are very specific, the technology is not exempt from interferences. Ion suppression or enhancement, which alters ion intensity/abundances, and signal to noise ratios, have become well recognized in mass spectrometric methods, but may go unrecognized when isolated to a single patient

specimen. For example, ion suppression can occur in the presence of compounds that are less volatile than the analyte of interest, through affecting analyte ionization. Salts, anions, ion-pairing agents (e.g., trifluoroacetic acid), drugs/metabolites, uncharacterized sample matrix components and co-eluting compounds, and reagent impurities are known to produce ion suppression or enhancement. In electrospray ionization, the primary source of ion suppression is due to changes in spray droplet formation in the presence of non-volatile compounds [20,21]. Another source of interference in mass spectrometry that may be overlooked includes compounds that are isobaric or isometric to the analyte of interest. An example of isobaric interferences was observed with endogenous nucleosides and ribavirin, in an LC-MS/MS method [22]. Plasticizers, reagents, lipids, and phospholipids in particular, are well recognized to introduce matrix effects, manifested as either suppression or enhancement of analytical response [23]. Consequently, internal standards, such as stable isotope-labeled compounds, are used to minimize the effects of ion suppression and improve the analytical accuracy of mass spectrometry [24]. Lastly, cross-talk can cause interference when different parent ions fragment and have identical product ion masses [25].

SPECIFIC EXAMPLES OF INTERFERENCES THAT AFFECT TDM

In this section specific examples are given regarding interferences affecting measurement of various therapeutic drugs.

Interferences in digoxin Measurement

Digoxin (Lanoxin) is a cardiac glycoside with positive inotropic effects, used to treat cardiac arrhythmias and congestive heart failure. Effects are dose-related, and TDM is required because the clinical symptoms of inadequate and excessive dose are similar. The traditional therapeutic range for digoxin, 0.5–2.0 ng/mL, is narrow among drugs that require TDM. It is also noteworthy that the upper limit of the therapeutic range overlaps somewhat with the threshold for toxicity. In congestive heart failure, an even more narrow range has been proposed (0.5–0.8 ng/mL), and patient outcomes begin to decline at concentrations greater than 0.9 ng/mL. The clinical expectations for maintaining a very narrow circulating concentration of digoxin raises questions about whether analytical techniques available for TDM of digoxin can perform with adequate accuracy and precision [26]. A very narrow therapeutic range also demands that pre-analytical variables be recognized and managed.

Pre-analytical variables

Digoxin has a high volume of distribution and requires several hours to distribute, after dosing. A common cause of an unexpectedly high digoxin is therefore based on inappropriate timing of specimen collection. Blood for digoxin analysis should be collected at least 8 h after a dose is administered.

Analytical variables

Both positive and negative interferences have been described for immunoassays designed for TDM of digoxin. It is usually assumed that negative interference is most dangerous to the patient management because it may contribute to inappropriate dose increases. Sources of analytical interferences that are recognized include digoxin metabolites, endogenous digoxin-like immunoreactive factor or substance (DLIF or DLIS), and exogenous drugs, including anti-digoxin Fab fragments used to treat digoxin overdose, aldosterone antagonists, other cardiac glycosides, and several herbal medicines. Some forms of interference can be effectively removed through physical separation, accomplished by ultrafiltration or chromatography. Analysis of ultrafiltrate, chromatographically distinct fractions, or direct analysis of independent specimen components such as via mass spectrometric detection, further reduces the potential for analytical interferences [27].

Tables 13.2 and 13.3 summarize examples of interferences described for common immunoassay technologies, in the absence or presence of digoxin, respectively. The type of interference described (positive, negative, or none) for FPIA, EMIT, MEIA-II and CLIA is indicated, along with whether or not ultrafiltration will resolve the interference. The data presented in these tables is intended for example purposes, and is inherently simplified. For example, the tables do not reflect that the presence and magnitude of interferences is closely related to the concentration of interfering substances and/or digoxin, and that specific reagents that utilize these technologies may differ. The package insert for a specific assay of interest should be consulted, and independent validations studies should be performed as needed, to fully characterize any interference, and its potential impact to a unique clinical setting. Each major source of interferences is described in more detail below.

Digoxin metabolites

Digoxin is extensively metabolized. Cross-reactivity of the metabolites with antibodies used to develop digoxin immunoassays was historically a source of analytical interference, such as with radioimmunoassays. In particular, the hydrolysis metabolites, digoxigenin, digoxigenin monodigitoxoside, and digoxigenin

TABLE 13.2 Interferences with digoxin immunoassays in the absence of digoxin.

Interferent	FPIA	EMIT	MEIA-II	CLIA	Resolved by ultrafiltration
DLIF	+	+			Yes
DRUGS					
Digitoxin	+	+	+	+	Partial
Anti-digoxin Fab		+	+	+	Yes
Aldosterone antagonists	+	+	+	+	No
HERBALS					
Chan Su	+	+	+		Partial
Danshen	+				Yes

TABLE 13.3 Interferences with digoxin immunoassays in the presence of digoxin.

Interferent	FPIA	EMIT	MEIA-II	CLIA	Resolved by ultrafiltration
DLIF	+	+	−		Yes
DRUGS					
Digitoxin	+		+/-		Partial
Anti-digoxin Fab		+/-	+/-	+/-	Yes
Aldosterone antagonists	+	+	−	+	Partial
HERBALS					
Chan Su	+	+	−		Partial
Danshen	+		−		Yes

bisdigitoxoside exhibited near equal cross-reactivity with digoxin in some assays. Because these metabolites exhibit physiological activity, it was proposed that this cross-reactivity could represent a total 'bioactive' digoxin concentration [28,29]. Newer assays exhibit little cross-reactivity to metabolites, suggesting that current assays are not subject to the same cross-reactivity. The concentration of active metabolites is also not thought to be sufficiently high to contribute significantly to the efficacy or toxicity of digoxin, in most patients. Nonetheless, awareness of cross-reactivity to digoxin metabolites may be relevant for application of immunoassay testing to patients that exhibit poor elimination kinetics, who may be at risk for accumulation of clinically relevant drug metabolites. Because metabolites do accumulate in urine, the analytical method selected for testing urine for digoxin should be evaluated carefully.

DLIF (Digoxin like immunoreactive factors)

The chemical structure of digoxin is steroid-like, and therefore exhibits high potential for endogenous counterparts. Indeed, a source of endogenous interferences in digoxin assays has been recognized in select populations, in the absence of digoxin exposure or administration. This steroid-like substance was called digoxin-like immunoreactive factor (DLIF), and digoxin-like immunoreactive substance (DLIS) in the mid 1980's, and was shown to cross-react with digoxin immunoassays. DLIF has been recognized most for patients with conditions of volume expansion. Vulnerable populations include infants and young children, pregnant women, patients with impaired renal or hepatic function, and persons that have exercised vigorously. It has also been reported to be elevated immediately after a myocardial infarction, and in the critically ill [28].

It is now thought that there are at least two classes of DLIF that may or may not cross-react with digoxin immunoassays, and exert activity at endogenous sodium-potassium ATPase [30]. The degree and type of interference may change in the presence of digoxin. In the absence of digoxin, clinically significant concentrations of apparent digoxin (no actual digoxin present) have been observed in samples collected from newborns and pregnant women, and analyzed by FPIA [31]. In the presence of digoxin, DLIF may lead to positive or negative interference, based on the specific analytical technology. Ultrafiltration or chromatographic separation remains the best means of resolving this type of

interference, when suspected. Due to the extensive characterization of DLIF, and recent improvements in immunoassay design, currently available immunoassays may exhibit minimal or no clinically significant interference from DLIF [32,33].

Anti-digoxin immune fragments

DIGIBIND and DigiFab are examples of pharmaceutical immune (Fab) products that bind digoxin with high affinity, and are used as antidotes, to treat digoxin overdose. These Fab products are responsible for variable amounts of analytical interference with digoxin assays, both in the absence and presence of digoxin. Technologies, such as CLIA, MEIA and EMIT, demonstrate concentration-dependent positive interference in the absence of digoxin, and concentration-dependent negative interference observed in the presence of digoxin, to the point of molar equivalency (neutralization). As might be expected, the presence of digoxin under conditions of Fab excess, results in positive interference [34].

Patients undergoing Fab-based decontamination treatment can be monitored by measuring free digoxin in ultrafiltrate, or by incorporating a pre-analytical protein-precipitation step to measure total digoxin (e.g., FPIA). Monitoring free digoxin concentrations in plasma ultrafiltrate is the most accurate and accepted approach. It is important to employ a method of analysis such as FPIA or MEIA that is free of matrix effects because ultrafiltrate exhibits different chemical/physical properties than plasma or serum. Ultrafiltrate is not an appropriate specimen for methods that depend on sample fluid dynamics, such as slide technologies (e.g., Vitros) [35].

Cardiac glycosides

The digitalis family is comprised of several cardiac glycosides with similar chemical structures, including the once popular drug digitoxin. Although not currently used in the US, digitoxin is used in European, Scandinavian, and other countries. While it would not be expected that digoxin would be co-administered with digitoxin, a patient may be transitioned from one drug to another, or may be using undisclosed medication obtained outside the primary clinic. The therapeutic dose and corresponding therapeutic concentration range of digitoxin is significantly higher (10−15 fold) than that of digoxin. Due to the higher therapeutic range, and structural similarity, even low cross-reactivity with a digoxin immunoassay could produce clinically significant interference. Only the CLIA digoxin assay has sufficiently low cross-reactivity with digitoxin to avoid analytical interferences. FPIA assays are particularly vulnerable to positive interference from digitoxin, in the absence of digoxin. MEIA assays are moderately susceptible to positive interference from digitoxin, in the absence of digoxin. Of note, negative interference is observed with MEIA when digoxin and digitoxin are present. Other cardiac glycosides, including oleandrin, used in folk medicines may exhibit the same patterns of positive interference in the absence of digoxin, and either positive or negative interference in the presence of digoxin. Interferences from non-digoxin cardiac glycosides may be at least partially resolved by measuring free digoxin in ultrafiltrate [36,37].

Aldosterone antagonists

Spironolactone and potassium canrenoate are used to treat hypertension and congestive heart failure, and are often co-prescribed with digoxin. These co-medications, along with their common active metabolite canrenone, are structurally similar to digoxin and have been described to produce positive interference in FPIA and CLIA digoxin assays. Negative interference has also been described for MEIA digoxin assays [38]. Newer generation MEIA III assays that employ monoclonal antibodies have improved resistance to the negative analytical interferences described. However, clinically significant positive analytical interference has been described for the MEIA-III assay in patients co-medicated with higher dose spironolactone (e.g., 100 mg/day). Similar positive interferences are described with EMIT digoxin assays as well. The impact of the analytical interferences on dosing can be significant, easily exceeding the therapeutic range of digoxin. One study demonstrated clinically significant differences in approximately 45% of patients co-medicated with digoxin and either spironolactone or canrenoate. Such interferences were not eliminated by measurement of ultrafiltrate [39]. As such, digoxin monitoring for patients co-medicated with aldosterone antagonists, is best pursued with chromatographic techniques.

Herbal medicines

Herbal medicines are a cornerstone of traditional Eastern medicine and lifestyle, and are readily available without prescription. These medicines are not well controlled in terms of potency or purity, and may not be reported by patients who use them. Several case examples and in vitro studies have demonstrated that select herbal medicines produce bi-directional analytical interferences in digoxin immunoassays. For example, positive and negative interferences have been described extensively for Chan Su and Danshen. Thus, Chan Su produced concentration-dependent positive interference in FPIA, EMIT, and MEIA digoxin immunoassays in the absence of digoxin. In the presence of digoxin, Chan Su produced positive interference in FPIA and EMIT, and negative interference in MEIA. Chan Su did not affect CLIA with or without digoxin present. Interferences with Chan Su were only partially resolved

by analysis of ultrafiltrate. Positive interference from Danshen has been reported in the absence of digoxin, when analyzed by FPIA, but not by EMIT, MEIA, or CLIA. As with Chan Su, positive interference has been observed with FPIA and EMIT, and negative interference with MEIA, when Danshen is found in the presence of digoxin; no effect was observed with CLIA. Danshen interferences were largely resolved by analysis of ultrafiltrate [40–42]. High concentrations of biotin have also produced positive interferences in digoxin immunoassays [43].

Case example [44]

A 19-year-old Taiwanese female presented to an emergency department with nausea, vomiting, dizziness, weakness, and numbness in the mouth. Noteworthy is her admission to having ingested cooked toad eggs approximately 1 h prior. Vitals and neurological exam were normal, but an electrocardiogram revealed a first-degree atrioventricular block. Serum digoxin concentration was measured by immunoassay. Despite no history of digoxin therapy, the concentration was 1.9 ng/mL; potassium concentration was 7.1 mmol/L. The patient was administered anti-digoxin Fab and admitted to the intensive care unit. Both the apparent digoxin concentration and potassium concentrations declined and the patient were released a few days later. The most likely cause of the digoxin cross-reactivity is that the toad eggs ingested contained ChanSu, a traditional Chinese aphrodisiac commonly made from toad venom.

INTERFERENCES IN CARBAMAZEPINE MEASUREMENT

Carbamazepine (Tegretol, Carbatrol) is a tricyclic anticonvulsant drug used for the treatment of partial and tonic-clonic seizures, trigeminal neuralgia, and manic-depression. The therapeutic range for carbamazepine is 4–12 μg/mL, and signs of toxicity, such as stupor, seizures, and respiratory depression may occur at concentrations exceeding 15 μg/mL. Carbamazepine is metabolized primarily by cytochrome P450 isozyme (CYP) 3A4, to the active metabolite, carbamazepine-10,11-epoxide. The 10,11-epoxide metabolite has similar activity as the parent drug, and may contribute significantly to efficacy of the drug in populations such as children, that accumulate the metabolite. The concentration of the metabolite may be higher than the parent drug concentrations in situations of carbamazepine overdose and in patients with renal failure (uremia); therefore, monitoring drug ratios of parent and metabolite may be useful for evaluating compliance and drug-drug interactions.

Pre-analytical variables

Drug-drug interactions are common with carbamazepine, due to relatively high protein binding (65–80%), and involvement of drug metabolizing enzymes, including CYP2C19 and CYP3A4. Carbamazepine is primarily eliminate via the kidneys, thus renal failure or insufficiency can lead to elevated results and increase the risk for toxicity.

Analytical variables

Major sources of analytical interference for carbamazepine include cross-reactivity from the 10,11-epoxide metabolite, and structurally similar drugs. Cross-reactivity of carbamazepine and its metabolite can vary in commercial immunoassays, from 0% for the Vitros assay to ~93.6% on the Dade Dimension. Other assays, such as MEIA (AxSYM) have moderate cross-reactivity (22%) [45]. This cross-reactivity can be utilized to calculate the total amount of epoxide present, but chromatographic methods are preferred [46]. Analytical interferences with carbamazepine immunoassays have been described for drugs that are structurally similar to carbamazepine, such as oxcarbazepine, which produce positive interference in carbamazepine analysis by EMIT [47]. Cross-reactivity with carbamazepine analogues that are currently in development, such as eslicarbazepine, is not yet characterized. Because this new drug is a purified isomer, it is important to recognize that non-chiral chromatographic assays would not be able to distinguish between use of eslicarbazepine and use of racemic licarbazepine. Non-carbamazepine analogues may also cross-react with carbamazepine assays. For example, Parant et al. [48] reported that the antihistamine drugs hydroxyzine and its metabolite, cetirizine, produced a false positive result for carbamazepine, using the particle-enhanced turbidimetric inhibition immunoassay (PETINIA), but not with EMIT, or turbidimetry (ADVIA Centaur) [48,49].

INTERFERENCES IN PHENYTOIN MEASUREMENT

Phenytoin (Dilantin) is administered to manage generalized tonic-clonic seizures, partial or complex-partial seizures, and status epilepticus. There is a good correlation between plasma concentration of phenytoin and its clinical effect, and a well-accepted therapeutic range is 10–20 μg/mL. Fosphenytoin (Cerebyx) is a prodrug that is rapidly converted by hydrolysis, to phenytoin. Total plasma phenytoin concentrations >20 μg/mL do not enhance seizure control and are associated with adverse effects such as nystagmus, ataxia,

psychosis, hyperglycemia, and hematological disorders; concentrations >35 μg/mL have been shown to precipitate seizure activity.

Pre-analytical variables

Drug-drug interactions are common with phenytoin, due to high protein binding (>90%), and involvement of drug metabolizing enzymes, including CYP2C9 and CYP2C19. Phenytoin is primarily eliminate via the kidneys, thus renal failure or insufficiency can lead to elevated results and increase the risk for toxicity due to accumulation of the parent drug. Samples collected after administration of fosphenytoin may lead to falsely elevated results due to in vitro metabolism of fosphenytoin, particularly in patients with elevated alkaline phosphatase activity [2].

Analytical variables

A major source of analytical interference for phenytoin is the primary metabolite, 5-(p-hydroxyphenyl)-5-phenylhydantoin (HPPH). Several phenytoin immunoassays cross-react with the metabolite HPPH, leading to falsely elevated results [50,51]. The glucuronide form of HPPH is eliminated in urine. Analytical interference due to HPPH is of particular concern for uremic patients, as well as any patients that have poor renal function and may thereby accumulate the metabolite [52].

Other sources of analytical interference reported for phenytoin include the nonsteroidal anti-inflammatory drug, oxaprozin (Daypro) which can exhibit cross-reactivity with TDx phenytoin assays to produce a falsely elevated result [53,54]. Fosphenytoin cross-reacts directly with several immunoassay formats, including FPIA, EMIT, and CLIA, to produce falsely elevated results. Of interest, the extent of cross-reactivity increases in the presence of phenytoin for FPIA (TDx), but is independent of phenytoin concentration for the CLIA (ACS:180) [55]. False negative results are also possible, as demonstrated by Brauchli et al., in a patient with monoclonal IgM lambda, and testing performed by an automated particle-enhanced turbidimetric inhibition immunoassay (see case example) [56].

Case example [56]

A 73-year-old female was admitted to a hospital due to confusion and recurrent convulsive facial contractions. The convulsions increased and extended to the right side of her body. An EEG identified epileptic zones in the occipital lobe, and treatment by phenytoin infusion was initiated. Despite consistent daily dosing, total serum phenytoin concentration was undetectable (<0.4 ng/mL) by the particle enhanced turbidimetric inhibition immunoassay (PETINIA: Dimension Analyzer, Siemens Diagnostics), in multiple samples. Other medications administered to the patient were detected in the same samples, eliminating the possibility of sample mixup. The possibility of drug-drug interactions was also eliminated. Because of the unexpected result, testing by alternate methods was pursued. Phenytoin was detected and concentrations were reasonable, when analysis was performed by FPIA or by chromatographic methods. Testing with the PETINIA was repeated, after a protein precipitation step, and phenytoin was detected as expected. It therefore was hypothesized that the false negative results were due to an interfering protein. A monoclonal IgMλ was detected in the patient serum by immunofixation that is likely to have been responsible for the analytical interference.

INTERFERENCES IN MEASUREMENT OF IMMUNOSUPPRESSANTS

Immunosuppressant drugs (cyclosporine A, tacrolimus, sirolimus, everolimus, mycophenolate mofetil) function to suppress the immune system by inhibiting T-cell activation and proliferation. Immunosuppressant drugs are used to treat autoimmune disease, allergies, multiple myeloma, chronic nephritis, and prevent rejection after organ transplantation. TDM for immunosuppressant drugs is routinely performed and dose adjustments made based on results, in the transplant setting. Blood concentrations below the therapeutic range can lead to under-dosing, which can put a patient at risk for graft rejection because the immune system is not suppressed. Blood concentrations that exceed the therapeutic range can lead to over-suppression of the immune system and increase the risk of opportunistic infections, malignancy, and organ toxicity.

Cyclosporine (Sandimmune, Neoral) and tacrolimus (Prograf) are both calcineurin inhibitors and are serious adverse effects such as renal dysfunction and toxicity, hypertension, and hyperlipidemia with excess exposure. The general therapeutic range is 100–400 ng/mL. The therapeutic range for tacrolimus is 5–20 ng/mL. Sirolimus (Rapamune) and everolimus (Zortress, Afinitor) are inhibitors the mammalian target of rapamycin (mTOR) and function to inhibit T-lymphocyte activation and proliferation and are associated with serious adverse effects such as anemia, leukopenia, thrombocytopenia, hyperlipidemia, and GI effects. The therapeutic range for sirolimus is 12–20 ng/mL and 3–8 ng/mL for everolimus. Mycophenolate mofetil (CellCept) is a prodrug that is rapidly metabolized to mycophenolic acid (MPA) in the liver. MPA is a reversible and

noncompetitive inhibitor of inosine monophosphate dehydrogenase (IMPDH), which is important for guanine nucleotide synthesis for T-cell proliferation. MPA is associated with adverse effects such as leukopenia, diarrhea, and vomiting. The therapeutic range for pre-dose MPA is 1.0–3.5 mg/L when combined with cyclosporine.

Pre-analytical variables

Cyclosporine, tacrolimus, sirolimus, and everolimus are highly bound to red blood cells and proteins; therefore, TDM is best performed with whole blood. MPA is not bound to red blood cells but highly bound to plasma proteins; therefore, TDM is performed with plasma. Changes in hematocrit may affect accuracy of whole blood testing results.

Bioavailability is extremely variable with most immunosuppressant drugs. These drugs are extensively metabolized, and eliminated primarily in the urine. As such, patients with impaired gastrointestinal, hepatic or renal function are subject to unpredictable pharmacokinetics. For example, liver transplant patients with hyperbilirubinemia will accumulate tacrolimus metabolites leading to a high positive bias in tacrolimus results by immunoassay [13]. Drug-drug interactions that lead to elevated or reduced concentrations of immunosuppressant drugs are also a significant concern.

Analytical variables

Immunosuppressant drugs can be monitored by immunoassays and chromatographic methods (HPLC, LC-MS/MS). LC-MS/MS methods are now commonly used to create multi-analyte panels and to increase specificity, as compared to immunoassay methods [57]. These methods are not free of interferences, and should be evaluated with the same scrutiny as an immunoassay or other analytical method [23]. Nonetheless, immunoassays remain a convenient means of testing, particularly in a hospital setting. Sources of analytical interferences for immunoassays include drug metabolites, and endogenous factors such as heterophilic antibodies and hematocrit. In addition, the structural similarity between sirolimus and everolimus leads to near-equivalent cross-reactivity with sirolimus immunoassays. Everolimus can be monitored by sirolimus immunoassay, but if a patient is co-medicated with both drugs, distinguishing everolimus and sirolimus requires a chromatographic approach [58–60]. Functional assays designed to monitor and optimize immunosuppressant therapy are available as well, particularly for MPA, but are not discussed here [61,62]. See Table 13.4 for a summary of drugs and drug metabolites that are reported to interfere with immunoassays for immunosuppressant drugs.

TABLE 13.4 Drugs and drug metabolites that may interfere with immunoassays for immunosuppressant drugs.

Drug	Metabolites	Drugs
Cyclosporine (Sandimmune)	31 (inactive) Hydroxylated-CYA (10% activity)	Amikacin Amphotericin B Azathioprine Carbamazepine Chloramphenicol Cimetidine Digoxin Disopyramide Erythromycin Furosemide Gentamicin Kanamycin A Lidocaine Mycophenolic Acid Phenobarbital Phenytoin Prazosin Prednisone Quinidine Rifampin Tobramycin Verapamil
Tacrolimus (Prograf)	8 (inactive) 31-O-desmethyltacrolimus (active)	
Sirolimus (Rapamune)	Hydroxy-sirolimus (unknown activity) Desmethyl-sirolimus (unknown activity) Sirolimus (ring-open form) (unknown activity)	
Everolimus (Zortress, Affinitor)	Hydroxy-everolimus (unknown activity) Dihydroxy-everolimus (unknown activity) Desmethyl-everolimus (unknown activity) Everolimus (ring-open form) (unknown activity)	
Mycophenolate mofetil (CellCept, Myfortic)	7-O-Glucuronide (MPAG) – (inactive) Acyl-Glucuronide (AcMPAG) – (active)	

Drug metabolites

There are 31 metabolites of cyclosporine, and one of the major metabolites has approximately 10% of the immunosuppressive activity of the parent compound, while the rest of the metabolites are inactive. Tacrolimus has one active metabolite, 31-O-desmethyl tacrolimus, and eight metabolites that are inactive. Sirolimus has seven inactive metabolites and everolimus has at least 20 inactive metabolites. The primary metabolite of MPA is MPA-glucuronide (MPAG) which is pharmacologically inactive.

Several immunoassay methods are known to cross react with inactive metabolites to produce results that are 20–60% higher than those obtained by chromatographic techniques such as HPLC or LC-MS/MS [63–65]. Immunoassay methodologies which utilize polyclonal antibodies exhibit the highest degree of positive bias, as compared to immunoassays based on monoclonal antibodies; however, LC-MS/MS is the preferred technology. For MPA, immunoassay results may be overestimated by cross-reactivity with the MPAG metabolite, as well as the prodrug mycophenolate mofetil [66,67].

Endogenous interfering substances

Heterophilic antibodies may interfere with immunoassays of any sort, and can be identified with various dilution and pre-treatment methods. Falsely elevated tacrolimus and cyclosporine results were reported in patients receiving these drugs (see case example), most likely due to endogenous anti-ß-galactosidase antibodies [68]. Hematocrit values have been shown to correlate with response in an MEIA assay for tacrolimus as well. Specifically, low hematocrit values are associated with overestimated tacrolimus concentrations by MEIA; the overestimation can be accounted for by correcting for hematocrit [14].

Case example [68]

A 53-year-old male who received a kidney transplant, was treated with tacrolimus and corticosteroids. Tacrolimus was monitored using the antibody conjugated magnetic immunoassay (ACMIA)-tacrolimus (Siemens Dimension). The tacrolimus results obtained for the renal transplant patient were consistent with expectations for the first three weeks post-transplant (<12 ng/mL). An unexpected tacrolimus result of 21.5 ng/mL was then observed, and dosing was suspended for a few days. During the period wherein tacrolimus was discontinued, blood tacrolimus concentrations persisted at >10 ng/mL. When tacrolimus was reintroduced, tacrolimus was measured by MEIA-tacrolimus (IMx), and results were consistent with clinical expectations. For comparison, samples were analyzed in parallel with antibody conjugated magnetic immunoassay (ACMIA on Dimension analyzer: Siemens Diagnostics) and results were approximately twice the MEIA concentrations. In the automated ACMIA-tacrolimus method (Dimension), whole blood is lysed and mixed with anti-tacrolimus-ß-galactosidase antibody conjugate, and magnetic particles to which unlabeled tacrolimus is bound. Tacrolimus in the patient blood competes with the tacrolimus bound to particles, for binding with the conjugate. The magnetic particles are separated from the reaction mixture and the tacrolimus-bound conjugate is detected, in the presence of enzyme substrate. In the case example, a dilution study was performed, that showed nonlinearity. In addition, the interference was resolved completely by treatment with polyethylene glycol, and by testing washed erythrocytes, suggesting that the interferent was a plasma component. Positive interference was observed with ACMIA-cyclosporine as well, despite the fact that the patient never received cyclosporine, which further suggests that the cause of the interference was endogenous anti-ß-galactosidase antibodies.

INTERFERENCES IN MEASUREMENT OF ANTIDEPRESSANTS AND MOOD STABILIZERS

In this section issues of interferences in measuring tricyclic antidepressants and mood stabilizing drug lithium are addressed.

Tricyclic antidepressants

Tricyclic antidepressants (TCAs), such as amitriptyline (Elavil), clomipramine (Anafranil), doxepin (Sinequan, Prudoxin, Zonalon, Silenor), imipramine (Tofranil), trimipramine (Surmontil), nortriptyline (Pamelor), and desipramine (Norpramin), are named for their three ring structure and used to treat various forms of depression, anxiety disorders, eating disorders, attention deficit hyperactivity disorder, enuresis in children, and chronic and neuropathic pain. Plasma concentrations of TCAs have a positive correlation with clinical improvement and toxicity. Serum TCA concentrations greater than 500 ng/mL can produce adverse anticholinergic symptoms, which include dry mouth, sedation, blurred vision, fever, urinary retention, agitation, confusion, and seizures. TCA toxicity from overdose, can be life threatening and involve cardiovascular complications, such as hypotension, tachycardia, and cardiac arrhythmias. Serum concentrations greater than 1000 ng/mL can be fatal. Chronically administered TCAs can contribute to cardiac toxicity by accumulating in cardiac tissues [15,69].

Pre-analytical variables

Several TCAs have active metabolites that may be important to identify and quantitate, as their concentrations contribute to its therapeutic efficacy. Some active metabolites are available as independent drugs. For example, nortriptyline is the active metabolite for amitriptyline and desipramine is the active metabolite for imipramine. Drug-drug or drug-herb interactions involving cytochrome P450 isozymes such as CYP2D6 and CYP2C19 commonly contribute to accumulation of active drug or drug metabolites that may lead to concentration-related toxicity [15,70].

Analytical variables

Immunoassays designed to detect TCAs are prone to interferences from other TCA drugs and drug metabolites, as well as structurally similar compounds from different drug classes. See Table 13.5 for a list of drugs that have been reported to interfere with immunoassays designed to detect tricyclic antidepressants. For example, cyclobenzaprine, a skeletal muscle relaxant, has a similar structure to TCA and can cause a false

TABLE 13.5 Drug analytes that may interfere with tricyclic antidepressant immunoassays.

Drug analytes
Carbamazepine
Carbamazepine-10,11-Epoxide
Cetirizine
Chlorpromazine
Cyclobenzaprine
Fluphenazine
Hydroxyzine
Maprotiline
Oxcarbazepine
Perphenazine
Phencyclidine
Prochlorperazine
Quetiapine Fumarate
Thioridazine

positive result for TCA by immunoassay due to antibody cross-reactivity [71]. Phenothiazine antipsychotics drugs, like thioridazine, have 3-ring structures and can also cross-react with TCA antibodies and produce a false positive results. There are other three-ring structured compounds that can produce false positive TCA results via cross reactivity with TCA immunoassays, which include phenothiazine antipsychotic drugs such as thioridazine [72], antiepileptic drugs such as carbamazepine and oxcarbazepine [73]; antihistamines such as cetirizine, hydroxyzine, and diphenhydramine [74]; and the antipsychotic drug quetiapine [75].

HPLC methods are well established for TCA testing; however this methodology is prone to interferences in peak separation and retention time, which can occur in patients on multidrug therapy. Thioridazine, an antipsychotic drug for schizophrenia therapy, can interfere with imipramine metabolism and analysis by HPLC [72]. Mass spectrometric methods provide high specificity and minimize interferences, and thus are considered the method of choice for identification, differentiation, and quantitation of tricyclic antidepressant drugs [76].

Case example [77]

A 17-year-old female with a history of bulimia was admitted to an emergency department after unknown drug ingestion. The patient was drowsy with dilated, reactive pupils, slurred speech, and ataxia. Electrolytes and liver function test results were normal. Urine toxicology testing was negative, but a serum toxicology screen was positive for TCAs by FPIA. An electrocardiograph was pursued that revealed only sinus tachycardia. Targeted chromatographic testing failed to identify any specific TCA, and the patient's condition improved rapidly. Subsequent testing revealed carbamazepine at 18.6 μg/mL in a blood sample collected at approximately 12 h post ingestion, which fell to 10 μg/mL in a sample collected at approximately 18 h post ingestion, but no TCAs, demonstrating that the initial screening result was a false positive.

Lithium

Lithium carbonate (Eskalith, Lithane) is a monovalent cation that is used as a mood-stabilizing agent for treatment of bipolar disorder, acute manic episode, and depression. Lithium TDM is recommended due to its narrow therapeutic range (0.6–1.2 mmol/L), unpredictable serum concentrations, and concentration-dependent toxicity. Concentrations of lithium, >1.5 mmol/L are associated with lethargy, muscle weakness, and tremors, and speech difficulties. Lithium concentrations >2.5 mmol/L can produce muscle rigidity, mental confusion, seizures, coma, cardiac arrhythmias, and death [15].

Pre-analytical variables

Patient specimens should not be collected in lithium-heparin tubes because the additive can increase the concentration of lithium in patient samples, causing a falsely elevated result. For this reason, serum is preferred over plasma. In addition, specimens that are grossly hemolyzed may cause a dilution effect on lithium concentrations, and should not be used for lithium analysis. Co-administration of diuretics and non-steroidal anti-inflammatory agents can cause drug-drug interactions and affect lithium concentrations in serum by altering lithium excretion. Lithium is excreted primarily in urine, where it is actively absorbed by the kidneys, and hence, serum concentrations of lithium are affected by the glomerular filtration rate [15].

Analytical variables

Lithium is most frequently measured by ion-selective electrodes (ISE). Automated colorimetric, photometric, and enzymatic methods, as well as elemental analysis using flame atomic absorption spectrometry (FAAS), flame atomic emission spectrophotometry (FAES), or inductively-coupled plasma mass spectrometry methods (ICP-MS) are also available and could be used to resolve a suspected interference with ISE [78]. Sources of analytical interferences are based on the technology. For ISE, sources of interference include ions, originating from other drugs and elements. Drugs, like quinidine, procainamide and its metabolite, N-acetylprocainamide (NAPA), can produce a positive interference with lithium results by ISE, particularly with the Beckman analyzer. The interference from the three drugs is additive. Quinidine can also produce a negative bias in lithium results by colorimetric methods, and valproic acid can produce a positive bias in lithium

results by the same method. A positive bias in lithium results is also caused by drug interferences from the cardiac drug, lidocaine, and the anticonvulsant drug, carbamazepine by ISE. Calcium concentrations, greater than 8.9 mmol/L, can cause a positive bias interference on ISE methods, and a negative bias interference by colorimetric methods. Potassium and sodium can also produce a negative and positive bias interference, by colorimetry, respectively [79].

CONCLUSIONS

In conclusion, TDM is a critical tool for optimization of drug therapy. Accuracy of laboratory testing to support TDM depends on a deliberate coordination of both pre-analytical and analytical sources of interferences. Timing of specimen collection, performance characteristics of analytical techniques and patient history guide interpretation of a TDM result. Any result that is inconsistent with clinical expectations should be critically evaluated before dose adjustments are made.

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CHAPTER

14

Limitations of immunoassays for screening of drugs of abuse in urine: issues of false positive and false negative results

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INTRODUCTION

Drugs of abuse testing is conducted both for medical purposes and legal causes such as workplace drug testing. Testing may be performed for detection of illicit drug use and/or to monitor medication compliance. Recent growth in the number of pain management and opioid dependency clinics has greatly increased demands for urine drug testing. Urine is the specimen of choice because it contains ample concentrations of the drug and its metabolite(s) and can be easily collected. As urine drug screening is used for many different purposes and patient populations, selection of appropriate cutoffs is of great importance.

For the past couple of decades, the common approach to urine drugs of abuse testing has consisted of a two-step process involving an immunoassay screen followed by confirmation of results by mass spectrometry (MS). Immunoassay screens use an antibody to detect the drug of interest, which allows for testing to be completed quickly (often in less than 20 min) and by automated analyzers or point of care devices. This relatively quick analysis time and ease of operation have made immunoassay screening a popular choice for emergency departments, physician offices and on-site workplace testing. However, immunoassays suffer from relatively low specificity due to interaction of antibody with molecules with similar structures resulting in false negative or positive results and can only be used to screen for the presence of a drug in the urine. Any positive immunoassay test is considered "presumptive positive" until it is confirmed by a more specific method such as MS. It should be noted that the amount of interference varies by assay depending on sensitivity, methodology used and cutoff concentration. For example, one reference laboratory found that the percentage of false positives for their immunoassay tests ranged from 0% (for ethanol and cocaine) to 34% (for opiates) [1]. False negatives are also a concern as they may lead to a false sense of security that drugs are not present in the specimen. For example, urine cannabinoids screens will not detect the presence of synthetic cannabinoids that may be present in a patient sample.

As mentioned above, MS is used to definitively confirm "presumptive positive" immunoassay screens. However, MS confirmation usually has a longer turnaround time and may not be available at all laboratories. Therefore, for clinical testing MS may not be performed depending on the scenario. However, MS confirmation is required for legal drug testing, such as for workplace drug testing. Federal drug testing programs follow guidelines from the Substance Abuse and Mental Health Services Administration (SAMHSA) which mandates the drugs to be tested. Private employers may test for additional drugs depending on their workplace drug testing protocols. However, as immunoassay drug screens are still commonly used, interpretation of these test results remains an important, but challenging topic. This chapter details the common causes of false positive and false negative results in drugs of abuse testing by immunoassays.

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ISSUES OF SPECIMEN ADULTERATION

As mentioned above, urine is the specimen of choice for drugs of abuse testing. Compared to blood, urine has much higher concentrations of drug metabolites which allows for longer windows of detection following drug consumption. Urine is also a much easier sample to collect. However, urine is more susceptible to specimen adulteration, which is the practice of exchanging or altering a specimen to change the test result. Depending on the situation, adulteration can be used to change the result to a negative or positive. The most common adulteration strategies seek to alter the specimen to invalidate the immunoassay into producing a negative result. This takes advantage of the two step drug screening process used by most labs, where specimens are only sent for definitive MS testing if they screen positive by immunoassay. Alternatively, patients being monitored for medication compliance in pain management programs who may sell or give their drug to others may add some of their prescribed drug to their urine in order to cause a positive result. Common adulteration strategies are summarized below:

- *Substitution of specimen* - One strategy to get a negative result on urine drug screening is to submit a substituted clean urine sample. This can be done by obtaining urine from someone else who is not taking any drugs of abuse or by purchasing "clean" dried urine powder and reconstituting it in water. Strategies to deter specimen substitution include witnessed collections or by monitoring the temperature of the sample immediately following collection. Some facilities have special washrooms that do not have access to hot water and add a dye to toilet water in order to detect sample reconstitution.
- *Addition of adulterant to fool immunoassay screen*— Many products are available online that claim to cause negative urine drug screens. These adulterants are designed to fool the immunoassay test by interfering with antibody binding or signal generation through changes in pH, salt content, etc. Some well-known adulterants include Stealth, Klear, Urine Luck and Urine Aid.
- *Specimen dilution* — Addition of water to urine specimens can dilute the drug of abuse to below the assay cutoff. Monitoring of creatinine, specific gravity and specimen temperature may catch specimen dilution. As mentioned above, removing the availability of hot water in washrooms would also help prevent specimen dilution.
- *Flush drug out of system prior to test* — Discontinuing drugs of abuse prior to drug testing will reduce their concentration in urine. Drinking copious amounts of water prior to testing may aid in removal of some drugs and natural dilution of urine samples. Flushing agents containing diuretics are also available for purchase online, which act to increase urine output and dilute the urine below the drug screen cutoff.
- Spiking — For patients being tested for medication compliance, spiking the urine sample with the drug will cause positive immunoassay screens for parent drugs. This can be detected by monitoring the parent drug and drug metabolite separately (e.g., Methadone and its metabolite EDDP).

IMMUNOASSAY INTERFERENCES

In this section commonly encountered interferences in various immunoassays used for drugs of abuse screening are addressed. Immunoassays are designed to detect a drug at a pre-determined concentration (cut-off values). However, confirmation of the drug may also have the same cut-off as screening cut-off or a different cut-off. Moreover, each drug has a specific detection window in urine. Screening and confirmation cut-off levels of various drugs along with window of detection in urine are listed in Table 14.1.

Amphetamines

The amphetamines are sympathomimetics consisting of amphetamine and drugs with similar structures including methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), and 3,4-methylenedioxyamphetamine (MDA). The amphetamines urine drug screen is a class-based screen, and so may react with one or more drugs within the group. Depending on the manufacturer, the assay may have higher specificity for amphetamine, methamphetamine, MDMA and/or MDA.

Amphetamines immunoassays are well known for their high false positive rate due to cross reactivity with other sympathomimetic amines and over the counter drugs. To help counteract false positives, many amphetamines immunoassays use higher cutoffs (e.g., 500 or 1000 ng/mL) to increase specificity. However, positive amphetamine immunoassay results should always be considered for confirmation by mass spectrometry. Some of the reasons for a positive amphetamines screen, besides illicit use, are listed below.

Amphetamine isomers/medications containing or metabolizing to amphetamines

Amphetamine and methamphetamine exist as optical isomers, D- and L-isomers. The D-amphetamine and

TABLE 14.1 Screening and confirmation cut-off concentrations of various drugs in urine.

Drug class/drug	Detection window	Screening cut-off	Confirmation cut-off
Amphetamine/methamphetamine[a]	2 days	500 ng/mL	250 ng/mL
MDMA/MDA/MDEA[a]	2 days	500 ng/mL	250 ng/mL for MDMA/MDA/MDEA
Cocaine[a] (as benzoylecgonine)	2–4 days	150 ng/mL[b]	100 ng/mL[c]
Opiates[a]	3 days	2000 ng/mL[b]	2000 ng/mL for either drug
Heroin[a] (as 6-acetyl morphine)	12–24 h	10 ng/mL	10 ng/mL
Marijuana[a]	2–3 days, single use 30 days, chronic use	50 ng/mL	15 ng/mL
Phencyclidine[a]	8 days	25 ng/mL	25 ng/mL
Barbiturates[e]	Short acting: 3 days Long acting: 15 days	200/300 ng/mL	200 ng/mL
Benzodiazepines[e]	Short acting: 2 days Long acting: 10 days	200/300 ng/mL	200 ng/mL
Hydrocodone/Hydromorphone[d]	3 days	300 ng/mL	100 ng/mL for either drug
Oxycodone/oxymorphone[d]	3 days	100 ng/mL	50 ng/mL for either drug
Propoxyphene[e]	3 days	300 ng/mL	300 ng/mL
Methadone[e]	3 days	300 ng/mL	100 ng/mL

MDA, 3, 4-methylenedioxyamphetamine; *MDEA*, 3, 4-methylenedioxyethylamphetamine; *MDMA*, 3, 4-methylenedioxymethamphetamine.
[a]*Included in original SAMHSA guidelines. For amphetamine analysis, methamphetamine is the target drug but for reporting positive methamphetamine, its metabolite amphetamine must be present at a concentration of 100 ng/mL or higher.*
[b]*Private employer may use 300 ng/mL cut-off for screening and confirmation.*
[c]*THC-COOH, 11-nor-Δ9-tetrahydrocannabinol- 9-carboxylic acid.*
[d]*From new SAMHSA proposed guidelines (2015).*
[e]*Non-SAMHSA drugs.*

D-methamphetamine are active central nervous system (CNS) stimulators and are the main abused form of amphetamines. The L-isomers demonstrate different effects than their D-counterparts. Although L-amphetamine retains some of the CNS stimulatory properties, it is at much lower potency than D-amphetamine. The L-methamphetamine has no CNS stimulation properties but causes vasoconstriction instead. Illicit amphetamine preparations contain d-isomers either alone or in a mixture with l-isomers. However, both isomers can also be present in urine from the use of certain prescription and over the counter medications.

Although most immunoassay screens are targeted toward the D-isomer, there is often some cross-reactivity with the L-isomers which can result in a (true) positive amphetamine urine drug screens [2]. Knowledge of amphetamine isomers and their sources is needed to correctly interpret urine drug screens, as patients properly taking any of the medications shown in Table 14.2 can test positive on both the immunoassay screen and MS confirmation. In some cases, determination of amphetamine isomers may be required to definitively determine illicit versus prescription amphetamine use [3]. It is important to note that most MS confirmation assays do not differentiate between l- and d-isomers as special chiral analysis is required. This type of testing is available only at some reference laboratories.

Case Study: An emergency medical team responded to the residence of a 77 year old man who was experiencing difficulty in breathing with oxygen saturation of 69%. The patient was given albuterol and ipratropium bromide that increased his oxygen saturation to 96% and improved his breathing. The patient was transported to the emergency department and was admitted to the intensive care unit. Approximately 12 h after admission he was unresponsive and expired. His medical history included heart failure, atrial flutter and bronchial asthma. His urine drug testing by gas chromatography/mass spectrometry (GC-MS) was positive for acetaminophen, nicotine, cotinine, caffeine, diltiazem, doxylamine and methamphetamine. However, chiral analysis of methamphetamine was not initially performed raising the possibility of D-amphetamine use by the patient. Thus, chiral analysis using trifluoroacetyl-*l*-propyl chloride as the chiral derivatizing agent was performed and the results identified the methamphetamine as the L-isomer. Further investigation revealed that the decedent frequently used a Vicks inhaler for his bronchial asthma [4]. This case demonstrates how most MS assays do not routinely differentiate between the

TABLE 14.2 Drugs containing or metabolizing to amphetamines.

Drug	Trade names	Prescribed for	Metabolites
Lisdexamphetamine	Vyvanse	Attention deficit-hyperactivity disorder (ADHD), Binge eating	L-amphetamine
Selegiline	Emsam	Monoamine oxidase Inhibitor, Parkinson's Disease	L-amphetamine, L-methamphetamine
D-amphetamine	Adderall Adderall XR Dexedrine Mydayis	ADHD	–
L-amphetamine	Adderall Adderall XR Evekeo Mydayis	ADHD, Narcolepsy, Exogenous Obesity	–
D-methamphetamine	Desoxyn	ADHD, Exogenous Obesity	D-amphetamine
L-methamphetamine	Vicks Vapor Inhaler	Nasal decongestion	L-amphetamine
Benzphetamine	Didrex	Exogenous Obesity	D-methamphetamine

Information compiled from Refs. [3,50].

d- and l-isomers for amphetamines. Prior to this analysis, the patient was suspected of illicit drug use. However, chiral analysis and investigation of the decedent's medication use determined that the methamphetamine identified by the GC-MS was the L-isomer from an over the counter medication (Vick's inhaler).

Interference from over the counter and prescription medication

Many over the counter medications are known to interfere in amphetamines immunoassays. The high prevalence of use of over the counter medications adds to the high false positive rate seen with amphetamines immunoassays.

- Ephedrine and pseudoephedrine are a major cause of false positives. Some assays have reformulated to reduce interference from these two drugs.
- Bupropion, an atypical antidepressant used for smoking cessation can also cause false positives. A retrospective chart review performed by Casey et al. [5] determined that up to 41% of their false positives could be due to bupropion in their Syva EMIT II assay.
- Ranitidine, a H2 receptor antagonist used to treat heart burn, is also implicated in false positives. Some manufacturers have reformulated the reagents for amphetamines immunoassays to improve the number of false results; however, there are assays that are still affected, and susceptible to cross reactivity from ranitidine [6].
- Trazodone and its metabolite m-chlorophenylpiperazine (m-CPP) (also available separately as a hallucinogenic drug) and trifluoromethyl-phenylpiperazine have been reported to cause false positives in the Ecstasy EMIT II assay [7].
- Fenofibrate has been implicated in false positives in assays for amphetamines (CEDIA) and MDMA (DRI EMIT) [8,9].

In addition, other drugs including herbal supplements may also interfere with amphetamine/methamphetamine immunoassays. These compounds are listed in Table 14.3.

Opioids

The opioids are a wide ranging class of drugs including morphine, codeine, heroin, oxycodone, fentanyl, and methadone. These drugs act at the opioid receptors within the nervous system and can cause analgesia, respiratory depression, constipation and euphoria.

Testing for opioids is complex, and there is no single immunoassay that can detect them all. Interpretation of urine drug screening results is further complicated by confusing nomenclature, specifically the difference between opioid and opiate. The term opioid refers to any drug that can interact with opioid receptors and so encompasses the entire class. The term opiate is a subclass of the opioids consisting of forms derived from the opium poppy, specifically morphine and codeine. Urine opiate screens are available and it is important to recognize that these screens only test for the presence of

TABLE 14.3 Drugs that interfere with amphetamine immunoassays.

Structurally similar Amines	Brompheniramine, Benzphetamine, Ephedrine, Isometheptene, Methylphenidate, Pseudoephedrine, Phentermine, Propylhexedrine, Phenylephrine, Phenmetrazine, Tyramine
Antidepressant/ Antipsychotic	Chlorpromazine, Bupropion, Desipramine, Doxepin, Fluoxetine, Perazine, Thioridazine, Trimipramine, Trazodone and its metabolite
Antihistamine	Ranitidine
Antimalarial	Chloroquine
Antispasmodic	Mebeverine
Beta-Blocker	Labetalol
Cardioactive	N-Acetyl procainamide (metabolite of procainamide)
	Mexiletine
Narcotic analgesic	Fentanyl
Non-Steroidal Antiinflammatory	Tolmetin
Tocolytic Agent	Ritodrine
Antiviral	Amantadine
Herbal supplement	Dimethylamine

opiates. However, because the terms opioids and opiates are often used interchangeably, a negative opiate result may be misinterpreted to conclude that no opioids (e.g., Fentanyl, oxycodone) are present in the sample. This represents a major sources of 'false negative' results when interpreting opiate urine drug screens.

In addition to the 'opiate' urine drug screen, individual immunoassays are also available to detect most opioids of clinical interest such as hydromorphone, hydrocodone, oxycodone, fentanyl, buprenorphine, methadone and its metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP). Interferences for these specific assays are discussed below.

Opiates screening assays

Similar to the amphetamines, the opiates screen is a class based screen. As discussed above, it primarily detects morphine and its prodrug codeine. However, depending on the assay there may be cross-reactivity with metabolites of codeine or morphine. Two minor metabolites specifically of interest are hydrocodone and hydromorphone, which can also be prescribed separately as drugs (Vicodin and Dilaudid, respectively). Heroin use is also detected by opiates screens either through cross reactivity with the primary metabolite 6-monoacetylmorphine or secondary metabolite, morphine. Therefore a positive opiate screen result, can indicate the presence of any of these drugs, but cannot distinguish between them. Immunoassays are available that target heroin, hydrocodone and hydromorphone, but this still only provides a presumptive result. Testing by MS is required for definitive identification.

Sources of false negatives for urine opiates screens fall into two main categories. The first, as mentioned earlier, happens when the urine opiates screen (which targets codeine and morphine) is mistaken as a test for all opioids (including fentanyl, oxycodone, methadone etc.). For these cases, having immunoassays specific for oxycodone, fentanyl, methadone, etc. are essential. The second source of false negatives is related to the reduced cross-reactivity of opiate metabolites (specifically hydrocodone and hydromorphone) in the opiate immunoassay compared to codeine and morphine. This is particularly important for monitoring of patients prescribed hydrocodone and hydromorphone, as they may screen negative using conventional cutoffs on a urine opiates screen even if they are taking their medication as directed [10]. In these cases, use of a hydrocodone/hydromorphone specific immunoassay or confirmation by mass spectrometry can resolve the conflict.

False positive opiate screens have been reported with the following drugs:

- Quinolone antibiotics, including levofloxacin, ofloxacin, pefloxacin and enoxacin, have been reported to cause false positives in several opiates assays [11,12].
- Rifampicin can cause false positives in the Roche KIMS Opiates assay [13].
- A recent case report suggested high concentrations of dextromethorphan caused a false positive result in the Syva EMIT assay [14].

Case Study: A 48 year old man spent 12 days in an inpatient psychiatry facility for treatment of depression which began subsequent to his abuse of cocaine after 90 days of sobriety. He was then transferred to a residential facility. Eleven days after his transfer to the residential facility, the patient was prescribed gatifloxacin for treating urinary tract infection. His urine drug screen before initiation of gatifloxacin therapy was negative. Six days after the start of gatifloxacin therapy, his urine screen was positive for opiates. The patient denied opiate abuse or consuming poppy seed containing food. GC-MS confirmation did not show the presence of any opiates. A second opiate drug screen performed 24 days later was

also positive for opiates by immunoassay, but was negative by GC/MS. However, when a third urine sample was analyzed 13 days after cessation of gatifloxacin therapy, the urine immunoassay screen was negative for opiates suggesting that the false positive opiates screen was due to gatifloxacin [15]. This demonstrates the 'presumptive' nature of immunoassay drug screens and the importance of MS confirmation for definitive testing.

Case Study: A 60 year old woman presents to her physician for a refill of her hydrocodone prescription. She has a history of chronic back pain which did not respond to non-opioid medication. She also admits to previous illicit drug use, but insists that she no longer uses illicit drugs. She reports using 2–3 tablets of hydrocodone per day to control pain and that her last dose was earlier that day. Before filling the prescription, her physician orders a urine drug screen which came back negative for opiates. The patient denies diversion of her hydrocodone medication. The physician requests MS confirmation of the immunoassay results. GC-MS analysis was positive for hydrocodone. This suggests that the initially immunoassay test was not sufficiently sensitive to detect the hydrocodone present in the patient's urine and demonstrates the utility of MS confirmation of false negative immunoassay results.

Methadone/EDDP screening

Methadone, and its major metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), are commonly prescribed for opioid dependency treatment or pain management. In this setting, urine drug testing is often performed to confirm medication compliance and it is useful to monitor the parent and metabolite separately in order to detect adulteration, such as spiking a urine specimen with methadone. Some manufacturers have separate methadone and EDDP assays that have minimal cross-reactivity with the other analyte allowing separate monitoring of each [16].

Several drugs have been identified that can cause false positives in methadone and/or EDDP assays.

- A case report described a false positive methadone result in a lateral flow immunoassay from the presence of diphenhydramine [17].
- Several drugs have been identified to cross react in the Roche KIMS methadone assay. These include quetiapine, promazine, chlorpromazine, levomepromazine, clomipramine, chlorprothixene, cyamemazine and alimemazine, cyamemazine, and doxylamine. [16,18–22],
- Doxylamine has been reported to cause false positives in Methadone screens [21].
- Tapentadol and its metabolites can cross react in the DRI Methadone assay with cross reactivities ranging from 0.6% to 4.4% [23]. However, tapentadol showed no interference with the Syva EMIT II Methadone assay [24].
- Verapamil and its metabolites have also been reported to cause false positives in the DRI Methadone assay [25,26].

Fentanyl screening

With the start of the opioid crisis in North America in 2013, demands for testing method for fentanyl have significantly increased. Several automated immunoassay kits are now available for rapid screening of fentanyl in urine. These kits predominantly use antibodies that primarily target parent fentanyl with some cross-reactivity with minor metabolite, despropionylfentanyl, and have little to no reactivity with norfentanyl, the major urinary metabolite of fentanyl [27].

Risperidone, and its metabolite 9-hydroxyrisperidone (paliperidone), have been shown to interfere in the DRI Fentanyl assay [28]. Some fentanyl analogues have been investigated for crossreactivity with commercially available fentanyl screens. Acetylfentanyl has 80% cross-reactivity in the DRI Fentanyl assay [28]. Helander et al. evaluated the cross-reactivity of 12 fentanyl analogues (acetylfentanyl, acrylfentanyl, butryfentanyl, 4-chloroisobutrylfentayl, 4-fluorobutyrfentanyl-4-fluorobutyrfentanyl, 2-fluorofentanyl, 4-fluorofentanyl, isobutyrulfentanyl, methoxyaetylfentanyl, 4-methoxybutyrlfentanyl, tetrahydrofuranfentanyl) in three fentanyl immunoassays (DRI, ARK and SEFRIA). With the exception of 4-methoxybutyrlfentanyl, all fentanyl analogues tested had some cross reactivity with the three assays [29].

Oxycodone screening

Oxycodone testing is commonly ordered as part of monitoring medication compliance. As mentioned earlier, opiates immunoassays have little to no reactivity with oxycodone. Therefore, oxycodone specific assays should be used to monitor for this drug.

Assays that detect both parent oxycodone and some of the major metabolites (noroxycodone, oxymorphone) are less likely to have false negative results in specimens with low oxycodone concentrations. A retrospective data analysis of a major reference lab determined that ~4% of patient samples screened negative by the oxycodone screen, but had oxycodone metabolites present when analyzed by LC-MS/MS [1]. Dixon et al. investigated the use of the DRI oxycodone assay for

detection of specimens at or near the cutoff concentration (100 ng/mL) [30]. They determined that the good cross-reactivity of oxymorphone (103%) in the assay allowed for positive screens even when parent oxycodone was below 100 ng/mL.

Case Study: A 40 year old male patient is receiving oxycodone to treat his chronic migraine headaches. Initially, he was prescribed 20 mg every 12 h which he reported was effective to treat his pain. However, over the past 6 months, the patient reported this dosage was no longer sufficient to treat his pain. His physician had decreased the dosing interval first from 12 to 8 h and then to every 6 h. Despite this, the patient would frequently call the physician office asking for early refills indicating he had finished the prescription faster than prescribed. The physician, concerned about possible diversion of the oxycodone, ordered a urine drug test. A screen performed by the laboratory was negative for amphetamines, opiates, benzodiazepines, and cannabinoids. The patient was confronted with these results, but vehemently denied diversion of the oxycodone. In further discussion with the laboratory toxicologist, the physician asked for MS confirmation of oxycodone and metabolites. The results were positive for oxycodone and its metabolite indicating the patient was taking his medication. This demonstrates the danger of misinterpreting the opiates immunoassay results and the value of consulting with laboratory personnel on test interpretation.

Buprenorphine screening

Buprenorphine is commonly used in opioid dependency treatment and pain management programs. It is metabolized to norbuprenorphine, which is then converted to the major urinary metabolite norbuprenorphine-glucuronide. Free buprenorphine and norbuprenorphine are excreted in urine, but at very low concentrations. Therefore, assays with antibodies targeting free norbuprenorphine (e.g., DRI buprenorphine) may have higher false negative rates compared to assays with antibodies targeting norbuprenorphine glucuronide (e.g., CEDIA). The CEDIA method has been shown to have higher sensitivity compared to the DRI assay, which may be attributable to the differing antibody specificity [31]. However, the same study showed that the DRI assay had far fewer false positives due to the presence of drugs other than buprenorphine than the CEDIA method. Pretreatment of urine samples with glucuronidase, to cleave the glucuronide moiety, can significantly improve sensitivity for assays targeting free buprenorphine or norbuprenorphine [32].

Drug cross-reactivity in buprenorphine assays are summarized below.

- Antipsychotic drugs amisulpride and sulpiride can cause false positives in the CEDIA buprenorphine assay [33]. Although the cross reactivity for each drug was estimated to be very low (0.002% for amisulpride and 0.00% for sulpiride), the high doses usually prescribed and the low cutoff of the buprenorphine assay increase the risk of a false positive.
- Metabolites of codeine and morphine, including dihydrocodeine, and morphine-3-glucuronide, can also cause false positives in the CEDIA buprenorphine assay [34]. For example, Berg et al. showed that consumption of 60 mg of codeine was sufficient to generate a false positive buprenorphine result. Similarly, high dose morphine has been reported to cause a false positive [35].
- Tramadol has also been reported to cause false positives [36].
- Hydroxychloroquine and chloroquine crossreact in the CEDIA buprenorphine assay, but not in the DRI assay [31].

Benzodiazepines

Similar to the amphetamines and opiates screens, benzodiazepines assays are class based screens. The benzodiazepines represent a large class of drugs used for a variety of purposes including sedation, anesthesia, anti-anxiety, and anti-convulsants. Immunoassays targeted toward the benzodiazepines cannot detect all members of this class of drugs with equal sensitivity, which represents a possible cause of false negatives.

Understanding benzodiazepine metabolism aids in interpretation of urine drug screens. Many benzodiazepines (e.g., diazepam, chlordiazepoxide) feed into a common metabolic route to nordiazepam and oxazepam, ending in oxazepam-glucuronide. Many immunoassays are calibrated to nordiazepam to detect the drugs metabolizing using this pathway, although exact identification of each metabolite requires MS. Benzodiazepines that do not metabolize to oxazepam, often are glucuronidated prior to urinary excretion. The glucuronide moiety may affect detection of certain benzodiazepines by immunoassays, specifically lorazepam and lormetazepam [37]. Addition of glucuronidase to the assays, which cleaves the glucuronide moiety, has allowed for improved detection of these drugs by immunoassay.

The patient population tested can also play a role in the number of false negatives in the benzodiazepine screens. Darraugh et al. evaluated the use of three benzodiazepines assays in a population of pain management patients [38]. Urine testing of patients taking benzodiazepines as part of their treatment regimen revealed that benzodiazepines immunoassay screens did not detect the drugs in 22% of specimens, even

with glucuronidase pretreatment of samples. The drug concentrations in the urine was detectable by MS, but was not high enough to trigger a positive result by immunoassay.

Over the past 10 years, several benzodiazepines have been identified as 'designer benzodiazepines' or novel psychoactive substances (NPSs). These are benzodiazepines that are showing up in the recreational drug supply but are (usually) not clinically available in Europe or North America. Therefore, there is very limited information on their cross-reactivity in benzodiazepines immunoassays and pose a possible source of false negatives. A recent study by Bergstrand et al. looked at the detectability of 13 benzodiazepine NPS (clonazolam, deschloroetizolam, diclazepam, estacolam, etizolam, flubromazepam, flubromazolam, flutozolam, 3-hydroxyphenazepam, meclonazepam, nifloxipam, phenazepam and pyrazolam) in four benzodiazepine urine drug screens (CEDIA, HEIA, EMIT II Plus and KIMS) [39]. In general, all 13 drugs were detectable in all four assays used; however, the % crossreactivity varied greatly between the assays. For example, 3-hydroxyphenazepam had the most variable cross-reactivity across the assays ranging from 48% with the EMIT II Plus assay to 464% with the HEIA assay. All assays were poor at detecting flutazolam, which had cross-reactivity ranging from 4% (EMIT II Plus) versus 41% (HEIA).

Drugs that have been identified to cause false positives in urine benzodiazepines screens include:

- Sertraline has been implicated for causing false positives in several benzodiazepines assays. The CEDIA assay has since been reformulated to reduce cross-reactivity with sertraline [40]. A two year retrospective chart review of drug screen results from the Naval Medical Centre in Portsmouth, Virginia suggested that sertraline can cause false positives in benzodiazepine screen assays in use at that institution (Abbott Architect and Aeroset) [41].
- False positives results have also been observed in Benzodiazepines screens done using "Triage 8" meters for patients on Efavirenz, due to cross-reactivity with its major metabolite 8-hydroxyefavirenz [42].

Cannabinoids

The term cannabinoid refers to any drug or molecule that can interact with the cannabinoid receptors in the human body. This includes signaling molecules from the endogenous cannabinoid system and exogenous drugs, the most common of which is Cannabis. The cannabis plant contains over 200 cannabinoids, but urine drug testing focuses on the main psychoactive cannabinoid, Δ9-tetrahydrocannabinol (THC). Urine drug screens use antibodies targeted to Δ9-carboxyTHC, the major urinary metabolite of THC.

THC and its metabolites are very lipid soluble, allowing them to accumulate in fatty tissues in the body. This significantly increases the excretion half-life of THC and is particularly pronounced in chronic users, where carboxy-THC can be detected in the urine for weeks to months after cannabis use is stopped [43].

A major source of false negatives with cannabinoids screening is the use of synthetic cannabinoids. Synthetic cannabinoids can bind endogenous cannabinoid receptors to cause similar psychoactive effects as THC, but have completely different structures (see Chapter 16). Thus, they are not detected by conventional cannabinoids screening immunoassays. However, as a group of NPS, synthetic cannabinoids are showing up more and more in the recreational drug supply and can have more serious side effects than cannabis use. Recently, immunoassay screens have been developed targeting specific synthetic cannabinoids JWH-018 and UR-144. Evaluation of these kits found that they had good performance at detecting JWH-018 and UR-144, there was limited cross-reactivity with other synthetic cannabinoids present in specimens [44]. One of the major challenges of detecting synthetic cannabinoids is how fast they change within the recreational drug market. The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) calls synthetic cannabinoids one of the most chemically diverse groups of NPS, with over 179 new forms identified between 2008 and 2017 [45]. Therefore, having immunoassays with broad cross-reactivity for synthetic cannabinoids would be extremely useful in helping detect these drugs in patient samples.

In recent years, some cannabinoid containing medications have become available, that depending on the active component can cause a true positive in a cannabinoids drug screen. Sativex, for example, is a pharmaceutical preparation of THC and cannabidiol (CBD) and will cause positive results on a cannabinoids screen. Dronabinol and nabilone are both synthetic cannabinoids, and are structurally different from THC so that they will not cross-react in cannabinoids screens [46].

False positive interferences for cannabinoids screens described in the literature include:

- Efavirenz has been reported to cause false positives cannabinoid screen results in the Rapid Response test Drugs of Abuse test strips, Triage Tox meter, and the CEDIA THC screen [47,48]. The interference is believed to be from the glucuronidated metabolite of efavirenz, as false positives are decreased after sample treatment with glucuronidase.

- Certain soaps, including baby washes used in hospital neonatal wards, can cause false positives for THC assays [49]. This is particularly of concern, as positive urine drug screen results in infants and children can lead to the involvement of social services. Mass spectrometry confirmation of positive cannabinoids screens must be performed in this population.

LIQUID CHROMATOGRAPHY COMBINED WITH MASS SPECTROMETRY FOR CONFIRMATION

Although gas chromatography combined with mass spectrometry (GC/MS) is still used widely for drug confirmation in last 20 years liquid chromatography combined with mass spectrometry (LC-MS) or liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) is gaining acceptance as a superior method for drug confirmation. A major advantage of LC-MS/MS over GC/MS is faster turn-around time because derivatization is not necessary as both polar and non-polar compounds can be analyzed using liquid chromatography (high performance liquid chromatography). More recently, the dilute and shoot approach for sample preparation has been adopted by several laboratories for rapid screening of drugs of abuse using LC-MS/MS using a single injection. This method enables diluting the sample with mobile phase solvent mixture, adding deuterated internal standards followed by direct injection to the LC-MS/MS analyzer. Theoretically this is a very easy methodology to employ by anyone, at the expense of instrument wear and tear. However, a more conservative method is to employ some mode of extraction of drug present in the urine sample either by liquid-liquid or solid phase (SPE) extraction. Following extraction, the extracts are concentrated by evaporation and then reconstituted with mobile phase prior to injection. This is more time consuming but produces a cleaner extract, which can reduce interferences and keep the instrument cleaner and can increase method sensitivity.

CONCLUSIONS

For many years, immunoassays have played an important role in urine drug screening. They are fast and can be performed at the point of collection. Even clinical laboratories have used immunoassay screening to identify samples for further mass spectrometry analysis. However, immunoassays can suffer from false positives and negatives, depending on the specificity of the antibody used and its cross-reactivity. In addition, immunoassays are the target of most of the adulteration strategies used to invalidate urine drug screening. Correct interpretation of immunoassay screens requires a good understanding of which drugs are targeted by the assay and what can interfere with the results. It is important to always remember that these tests are screens, and definitive mass spectrometry testing should be performed if there is any question or concerns about the results. Consultations with the local laboratory and toxicologist are important tools in helping understand and accurately interpret urine drug screen results.

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CHAPTER

15

Challenges in confirmation testing for drugs of abuse

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INTRODUCTION

In order to accurately interpret drug testing results, more information than the result itself (typically positive or negative) is required. Some of the factors influencing interpretation include: sample tested, purpose of test request, scope of testing performed (i.e., what drugs are included), knowledge of components and analytical parameters of the initial and confirmation methods used, presence of other chemicals in the sample, physiological state of the donor, and metabolism of the drug(s). This chapter will focus on the confirmatory testing typically performed using mass spectrometry after the immunoassay screening described in Chapter 14 and challenges in interpreting such results including positive, negative, or in some cases questionable results.

SPECIMEN SELECTION

Although urine is the sample most commonly used for drugs of abuse testing a variety of other samples including serum/plasma/blood, hair, oral fluid and sweat can also be used. Advantages of urine specimens include non-invasive collection, relatively easy, cost-effective proven technology, and a moderate window of detection for most of the drugs of interest. A primary disadvantage of urine is the lack of correlation between drug concentration and impairment. In situations in which correlation between drug concentrations and pharmacological effects or impairment is desirable the appropriate sample is serum, plasma, or blood. The use of oral fluid offers the advantages of ease of collection and less possibility of sample adulteration, but the window of detection for most drugs of interest differs from those reported in urine. Hair offers the advantage of the longest window of detection as the presence of a drug in hair reflects use or exposure of several weeks, even months, past. A disadvantage of hair as a sample is that testing methods are fairly cumbersome often requiring significant sample preparation. Although this chapter will focus on the interpretation of results from testing urine samples some of the information may be applied to results obtained using other samples. When interpreting results from samples other than urine some additional factors to consider include analytes detected (parent drug or metabolite(s)), cutoff concentrations, windows of detection, methods of analysis, and sample handling techniques [1].

PURPOSE OF DRUG TESTING

The purpose of testing is another factor to consider when interpreting results. Drug testing may be performed for different reasons including the setting and possible medical or legal implications. For example, in the clinical emergency setting testing is usually requested to assist in determining whether the symptoms are caused by one or more drugs versus trauma or disease. In the pain management setting testing may be used to monitor patient compliance and/or identify use of other drugs. Another example is the use by social services of newborn meconium drug screening results in investigations involving parental custody. The purpose of testing may dictate whether confirmation testing by a more sensitive and specific technique should be performed or not. In workplace and forensic drug testing, confirmation is either mandated by law or by consensus within the forensic testing profession. In the clinical

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setting confirmation may not be required, particularly if treatment decisions are based on a combination of patient presentation and initial screening results. When confirmation is required it is typically performed only for positive screening results but in some situations such as pain management monitoring negative results may require confirmation [2].

The scope of a drug test, i.e., which drugs are detected/reported, is affected by several factors, including the intended purpose of the testing. The Substance Abuse and Mental Health Services Administration (SAMHSA) in the Department of Health and Human Services (HHS) administers the regulation of the federal workplace drug-testing program. The drugs/metabolites to be tested in this program are established by law. For clinical samples factors determining the scope of testing include prevalence of local usage, clinician requests, complexity and availability of testing methods and instrumentation, and personnel requirements.

Specific components of the confirmation testing procedure may affect drug-testing results and should be considered when interpreting results. Knowledge of analytical parameters of the confirmation procedure such as specificity, sensitivity (limit of detection - LOD and limit of quantitation -LOQ), upper limit of linearity (ULOL), and interferences aids the interpretative process. Laboratories should determine these parameters as part of the initial validation of a method and the periodic revalidation typically required by regulatory and accreditation agencies. Knowledge of the sensitivity and specificity of the assay is essential for interpretation of results and implementation of follow up action as false positive (incorrectly reporting the presence of a drug) and false negative (failure to detect the presence of a drug) results have serious consequences. The specificity of immunoassays is crucial for screening procedures and has been discussed in Chapter 14. Confirmation procedures are typically very specific for targeted compounds but may be subject to interference from compounds with very similar structures. The specificity of screening procedures may depend on the intended use of the test. For example, in the clinical setting it may be desirable to detect all of the compounds of a given class, but in the workplace setting detection of only the compounds specified by law or contract is allowed. The practical sensitivity for immunoassays is the cutoff concentration although they have the ability to detect drugs below these levels. The cutoff concentrations for confirmation of many drugs are mandated by SAMHSA (Substance Abuse and Mental Health Services Administration) guidelines but laboratories have the ability to detect these drugs at lower concentrations. SAMHSA-certified laboratories are required to have a minimum sensitivity (expressed as the LOQ) of 40% of the cutoff concentration and although laboratories can often detect lower concentrations some choose to maintain the LOQ at this concentration. For reconfirmation of positive results these laboratories are allowed to use the LOD (i.e., the lowest level at which a compound can be detected but not accurately quantitated) because of the possibility of deterioration of the drug during storage between time of initial reporting and subsequent reconfirmation. Regardless of the sensitivity of an assay it is important to recognize that neither immunoassay nor mass spectrometry based methods can measure to zero [1].

Deliberate attempts to mask or destroy drugs present in urine obviously affect the interpretation of drug testing results. The tests performed to detect dilution, substitution, or adulterations are referred to as *specimen validity tests* (SVT) and interpretation of these results will be discussed later in this chapter.

The physiological state of the donor may affect interpretation of drug testing results. Any condition or disease affecting the absorption, metabolism, or excretion of drugs can impact drug testing results. Because the primary site of drug metabolism for most drugs is the liver and the primary mode of excretion is most often urinary, hepatic or renal conditions affect drug testing results. Some drugs are excreted unchanged while others undergo chemical modification such as demethylation, oxidation, or reduction. Often drugs or metabolites have glucuronide or sulfate molecules attached via the process of conjugation to form a more water-soluble compound excreted in urine.

Most drugs of interest appear in urine as a combination of unconjugated (free) and conjugated parent drug and metabolites. Each of these has a different excretion pattern with some detectable for a few hours and others for days to weeks. Thus the choice of compounds (parent drug and/or metabolite(s)) in the drug panel can influence the results obtained [2].

TESTING PROCESS FOR DRUG CONFIRMATION

In the workplace and forensic settings, confirmation is usually performed using gas chromatography/mass spectrometry (GC/MS) or liquid chromatography tandem mass spectrometry (LC/MS/MS). Although confirmation procedures are specific for each drug/metabolite, there are common features that will be discussed below. The conditions of collection and transport to the laboratory can affect the ultimate drug test results but these actions may not be under the laboratory's control. For regulated workplace drug testing the laboratory may take action including rejection of the specimen based on errors such as sample misidentification,

improper or broken security seal, specimen leakage, and incomplete chain of custody documentation.

The testing process for gas chromatography/mass spectrometry (GC/MS) confirmation procedures typically includes the following steps: sample pretreatment, extraction, derivatization, chromatographic separation, and mass spectrometric detection. Liquid chromatography combined with tandem mass spectrometry (LC/MS/MS) procedures typically require much less sample extraction and may consist of simple dilution of the sample without any prior extraction followed by injection onto the column ("dilute and shoot"). Sample pretreatment includes addition of internal standard (IS) and may include sample dilution and chemical treatment such as hydrolysis, periodate oxidation, or oxime derivatization. Errors in any of these processes can lead to inaccurate results. Typically pipetting of the donor, standard(s), and control(s) samples is the initial step followed by addition of IS to all samples. The most commonly used internal standards are compounds with physical-chemical properties similar to the drug which are deuterated compounds where deuterium replaces hydrogen atoms. Ideally an IS has an extraction recovery, retention time, and ionization response similar to the target analyte, and only a slight difference in mass. Internal standards are used to determine drug analyte concentrations (using ratios of drug to IS response) and to account for any conditions that can adversely affect drug recovery. Because the IS and drug of interest will be affected the same during the pre-analytical and analytical processes the ratio of drug analyte to IS remains constant even when conditions cause absolute drug loss. Some of the factors affecting recovery include: extraction efficiency, pipetting variance, gas or liquid flow rates, column variance, or sample injection volume. Addition of the same amount of IS to every sample in the batch is critical. HHS-certified laboratories are required to monitor internal standard recovery with acceptable limits being 50–200% of the IS area of calibrator(s) or control(s). Use of deuterated IS solution contaminated with non-deuterated analyte can lead to inaccurate concentrations and potential false results. IS solutions should be monitored for the presence of the non-deuterated analyte of interest. Error in addition of IS solution is another potential source of inaccurate results.

Urinary excretion of drugs and metabolites as conjugated compounds enhances their water solubility but increases the difficulty of extraction using an organic solvent. Hydrolysis of the conjugate prior to extraction is necessary for these drug analytes. The 3 most common modes of hydrolysis are: enzymatic such as β-glucuronidase, acidic such as using hydrochloric acid (HCl), or basic such as using sodium or potassium hydroxide (NaOH, KOH). Hydrolysis using a strong acid or base and higher temperatures is usually faster but can destroy the drug analyte or produce interfering artifacts. A control containing the conjugated drug should be included in the batch, to demonstrate the effectiveness of the hydrolysis step. The discussion of specific drug analytes requiring a hydrolysis step or other chemical pretreatment such as periodate oxidation or oxime derivatization will be included in the following sections of this chapter.

After sample pretreatment the next step in confirmation procedures is extraction of the drug analyte from the urine and subsequent concentration by drying and reconstitution. The two most common techniques used for extraction of drug analyte(s) from urine are liquid-liquid and solid-phase extraction columns. Analytical factors to be considered for liquid-liquid extraction procedures include the choice of solvent, pH, and buffer. The drug analyte is extracted into an organic solvent after adjustment of pH to maximize the extraction efficiency for that particular analyte. Some procedures include back extraction from the organic solvent into an aqueous solution and then re-extraction of the analyte into an organic solvent. The solvent extract can be concentrated by evaporation for subsequent reconstitution, possible derivatization, and then GC/MS analysis.

The second type of procedure commonly used for drug analyte extraction from urine is the use of solid-phase extraction (SPE) columns of various types and phases. Columns are used to extract drug analyte(s) from urine through a process of adsorption onto the column matrix, removal of other compounds during a wash procedure and elution into an organic solvent. Three types of SPE columns are normal-phase, reverse-phase and ion-exchange. Reverse-phase and ion-exchange columns are widely used in forensic toxicology laboratories. Most SPE procedures include common steps of pH adjustment of the sample and conditioning of the column in order to maximize retention of the drug analyte onto the column. The sample is applied to the column and potential interfering compounds are washed through followed by elution using an organic solvent. Often the SPE columns used for drug testing are mixed-mode, containing both ion-exchange and reverse-phase properties. In this case, the elution solvent is often methanol containing a 2–5% solution of acid or base depending on the properties (acidic or basic) of the analyte drug(s).

After a compound (drug or metabolite) has been extracted from urine the extract is typically dried and reconstituted for injection into the gas chromatograph (GC). Frequently it is necessary to convert the drug to a more volatile compound and this is accomplished by reconstitution with a derivatizing reagent. Derivatized compounds are typically more volatile, more stable to thermal decomposition, and less likely to adsorb to GC column walls or solid supports. If the derivatizing

reagent is optically active, it can be used to separate *d*- and *l*-stereoisomers of a drug such as methamphetamine. The most popular derivatization method is silylation in which active hydrogens on the target analyte are replaced with a trimethylsilyl, or TMS, group. N,O-bismethylsilyltrifluroacetamide (BSTFA) and N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA), N-Methyltrimethylsilyltrifluoroacetamide (MSTFA), and trimethylchlorosilane (TMCS), are some silylating reagents. Another derivatization method is acylation, which converts amino, hydroxyl, and thiol groups to esters, thioesters, and amines, respectively. Commonly used acylation agents include acetic anhydride, trifluoroacetic anhydride (TFAA), heptafluorobutyric anhydride (HFBA), 4-carbethoxyhexafluorobutyryl (4-CB), and pentafluoropropionic acid anhydride (PFPA). A third derivatization method is alkylation in which the active hydrogen in a carboxylic acid, hydroxyl, thiol, or amino functional group is replaced with an alkyl group that decreases polarity of drugs. Alkylation reagents include trimethylanilinium hydroxide (TMAH) and tetrabutylammonium hydroxide (TBH) [3].

CONFIRMATION OF AMPHETAMINES

Federally regulated workplace drug testing has always included testing for amphetamine and methamphetamine, and in October 2010 screening for the synthetic stimulant 3,4-methylenedioxymethamphetamine (MDMA; Ecstasy) with confirmation testing for MDMA, 3,4-methylenedioxyamphetamine (MDA), and methylenedioxyethylamphetamine (MDEA) was added. On October 1, 2017 MDEA was removed as a drug to be tested for federal workplace testing but may still be tested for nonfederal workplace samples. The current cutoffs for amphetamines (amphetamine and methamphetamine) and MDMA MDA for federal workplace testing is 500 ng/mL for screening and 250 ng/mL for confirmation [4]. These compounds and others with similar structures and properties such as pseudoephedrine, ephedrine, propylhexedrine, phenylephrine, phenmetrazine, and phentermine available by prescription or as over-the-counter (OTC) treatments for nasal congestion and appetite suppression are known as sympathomimetic amines. Additional designer amphetamines such as methiopropamine, 4-fluoroamphetamine, 4-fluoromethamphetamine, and 4-methylamphetamine are together with cathinones referred to as "bath salts" (Please see Chapter 16).

Several approaches have been taken to minimize the occurrence of false-positive results with amphetamines immunoassays [5]. These approaches include performing a second immunoassay screen from a different manufacturer using an immunoassay with different cross-reactivity or pretreatment of samples with sodium periodate to eliminate interference from sympathomimetic amines. Another method for differentiating true positives from false positives is neutralization of the signal in a true-positive sample by the addition of antibody to the target analyte [6]. Using serial dilution testing and optimal slope cutoffs (determined by ROC analysis) enabled Woodworth et al. to differentiate samples containing amphetamine/methamphetamine from those containing cross-reacting compounds and to increase the positive predictive value of the immunoassay [7]. Immunoassay screening of drug classes including amphetamines is being replaced with definitive screening by liquid chromatography coupled with mass spectrometry in either single (LC/MS) or tandem (LC/MS/MS) mode capable of detecting multiple analytes including designer amphetamines and other amphetamine −like stimulants. Development of simpler and faster sample preparation procedures such as "dilute, filter, and shoot" or "dilute and shoot" and the increased capability of LC/MS and LC/MS/MS have facilitated development of many definitive multianalyte screening methods [5]. Another approach is to combine both immunoassays and mass spectrometry procedures for performing drug screening [5]. For federal workplace drug testing LC/MS/MS has been recognized as an acceptable alternate technology to immunoassays for initial screening [4].

This chapter will focus on results obtained by GC/MS confirmation procedures following positive screening results using immunoassays targeting amphetamines and/or MDMA. Confirmatory testing of amphetamines can be performed in a single method that includes all four/five analytes (amphetamine, methamphetamine, MDMA, MDA/MDEA) or as separate methods for amphetamine/methamphetamine and MDMA/MDA/MDEA.

Reports of positive amphetamines or MDMA/MDA results typically include a positive result for the screening class (amphetamines or MDMA/MDA) and identification of the compound(s) that were confirmed − amphetamine and/or methamphetamine for positive amphetamines results and MDMA, MDA, MDEA (if included in panel) for positive MDMA/MDA results. Interpretation of these results should consider several factors including possible further testing for optical isomers and investigation of medications and supplements ingested. Amphetamine and methamphetamine have optical isomers designated *d* (or +) for dextrorotatory and *l* (or -) for levorotatory; the *d* isomers, the more physiologically active compounds, are the intended targets of immunoassays. Because GC/MS procedures using non-chiral derivatives and non-chiral columns do not differentiate the *d* or *l* isomers of amphetamine and methamphetamine it is necessary to perform isomer

resolution to determine that a positive result is due to the presence of the *d* isomer. Primary examples are the presence of *l*-methamphetamine in Vicks inhaler, which can't be distinguished from use of illicit methamphetamine (*d* isomer or racemic mixture depending on method of production), the excretion of *l* methamphetamine and *l* amphetamine by patients taking selegiline for Parkinson's, and the excretion of *d* and *l* amphetamine and methamphetamine by patients taking the analgesic famprofazone [8]. Studies have shown that heavy use of the inhaler can cause false-positive results but that when the inhaler was used as directed no false positive results were obtained [9]. Isomer resolution can be accomplished using a chiral, optically active column or chiral derivatizing reagents [10]. Use of chiral derivatizing reagents allows analysis on instrument/column systems used for other routine analyses but has the potential disadvantage of possibly obtaining four isomers instead of two if the derivatizing agent is not optically pure. N-trifluoroacetyl-l-propyl chloride (l-TPC) and (-)-methylchloroformate can be used to distinguish methamphetamine enantiomers with GC/MS without a chiral column. Chiral columns such as N-3,5-(dinitrobenzoyl)phenyl-glycine or beta cyclodextrin products can differentiate *d* and *l* isomers, or enantiomers, of amphetamines for GC and liquid chromatography (LC) methods [3]. The generally accepted interpretation of isomer resolution results is that greater than 80% of the *l* isomer is considered consistent with use of legitimate medication or conversely greater than 20% of the *d* isomer (and total concentration above the cutoff) is considered evidence of illicit use [9].

Positive amphetamines results can be obtained due to ingestion of medications containing amphetamine, methamphetamine, or compounds metabolized to these compounds.

Amphetamine [Dexedrine (*d*-amphetamine), Adderall (*d* and *l*-amphetamine), etc] and methamphetamine [Desoxyn (*d*-methamphetamine)] are the active compounds of medications prescribed for appetite suppression, narcolepsy, and attention deficit disorder and ingestion of these drugs will result in true positive results due to excretion of these compounds in the urine. In addition, compounds known to be metabolized to amphetamine and/or methamphetamine include selegiline, amphetaminil, benzphetamine, clobenzorex, dimethylamphetamine, ethylamphetamine, famprofazone, fencamine, fenethylline, fenproporex, furfenorex, mefenorex, and prenylamine [11]. Some weight loss or nutritional supplements contain fenproporex and use of these supplements has resulted in detection of *d*-amphetamine in the urines of users [12]. In January 2006 the FDA warned consumers that Brazilian dietary supplements Emagrece Sim and Herbathin contain active drug ingredients [9,13].

Another important consideration when interpreting confirmation results for amphetamines is the possibility of false negative results due to destruction of the compounds or false positive results due to generation of the compounds during the procedure [9]. All GC/MS confirmation procedures for amphetamines and related compounds should include preventative measures to avoid loss of these volatile compounds during the evaporation step of extraction or during analysis. Procedures to reduce/eliminate loss of amphetamines during evaporation include lowering the temperature for evaporation, performing incomplete evaporation or adding methanolic HCl prior to evaporation in order to produce more stable hydrochloride salts [11,14,15]. Derivatization using compounds such as heptafluorobutyric anhydride (HFBA), pentafluoropropionic anhydride (PFPA), trifluoroacetic anhydride (TFAA), 4-carboxyhexafluorobutyryl chloride (4-CB), N-methyl-N-*t*-butyldimethylsilyl trifluoroacetamide (MTBSTFA), N-trifluoroacetyl-1-prolyl chloride (TPC), 2,2,2-tri chloroethyl chloroformate, and propylchloroformate decreases the volatility of amphetamines in addition to improving chromatography and quantitation, and forming higher molecular weight fragments yielding different mass ions and ion ratios than potentially interfering compounds [9]. Contaminants in the heptafluorobutyryl derivatizing reagent have been shown to give methamphetamine peak interferences when ephedrine is present in the sample [16]. In 1993 it was demonstrated that concentrations of methamphetamine less than 50 ng/mL can be generated from high levels of pseudoephedrine or ephedrine in injection ports at a temperature of 300 °C after derivatization with 4-CB, HFBA, and TPC [17]. False positive results in the federal drug testing program due to generation of methamphetamine resulted in the implementation of the requirement that the metabolite amphetamine must be present at a concentration of 100 ng/mL (250 ng/mL prior to October 2010) or higher in order to report a positive methamphetamine [4]. Lowering the injector temperature and periodate pretreatment of samples are two procedures used to prevent generation of methamphetamine. Periodate treatment (0.35 M sodium periodate for 10 min at room temperature) eliminates formation of methamphetamine by selective oxidation of pseudoephedrine, ephedrine, phenylpropanolamine and norpseudoephedrine at concentrations as high as 1,000,000 ng/mL in the presence of amphetamine and methamphetamine [18]. Inclusion of a quality control sample containing a high concentration of sympathomimetic amines in each confirmation batch can be used to monitor the effectiveness of the periodate oxidation procedure [9].

As previously noted there is a HHS requirement that the metabolite amphetamine must be present at a

concentration of 100 ng/mL in order to report a positive methamphetamine. Jemionek et al. reported the detection of low concentrations of methamphetamine in urine specimens containing high concentrations of amphetamine suggesting that the methamphetamine was either a minor by-product of the amphetamine manufacture or the product of a minor metabolic product of amphetamine elimination [19].

Compounds with structures similar to amphetamine and methamphetamine could potentially interfere with GC/MS confirmation procedures. Laboratories that are HHS-certified must perform interference studies for amphetamine confirmation assays by analyzing samples containing interferents (phentermine at 50,000 ng/mL and phenylpropanolamine, ephedrine, and pseudoephedrine at 1 mg/mL) in the presence of and without amphetamine and methamphetamine at 40% of the cutoff [20]. Hydroxynorephedrine, norephedrine, norpseudoephedrine, phenylephrine, and propylhexedrine are other compounds with structures similar to amphetamine and methamphetamine that may be tested for interference [21]. When testing for designer amphetamines such as MDMA and MDA the laboratory must be aware of the metabolism and excretion patterns for these drugs because some of the metabolites (3,4-dihydroxymethamphetamine (HHMA); 3,4-dihy droxyamphetamine (HHA); 4-hydroxy-3-methoxy methamphetamine (HMMA); and 4-hydroxy-3-methoxy amphetamine (HMA)) are excreted as glucuronide and sulfate conjugates. In order to obtain adequate recovery of these metabolites a hydrolysis procedure should be included as part of the confirmation testing [9,21,22].

CONFIRMATION METHODS FOR BENZOYLECGONINE

Cocaine, a naturally occurring alkaloid obtained from the South American shrub *Erythroxylum coca*, is rapidly metabolized by cholinesterases to ecgonine methyl ester and benzoylecgonine that are excreted in urine. Both immunoassay screening and GC/MS confirmation procedures target benzoylecgonine. Because no compounds causing false positive benzoylecgonine confirmation results have been reported the interpretation of positive results should focus on explanations of cocaine introduction into the body. Two possible unintended sources to consider are use of cocaine as a local anesthetic in ENT surgery and ingestion of tea prepared from coca leaves. A positive result due to use of a cocaine solution as a local anesthetic can be verified by the physician performing the procedure. Although teas made from coca leaves are illegal in the US they could be obtained unintentionally during travel in South America or purchased in ethnic or other stores selling herbal teas such as Health Inca and Mate De Coca, 2 coca-leaf teas previously removed from the market.

In October 2010, the confirmation cutoff concentration used in federally-regulated workplace drug testing programs was lowered to 100 ng/mL for GC/MS confirmation methods. Much lower cutoffs are often found in clinical and non-regulated workplace settings. Several LC/MS/MS methods have been described for measurement of cocaine and/or benzoylecgonine in plasma, oral fluid, and hair [23–29].

False negative benzoylecgonine results could be caused by intentional destruction from a chemical adulterant or hydrolysis due to nonspecific esterases or spontaneously occurring at pH above 7. Destruction by hydrolysis can be slowed by freezing the specimen [2]. In order to avoid benzoylecgonine loss sample preparation techniques for confirmation testing should not be performed at a high pH. A typical extraction is performed at pH 6.0. Liquid-liquid extractions using organic solvents such as diethyl ether, chloroform, or a chloroform-isopropanol mixture can be used but many laboratories use SPE.

CONFIRMATION OF OPIOIDS

Opiates and opioids have important therapeutic uses in pain management and other purposes. The term *opiates* refers to naturally occurring alkaloids of the poppy plant, *Papaver somniferum*, notably morphine and codeine, and semi-synthetic alkaloids with similar structures. The semi-synthetic alkaloids of this class include heroin and the prescriptive medications buprenorphine (Buprenex), hydrocodone (Vicodin), hydromorphone (Dilaudid), oxycodone (Percodan, OxyContin), and oxymorphone (Opana). The term *opioids* refer to compounds that have affinity toward opioid receptors and exhibit pharmacological properties similar to morphine, but may be structurally unrelated to morphine. These include fentanyl (Duragesic, Sublimaze), meperidine (Demerol), methadone, pentazocine (Talwin), propoxyphene (Darvon), and tramadol (Ultram). Thus, it can be said that all opiates are opioids, but all opioids are not opiates [1].

On October 1, 2017 the federal workplace drug testing program added testing for hydrocodone, hydromorphone, oxycodone, and oxymorphone at screening concentrations of 300 ng/mL for hydromorphone/hydromorphone and 100 ng/mL for oxycodone/oxymorphone. Confirmation cutoff concentrations for all 4 opioids is 100 ng/mL [4].

As discussed in Chapter 14 the immunoassay screens for opiates are designed to detect morphine and codeine and have varying sensitivity to other related members of this class. These compounds were targeted because of

the desire to detect heroin abuse. With the emergence of pain management testing, the increasing abuse of other opiates, and inclusion of opioids in federal workplace testing immunoassays targeting the opioids hydrocodone, hydromorphone, oxycodone, and oxymorphone are available. Immunoassays to detect methadone and propoxyphene have been available for decades. An assay for buprenorphine was developed when it was introduced as a treatment option for heroin dependency and immunoassays for fentanyl and its metabolites are also now available. Clinicians often mistakenly think that opiate assays (screening and confirmatory) also detect any opioids including methadone, oxycodone and fentanyl and do not realize that the tests have to be ordered separately when detection of these drugs is desired [2]. Confirmation of opioids after immunoassay screening is often performed using LC/MS/MS.

Knowledge and understanding of the complex metabolism of opiates is essential for the interpretation of opiate results. Fig. 15.1 illustrates the metabolism and relationships of several, but not all, opioids and metabolites. Metabolism of opioids typically includes a combination of conjugation via glucuronidation and sulfation and/or oxidation or reduction prior to excretion in urine. The metabolism of several opioids will be discussed below in order to provide information necessary for the interpretation of test results.

Heroin

The primary concern in workplace drug testing of opiates has traditionally been the detection of heroin. Heroin (diacetylmorphine) is rapidly metabolized to 6-acetylmorphine; also called 6-monoactylmorphine (6-AM; 6-MAM) and subsequently to morphine. Testing for 6-AM in addition to morphine and codeine is now required in the federally regulated drug testing system.

Morphine

Approximately 85% of a morphine dose is excreted as parent morphine (75% morphine-3-glucuronide and 10% unconjugated (free) morphine) with another 5% undergoing demethylation to normorphine (4% conjugated normorphine and 1% free normorphine) [30]. Minor metabolites that have been identified include morphine-6-glucuronide, morphine-3-ethereal sulfate, and morphine-3,6-diglucuronide [31]. Hydromorphone has been identified as a minor metabolite in patients chronically treated with morphine with ratios of hydromorphone to morphine from 0.2 to 2.2% [32]. Oxymorphone was not detected in urine from high-dose morphine and high-dose hydromorphone patients indicating that oxymorphone is not a metabolite of morphine or hydromorphone [33,34].

Codeine

Codeine is demethylated to morphine and norcodeine with some individuals (approximately 7% of Caucasians and 1–3% in other ethnic groups) being poor metabolizers because of a CYP2D6 deficiency [35]. The urinary excretion pattern for a dose of codeine includes

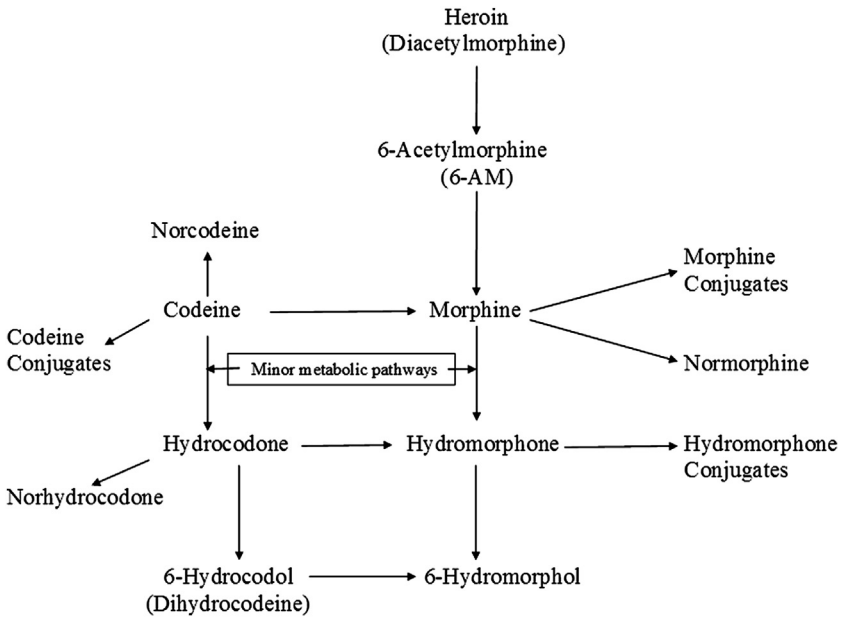


FIG. 15.1 Metabolism of selected opioids.

the following approximate percentages: 32–46% conjugated codeine, 5–17% free codeine, 10–21% conjugated norcodeine, 5–13% conjugated morphine, trace amounts of free norcodeine and morphine [29]. Oyler et al. reported that hydrocodone can be produced as a minor metabolite of codeine and may be excreted in urine at concentrations as high as 11% of the codeine concentration [34,36].

Hydrocodone

Hydrocodone is initially metabolized to hydromorphone and norhydrocodone with the urinary excretion pattern of a hydrocodone dose giving these approximate levels: 12% free hydrocodone, 5% norhydrocodone, 4% conjugated hydromorphone, 3% 6-hydrocodol, and 0.1% as conjugated 6-hydromorphol [30].

Hydromorphone

Approximately 36% of a dose of hydromorphone (30% conjugated hydromorphone and 6% free hydromorphone) is excreted without undergoing oxidation or reduction. Cone et al. reported metabolism of hydromorphone via reduction to trace amounts of conjugated 6α- and 6β-hydromorphol [37].

Oxycodone

Oxycodone is extensively metabolized with the following approximate levels reported from a single dose: 8% free oxycodone, 23.1% free noroxycodone, 10.4% conjugated oxymorphone, 0.3% free oxymorphone,8.6% conjugated noroxymorphone,5.6% free noroxymorphone, 6.0% α-oxycodol, 1.9% β-oxycodol, 6.8% α-noroxycodol and 1% as β-noroxycodol [38].

Oxymorphone

The urinary excretion pattern of oxymorphone includes approximately 44% as conjugated oxymorphone, 1.9% free oxymorphone, and 3% as 6-oxymorphol (2.9% free and conjugated 6β-oxymorphol and 0.1% 6α-oxymorphol) [30].

Methadone

Methadone, a synthetic opioid used as a treatment for heroin (and other opioids) dependence and for chronic pain, is subject to wide variations in inter- and intra-individual pharmacokinetics. The primary methadone metabolite is 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), which is inactive. Approximately 5–50% of a dose is eliminated in the urine as methadone and 3–25% as EDDP. Detection of both methadone and its primary metabolite, EDDP, should be included when using urine drug testing to monitor methadone treatment for addiction or pain management. When immunoassay is used as the testing technique, two separate assays are required because the immunoassays for methadone and EDDP demonstrate little or no cross-reactivity to each other [1].

Interpretation of opioid results

Because of the complex metabolic relationships described previously (Fig. 15.1) interpretation of opioid results is not always straightforward. One may not be able to definitively determine ingestion of certain compounds versus the presence of these compounds as metabolites of ingestion of other compounds. Some factors to be considered when interpreting these results are discussed below.

Heroin use can be confirmed by the detection of morphine and 6-AM, the metabolite unique to heroin. The absence of 6-AM does not confirm non-use of heroin because it has a shorter half-life than morphine. More difficult to explain is a positive 6-AM in the absence of morphine, an occurrence reported in some samples since 6-AM screening became part of the federal testing program in October 2010. Speculative explanations include the existence of a unique metabolic pathway in some individuals or the simultaneous introduction of small amounts of morphine and generation of 6-AM by the laboratory during the testing process (discussed later in this section). In addition to heroin or morphine use there are other possible considerations for the interpretation of positive morphine results. Poppy seeds contain morphine naturally and their ingestion may cause a positive opiates result regardless of the testing methods (immunoassay or chromatography). In an effort to eliminate or reduce the number of positive results due to poppy seed ingestion, the Department of Defense raised the opiate cutoff levels from 300 to 2000 ng/mL in 1994. In December 1998, federally mandated workplace programs followed suit.

Lower cutoffs continue to be used in clinical and non-regulated settings. In the pain management setting testing is performed not only to confirm use of the medication as prescribed but also to determine if the patient is taking other opioids. Sometimes the presence of a compound not previously reported as a metabolite is interpreted as use of the compound. For example, before hydromorphone was identified as a minor metabolite in patients chronically treated with morphine the presence of hydromorphone in the urine of a patient treated with morphine could be interpreted as ingestion of hydromorphone [32]. There have been attempts to use ratios

of parent and metabolite concentrations to interpret opioid results but this is difficult [39].

The variable pharmacokinetics and potential drug-drug interactions associated with methadone contribute to a range of methadone and EDDP concentrations in the urine of patients being monitored. Compliant patients are expected to be positive for both methadone and EDDP, but a sample that is negative for methadone and positive for EDDP could indicate the patient is a rapid metabolizer. Negative results for both methadone and EDDP typically indicate that methadone was not ingested and could result from bingeing. These results could also occur if levels of both methadone and EDDP are below the cutoff concentrations as would occur in a dilute urine sample. A technique known as spiking has been used by patients to mimic compliance when diverting methadone for sale. Since this involves the addition of a small amount of the dose to the urine after collection (before testing), this yields a strongly positive result for methadone but a negative EDDP. It should be noted that this is also attempted with other drugs (such as buprenorphine) used in rehabilitation programs [1].

Because of the complex metabolism of opioids and the similarity in structures another factor to consider when interpreting opioid results is the potential for interferences in the GC/MS or LC/MS/MS confirmation procedures. HHS-certified laboratories must perform interference studies for opioids confirmation assays by analyzing samples containing interferents (hydrocodone, hydromorphone, oxycodone, oxymorphone, and norcodeine at 5,000 ng/mL) in the presence and absence of morphine and codeine at 40% of the cutoff [20]. Similarly, interference studies for 6-acetylmorphine assays must test free morphine, codeine, hydrocodone, hydromorphone, oxycodone, oxymorphone, and norcodeine at 5,000 ng/mL as potential interferents [20]. Also assays for hydrocodone, hydromorphone, oxycodone and oxymorphone must test for the following interferents: 6-AM at 200 ng/mL; free morphine and codeine at 40,000 ng/mL; norcodeine and norhydrocodone at 5000 ng/mL; noroxycodone and noroxymorphone at 1000 ng/mL [20]. Several of these potential interferents are keto opiates and a dual derivatization procedure (oxime derivatization step using reagents such as hydroxylamine and methoxylamine followed by trimethylsilyl derivatization) produces compounds that separate chromatographically from codeine and morphine and prevent interference [40]. Similar procedures eliminate potential interferences with the measurement of 6-AM [41,42]. In addition to interference studies the initial validation study by HHS-certified laboratories using LC/MS/MS methods for opioids (and any other analytes) must include evaluation of matrix effects that assesses the entire confirmatory test process. A matrix effect study should be performed for any LC/MS/MS procedure and the recommended approach to such a study is described by Matuszewski, Constanzer, and Chavez-Eng [43].

As previously discussed opiates such as morphine and codeine are excreted primarily as conjugated parent compound or metabolites. Incomplete recovery of these compounds during the confirmation procedure can lead to falsely decreased concentrations or even false negative results. Hydrolysis to convert the conjugate to free drug prior to extraction is necessary when testing for opiates. Wang et al. reported that acid hydrolysis, which liberated >90% of morphine and hydromorphone glucuronides was preferable to hydrolysis using β-glucuronidase from various sources [44,45]. This report of inefficient enzyme hydrolysis is in disagreement with other reports, perhaps due to the fact that many factors including buffer pH and composition, ionic strength of the solution, preparation in silylized glassware, temperature and length of incubation can affect enzymatic hydrolysis [40,46–54]. It is important to note that acid hydrolysis will lead to the degradation of 6-AM and its extraction is typically carried out separately from the extraction of morphine and codeine. To prevent loss of 6-AM extraction is typically performed using silanized glassware. Furthermore, once extracted, the analytes are subject to additional losses and should be analyzed immediately.

The National Laboratory Certification Program (NLCP) reported 2 instances of a laboratory generating 6-AM when an acetyl donor such as ethyl acetate or acetic acid was used during extraction, derivatization, and/or confirmatory analysis of specimens containing morphine [55]. One sample was a proficiency testing sample containing 5000 ng/mL free morphine and the other sample was a donor specimen containing more than 200,000 ng/mL morphine. Laboratories were advised not to use compounds that could serve as acetyl donors during 6-AM confirmatory procedures. In order to demonstrate the absence of generation of 6-AM in samples containing high concentrations of morphine laboratories should include a control containing approximately 90% conjugated and 10% free morphine. Because this situation would not occur in the absence of morphine it would not explain a result of positive 6-AM and negative morphine unless morphine was also introduced during the testing process (i.e. as a contaminant in the deuterated morphine IS).

CONFIRMATION OF MARIJUANA METABOLITE

Cannabinoids are a group of compounds present in marijuana, a recreational drug that originates from the

dried leaves and flowers of *Cannabis sativa*. The principal psychoactive cannabinoid is Δ^9-tetrahydrocannabinol (THC). More than 100 THC metabolites have been identified and more of the dose is excreted in feces than in urine. The major urinary metabolites are 11-nor-tetrahydrocannabinol-9-carboxylic acid (THCA) and its glucuronide conjugate. Screening immunoassays for cannabinoids detect multiple metabolites of marijuana with a cutoff concentration of 50 ng/mL used for regulated drug testing and as low as 20 ng/mL in the clinical and non-regulated setting. GC/MS confirmation identifies and quantifies THCA in urine using a cutoff of 15 ng/mL in regulated drug testing. GC/MS confirmation procedures include a basic hydrolysis step of the glucuronide conjugate, using a reagent such as NaOH.

Interpretation of a positive marijuana metabolite result using a cutoff of 50 ng/mL indicates use but it is difficult to determine the time of use. Due to the lipophilicity of THC and its metabolites, marijuana may be detected in urine for several weeks post-exposure, particularly with chronic use. Chronic marijuana users may produce positive results for longer periods of time because of accumulation of cannabinoid metabolites in fatty tissue followed by slow release. This variable release from tissue and differences in hydration status can lead to a false assumption of new marijuana use if a negative result is followed by a positive result. Creatinine normalization (dividing the THCA result by the creatinine result) has been used to address this problem. For less-than-daily users, an increase of greater than 50% in the normalized THCA concentration is considered indicative of new marijuana use. For chronic users, the excretion pattern is more complicated as they have smaller decreases in excretion later in the elimination phase. Increase of greater than 150% for these users has been suggested as an indicator of new use. Marijuana smoke can be inhaled by a nonsmoking individual but studies have shown that it is virtually impossible that this passive inhalation can cause a positive result when using a screening cutoff of 50 ng/mL [1].

CONFIRMATION OF PHENCYCLIDINE

Although phencyclidine (L-phenylcyclohexylpiperidine, PCP) is classified as a dissociative anesthetic it also exhibits hallucinogenic, stimulant, depressant, and analgesic properties.

The cutoff concentration for immunoassay screening and GC/MS confirmation of PCP in regulated drug testing is 25 ng/mL. Compounds causing false positive immunoassay screening results are discussed in Chapter 14 and no compounds causing false positive GC/MS results have been reported.

CONFIRMATION OF BENZODIAZEPINES

Benzodiazepines, among the most frequently prescribed drugs in the US, include alprazolam (Xanax), chlordiazepoxide (Librium), clonazepam (Klonopin), diazepam (Valium), estazolam (ProSom), flunitrazepam (Rohypnol), flurazepam (Dalmane), lorazepam (Ativan), midazolam (Versed), oxazepam (Serax), prazepam (Centrax), quazepam (Doral), temazepam (Restoril), and triazolam (Halcion). With the exception of flunitrazepam, those listed are available for use in the United States. It should be noted that a number of others are available in other countries and occasionally make their way into this country.

After oral administration, benzodiazepines are well absorbed and distributed throughout the body. They are extensively metabolized in the liver via dealkylation, reduction, and hydroxylation, followed by conjugation. The major urinary products of benzodiazepines are the glucuronide metabolite conjugates. Interpretation of urinary metabolite results is difficult because the metabolism of different benzodiazepines often results in common metabolites such as oxazepam, temazepam, and nordiazepam. The cytochrome P450's 3A3 and 3A4 enzymes mediate the hydroxylation and dealkylation reactions, so co-administration of drugs that inhibit or induce these enzymes affects benzodiazepine metabolism. Similarly, any drugs that alter glucuronyl transferase activity may affect benzodiazepine metabolism.

Benzodiazepines immunoassays target oxazepam or nordiazepam and the variable cross-reactivity to other benzodiazepines. Those benzodiazepines that are not metabolized to oxazepam or nordiazepam, such as flurazepam, alprazolam, lorazepam, clonazepam, and triazolam, may not be detected by immunoassays. To date, no immunoassay detects every benzodiazepine nor all of the metabolites and as a result, false negatives occur. Because metabolites are excreted as glucuronide conjugates, the sensitivity of immunoassays may be improved by using a hydrolysis step prior to immunoassay testing. This is cumbersome and not realistic in many settings. It is thus important that the laboratory and clinician clearly understand which benzodiazepines and metabolites are detected.

Methods for confirmation testing of benzodiazepines include GC and LC with MS detection [56–58]. Confirmation methods using GC/MS generally report cutoff concentrations of 50 ng/mL. Most procedures detect oxazepam, nordiazepam, temazepam, α-hydroxyalprazolam, and diazepam at a minimum. The first step in confirmation testing is hydrolysis of the conjugated metabolites using either glucuronidase or HCl. HCl hydrolysis cleaves the benzodiazepine ring to form benzophenone that can be separated and identified.

Disadvantages of benzophenone producing methods include inability to identify specific benzodiazepines present and the inability to detect triazolobenzodiazepines such as alprazolam. For this reason, many laboratories have turned to LC/MS/MS and some of these methods permit the identification of multiple benzodiazepines and metabolites at low concentrations. As mentioned previously interpretation of results to determine benzodiazepines ingested is challenging since several of these drugs are metabolized to the same metabolites. Depending on the method and drug ingested it may not be possible to determine exactly which drug was ingested.

Flunitrazepam (Rohypnol) is a potent sedative hypnotic that is banned in the United States but is prescribed in other countries for the treatment of insomnia. It is important to remember that benzodiazepines immunoassays vary considerably in their ability to detect flunitrazepam and many have very little cross-reactivity with the drug or its metabolites. There are however, specific immunoassays designed specifically for flunitrazepam detection. Confirmation testing using GC/MS or LC/MS/MS typically targets detection of flunitrazepam and its principal metabolite, 7-aminoflunitrazepam [1].

CONFIRMATION OF BARBITURATES

Barbiturates are typically classified by their duration of action. Common barbiturates include phenobarbital (long acting), butalbital (intermediate acting), pentobarbital and secobarbital (both short acting), and thiopental (ultra-short acting). Abuse of barbiturates decreased significantly as the use of benzodiazepines increased.

All barbiturates are strong inducers of hepatic microsomal enzymes; and for this reason, they are often found to influence the metabolism of co-administered drugs. For example, phenobarbital induces uridine diphosphate glucuronyl transferase (UGT) enzymes and the CYP2C and CYP3A subfamilies of cytochrome P450. Other drugs metabolized by these enzymes would be metabolized more rapidly when co-administered with phenobarbital. Similarly, secobarbital is a strong CYP2A6 and CYP2C8/9 inducer. Thus use of barbiturates could conceivably cause false negative results for other drugs due to rapid metabolism of the drug(s).

The immunoassays for barbiturates are designed to detect secobarbital, usually with a screening cutoff of 200 or 300 ng/mL. Cross-reactivity to other barbiturates varies, but is generally sufficient to allow their detection. Acceptable confirmation methodologies include GC, HPLC, GC/MS, or LC/MS/MS [59–61]. The most common barbiturates included in a confirmation panel are amobarbital, butalbital, pentobarbital, phenobarbital, secobarbital, and less often, butabarbital. Interpretation of the results is simpler than that of opiates and benzodiazepines.

SPECIMEN VALIDITY TESTING

Drug abusers try to avoid detection by drug testing in many ways including use of products designed to prevent detection of drugs present in a urine sample. Some products advertised to "beat a drug test" require ingestion (usually in conjunction with large amounts of water) prior to submission of a urine sample while other products are to be added to or substituted for the urine sample itself. Several of these products were discussed in Chapter 14. Collection procedures have been designed to prevent such substitution, dilution, or adulteration of the specimen. These procedures include collection site preparation (i.e., no hot water, addition of a coloring agent to the toilet, no coats or purses, etc.) and use of collection cup temperature monitoring devices. Tests of specimen validity can be performed in an attempt to detect tampering with the samples.

HHS-certified laboratories are now required to perform specimen validity testing to identify dilute, substituted, adulterated or invalid samples submitted for regulated drug testing (Table 15.1). This testing includes creatinine, specific gravity (when the creatinine is below 20 ng/mL), pH, and nitrites measurements. Testing for known adulterants such as glutaraldehyde, pyridinium chlorochromate, or oxidizing chemicals as a class is optional. Criteria for the testing process is very similar to that of testing for drugs, that is, the results must be obtained using approved initial and confirmatory tests on two separate aliquots of urine. A sample is reported as *adulterated* when test results show the presence of a substance that is not a normal constituent or an endogenous substance present in an abnormal concentration. A sample is reported as *substituted* when creatinine and specific gravity values are outside the physiologically possible ranges of human urine indicating submission of a non-urine specimen. A result of *substituted* or *adulterated* indicates intention to circumvent testing for drugs and, in some settings, is treated the same as a positive drug result. A result is reported *dilute* when the creatinine and specific gravity values are lower than expected but are still within the physiologically producible ranges of human urine. A dilute specimen does not necessarily indicate specimen tampering because it can result from consumption of large amounts of fluid or by addition of liquid to the urine specimen.

The result of *invalid* can apply to a number of situations related to the ability to perform all tests satisfactorily. A result is reported as invalid if the laboratory is

TABLE 15.1 Reporting specimen validity test results by HHS-certified laboratories.

Test(s)	Result(s)	Reported as
pH	<4.0 or ≥11.0	Adulterated
	≥4.0 & <4.5	Invalid
	≥9.0 & <11.0	Invalid
Nitrite	≥500 mcg/mL	Adulterated
	≥ 200 & ≤ 500 mcg/mL	Invalid
Creatinine & Specific Gravity	≥2.0 & <20 mg/dL >1.0010 & <1.0030	Dilute
Creatinine & Specific Gravity	<2.0 mg/dL	Substituted
	≤1.0010 or ≥ 1.0200	
Creatinine & Specific Gravity	<2.0 mg/dL	Invalid
	Acceptable (1.0011 −1.0199)	
Creatinine & Specific Gravity	≥2.0 mg/dL ≤1.0010	Invalid

Definitions

Adulterated specimen: a urine specimen that has been altered, as evidenced by test results showing either a substance that is not a normal constituent or showing an abnormal concentration of an endogenous substance. Program cutoffs have been established for pH, nitrite and other adulterants to distinguish between acceptable and adulterated urine specimens.

Substituted specimen: a specimen that has been submitted for testing in place of the donor's urine, as evidenced by creatinine and specific gravity values that are outside the physiologically producible ranges of human urine. Program cutoffs have been established for specific gravity and creatinine to distinguish between acceptable, dilute, and substituted specimens.

Dilute specimen: a urine specimen with creatinine and specific gravity values that are lower than expected but are still within the physiologically producible ranges of human urine. Donors may deliberately dilute specimens by consuming large amounts of water or other liquid or by adding liquid to their urine specimens. A dilute finding is *not* direct evidence of specimen tampering.

Invalid result: refers to the result reported by a laboratory for a urine specimen that contains an unidentified adulterant, contains an unidentified interfering substance, has an abnormal physical characteristic, or has an endogenous substance at an abnormal concentration that prevents the laboratory from completing testing or obtaining a valid drug test result.

Source: National Laboratory Certification Program Manual for laboratories and inspectors. December 2018.

unable to complete testing or obtain a valid drug test result due to the specimen containing an unidentified adulterant or interfering substance, or having an abnormal physical characteristic or endogenous substance at an abnormal concentration. Reporting the specimen validity test results (including an invalid result) does not preclude reporting the drug test results. Thus a result of dilute, adulterated, substituted, or invalid can be reported in conjunction with positive results for specific drugs if the criteria for identification and reporting of the positive result are met. To reiterate selected drugs may be reported positive even if the laboratory is unable to complete testing for all of the drugs in the panel. For example, a result of *THC positive*, *invalid result* means that the laboratory was able to confirm the presence of THC (and hence legally defend this result) but was unable to complete the testing for one or more of the other drugs in the panel for one of the reasons previously mentioned. Regulated samples reported as positive for any drug, substituted, adulterated, and/or invalid must be retained in secured frozen storage for a minimum of 1 year [1].

CONCLUSIONS

In order to accurately interpret drug-testing results more information than the result itself (typically positive or negative) is required. A positive result for a drug or a class of drugs does not automatically indicate intentional use of an illegal substance or non-prescribed ingestion of medication. Some of the factors influencing interpretation include: sample tested, purpose of test request, scope of testing performed (i.e., what drugs are included in the drug panel), knowledge of components and analytical parameters of the initial and confirmation methods used, presence of other chemicals in the sample, physiological state of the donor, and metabolism of the drug(s). All of these factors should be considered when decisions involving interpretations of results are made particularly considering the possible subsequent consequences.

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CHAPTER

16

Issues of false negative results in toxicology: difficult in detecting certain drugs and issues with detection of synthetic cathinone (bath salts), synthetic cannabinoids (spice), and other new psychoactive substances

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INTRODUCTION

Clinicians rely on accurate drug testing in the clinical laboratory to provide quality patient care. In the last decade, the relevant comprehensive abilities of drug testing laboratories have been challenged with the continued emergence and proliferation of new/novel psychoactive substances (NPS). NPS are illicit synthetic compounds manufactured clandestinely and distributed throughout the world. NPS can be divided into various categories including synthetic cathinones (bath salts), synthetic cannabinoids (spice), tryptamines, piperazines, as well as analogues of medications such as amphetamines, fentanyl and other opioids. In a clinical laboratory, false negatives for NPS and other illicit substances may prevent health care professionals from effectively treating patients with substance use disorders or understanding the cause of illness in the emergency room patient. The general lack of knowledge and absence of analytical reference materials and standards have led to the inability to detect NPS presence in biological fluids and subsequent false negatives reporting from clinical laboratories [1].

Traditional analytical assays utilize targeted methodologies that identify exact compounds or drug classes within the confines of the assay's sensitivity and specificity. Techniques often include specific immunoassays and various mass spectrometry techniques, including liquid chromatography with tandem mass spectrometry (LC-MS/MS), for qualitative and/or quantitative determination at lower limits of detection and increased specificity. Various mass spectral libraries are available to assist with the identification of unknown compounds as well as software that can help to predict the mass spectral patterns of drugs and their metabolites. However, due to the lack of drug standard materials the laboratories may not be able to accurately determine an unknown compound. Laboratories can only positively identify a compound in biological fluids if it is anticipated at the time of method development and validation.

This chapter provides a brief background and overview on the notable synthetic cannabinoids, cathinones and fentanyl analogue NPS classes. Analytical detection challenges and potential solutions will be discussed in context of these classes however, the same generalized discussion points apply to all NPS.

ABUSE OF NPS

Synthetic psychoactive substances have been a threat to public health for over a century. Today's NPS are considered to be drugs not covered under the 1961 and 1971 United Nations drug control conventions [2]. Over the last decade NPS continue to appear at an

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unprecedented rate. They are readily attained at local retailers (i.e. head shops) and from online sources under common names such as, 'bath salts', 'herbal highs', 'research chemicals', and 'incenses' [2–6]. These drugs are often referred to as "legal highs" due to the difficulty in detecting them and subsequent delay in scheduling by drug enforcement agencies [4,6].

The United Nations Office on Drugs and Crimes reported the emergence of 739 NPS between 2009 and 2016 [6]. Of those, only 80 were detected each year spanning 2009–15 and 60 seem to have completely disappeared [6]. Due to the difficulty in detecting NPS, it is entirely possible these numbers are underestimated. On August 15, 2018 a massive overdose of >70 individuals in New Haven, Connecticut was reported to be caused by a common synthetic cannabinoid marketed under the brand name K2 laced with fentanyl [7]. This brand of synthetic cannabinoid has been known to contain NPS of the JWH type, commonly JWH-018 and/or JWH-073 [8]. Another NPS closely related to fentanyl known as U-47700 or Pink was emergently scheduled to class I on November 14th, 2017 by the United States Drug Enforcement Administration after several fatalities were reported [9].

These events underscore the dangers NPS continue to pose to public health and safety. In order to keep pace with the rapidly evolving NPS landscape and support quality patient care, clinical laboratories need to improve detection techniques and methodologies. Of note, much of the NPS production is due to unregulated industrial scale pharmaceutical manufacturing operations in China and other parts of Asia [2].

Rise of synthetic cannabinoids, cathinones and fentanyl analogues abuse

New analogues of synthetic cannabinoids, cathinones and fentanyl continue to emerge. Within these drug classes, structural changes are made frequently, which in turn, changes the drug and the ability to detect them with analytical technologies reliant on pre-specified chemical structures.

Synthetic cannabinoids were formulated as research candidates for pharmaceutical therapeutic agents following the discovery of the cannabinoid receptors in the mid-1980s and first appeared on the illegal drug market in the mid-2000's [10]. Synthetic cannabinoids are Cannabinoid receptor type 1 (CB1) and type 2 (CB2) agonists designed to mimic the effects of cannabis [6]. Their toxicity often lies in the extreme potency of their agonist activity, relative to the psychoactive component of cannabis, delta-9-tetrahydrocannabinol (THC).

Spice compounds have undergone significant chemical structure modifications in an effort to evade detection and regulation. Typically for synthetic cannabinoids and cathinones, new analogs of these drugs appear just after the preceding drug comes under regulation. Generally, the regulations are limited by the structures of the drugs. Therefore, modifications of a side chain or a substitution of one constituent for another provides just enough change to now be regarded as being beyond the scope of regulation. One of the predominate Spice compounds, JWH-018, is a naphthoylindole alkylated at the indole nitrogen [11]. This compound was quickly developed into a naphthoylindazole THJ-018, indazole carboxamide AKB-48 and, more recently, another indazole carboxamide MDMB-CHIMICA. This phenomenon of molecular structure manipulation has led to hundreds of compounds detected in products on the street [2]. It is important to note that Spice compounds do not highly interact with cannabinoid-receptor independent pathways, like the phytocannabinoid cannabidiol (CBD). CBD activity in traditional cannabis is responsible for some of the reported positive health benefits, such as its anxiolytic effects [12]. Chemical structures of some commonly abuse synthetic cannabinoids are shown in Fig. 16.1.

Cathinone, a monoamine alkaloid, was first discovered in 1887 as a natural component in the khat plant [13]. In recent years, the abuse of synthetic cathinones has increased and is now second in prevalence among NPS relative to synthetic cannabinoids. Potency of these synthetic psychostimulants are reported to be greater than amphetamine, but less than methamphetamine [14]. In fact, most synthetic cathinones are amphetamine, methamphetamine, or 3,4-Methylenedioxymethamphetamine (MDMA) type analogues [15,16]. The chemical structure of a cathinone is defined by a characteristic β-keto group on the side chain of the corresponding phenethylamine [5]. Cathinone has been the primary model for synthetic cathinone or bath salt production [5]. MDMA is another example where modification has led to the

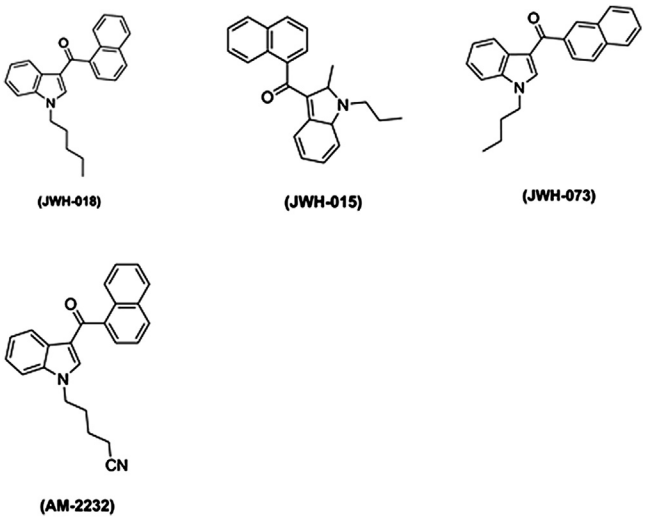


FIG. 16.1 Chemical structures of common synthetic cannabinoids JWH-108, JWH-015, JWH-073 and AM-2232.

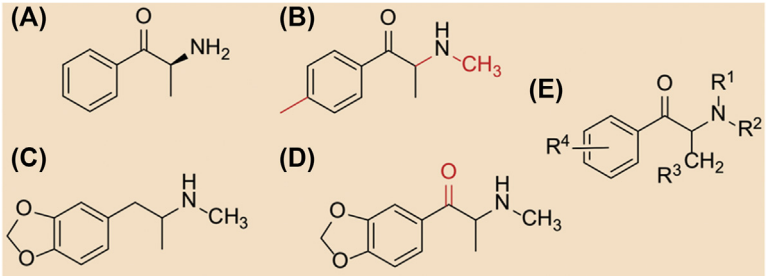


FIG. 16.2 Chemical structures of cathinone (A), mephedrone (B), MDMA (C) and methylone (D). Differences between controlled substances (i.e., cathinone and MDMA) and synthetic derivatives of cathinones (i.e., mephedrone and methylone) are highlighted in red. The molecular structure of generic cathinone derivatives is represented in structure (E). The 'R' groups indicate locations of the molecule where modifications can occur to produce a wide range of cathinone derivatives.

commonly abused bath salt methylone, as illustrated in Fig. 16.2 above. The most commonly abused synthetic drugs in this class have been reported to be 4-methylmethcathinone (mephedrone), 3,4-methylenedioxy-N-methylcathinone (methylone), and 4-methylenedioxypyrovalerone (MDPV). However, after MDPV and mephedrone's rapid rise in abuse, DEA scheduling has drastically decreased their prevalence and new synthetic cathinones like alpha-PVP and ethylone appeared the following year [15,17]. Chemical structure of cathinone and commonly abuse synthetic cathinones are listed in Table 16.1.

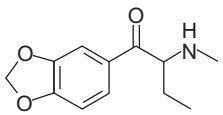
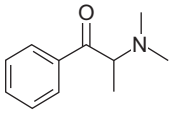
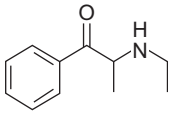
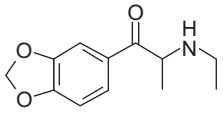
Synthetic fentanyl analogues are the newest NPS threat to public safety and present similar analytical challenges as the synthetic cannabinoids and cathinones. Several fentanyl analogs were developed between the 1960s and 1990s for pharmaceutical research purposes but have been rediscovered by clandestine laboratories and are now being produced on a large scale for illegal sale. Examples of such compounds include acetylfentanyl, butyrfentanyl, furanylfentanyl and ocfentanil [6]. In 2017, the EU early warning system reported that of the 28 different synthetic fentanyls identified, 18 were reported since 2016 [2]. Most commonly these substances appear in powder form but, also exist in tablet and liquid forms [2].

Compounding on the opioid crisis, these fentanyl analogues are hundreds to thousands of times more potent as compared to morphine [18]. They have appeared in counterfeit prescription medicines and as additives in street drugs such as cocaine and heroin causing drug users to inadvertently overdose [2]. Of all the NPS classes, synthetic opioids and fentanyls provided the greatest risk of mortality. Fentanyl, already 50–100× more potent towards the μ-opioid receptor as morphine, is often used as a model in synthesis of these new compounds. The chemical modifications in some of the commonly encountered synthetic fentanyl analogues are shown in Fig. 16.3.

ANALYTICAL CHALLENGES

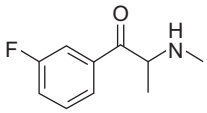
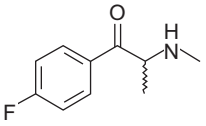
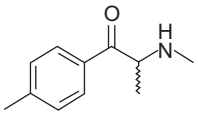
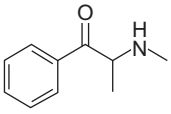
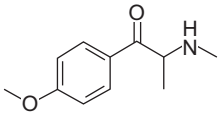
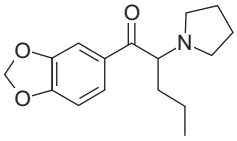
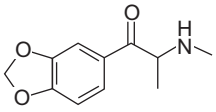
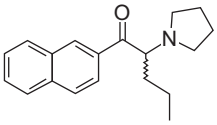
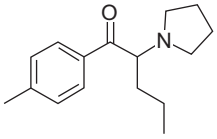
Analytical assays for NPS are complex due to the expansive number of compounds and the absence of chemical standard materials for parent drugs and their metabolites. In addition, analysis is further

TABLE 16.1 Names and chemical structures of synthetic cathinones used as recreational drugs.

Common name and abbreviation	Chemical name	Chemical structure
Butylone (β-keto-N-methylbenzodioxolylbutanamine [βk-MBDB])	1-(1,3-Benzodioxol-5-yl)-2-(methylamino)butan-1-one	
N,*N*-Dimethylcathinone (methamfepramone)	(*RS*)-2-dimethyloamino-1-phenylpropan-1-one	
N-Ethylcathinone (EC)	(*RS*)-2-(ethyloamino)-1-phenyl-propan-1-one	
Ethylone (4β-MDEA)	(*RS*)-1-(1,3-benzodioxol-5-yl)-2-(ethyloamino) propan-1-one	

Continued

TABLE 16.1 Names and chemical structures of synthetic cathinones used as recreational drugs.—cont'd

Common name and abbreviation	Chemical name	Chemical structure
3-Fluoromethcathinone (3-FMC)	(*RS*)-1-(3-fluorophenyl)-2-(methylamino)propan-1-one	
4-Fluoromethcathinone (4-FMC)	(*RS*)-1-(4-fluorophenyl)-2-(methylamino)propan-1-one	
Mephedrone (4-methylmethcathinone; 4-MMC)	(*RS*)-2-methyloamino-1-(4-methylphenyl)propan-1-one	
Methcathinone (ephedrone)	α-(Methyloamino)-1-phenyl-propan-1-one	
Methedrone (4-methoxymethcathinone, βk-PMMA)	(*RS*)-1-(4-methoxyphenyl)-2-(methyloamino) propan-1-one	
3,4-Methylenedioxypyrovalerone (MDPV)	(*RS*)-1-(benzo[*d*][1,3]dioxol-5-yl)-2-(pyrrolidin-1-yl) pentan-1-one	
Methylone (3,4-methylenedioxy-*N*-methylcathinone; βk-MDMA)	(±)-2-Methylamino-1-(3,4-methylenedioxyphenyl) propan-1-one	
Naphyrone (naphylopyrovalerone)	1-(Naphthalen-1-yl)-2-(pyrrolidin-1-yl)pentan-1-one	
Pyrovalerone	(*RS*)-1-(4-methylphenyl)-2-(pyrrolidin-1-yl) pentan-1-one	

Source: Zawilska JB, Wojcieszak J. Designer cathinonoes-an emerging class of novel recreational drugs. Forensic Sci Int 2013;231:42—53. Table 1 in the original published article. ©Elsevier. Reprinted with permission.

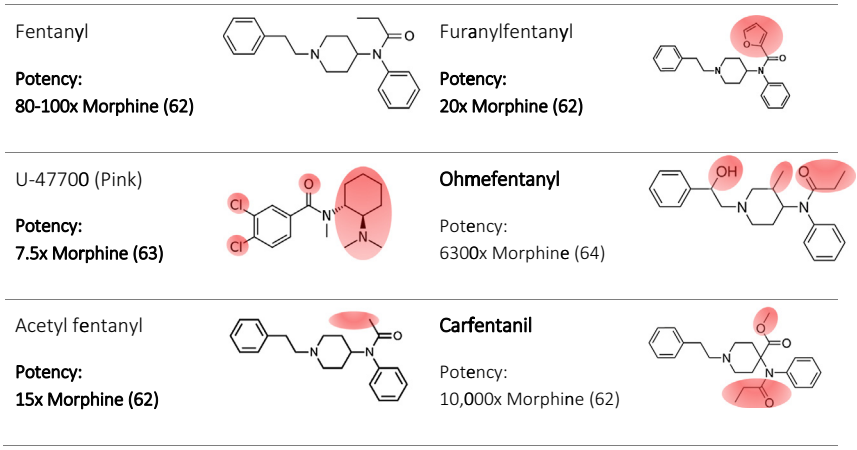


FIG. 16.3 Chemical structures of fentanyl and commonly abused fentanyl analogs. *Examples of Chemical modifications leading to new synthetic fentanyl analogues. Structures were obtained from the PubChem database [62]. Potency relative to morphine is followed by references in parenthesis [63,64,65].*

compounded by low drug concentrations in biological fluids and analyte stability. When preparing to develop a relevant up-to-date assay the laboratory scientist will typically focus on resources published in the last six months to one year. Primary resources for this information should include industry-observed pattern reports such as those from the National Forensic Laboratory Information System (NFLIS), United Nations Office on Drugs and Crime, United States Drug Enforcement Administration (DEA), European Monitoring Center for Drugs and Drug Addiction (EMCDDA), and peer-reviewed scientific literature.

Reference standards for the appropriate analytical target

As mentioned above, the most significant challenge to developing relevant assays is the availability of verified reference standards for the correct chemical target (i.e., parent and/or metabolites). Clinical laboratories require verified standards to develop and validate methodologies that are precise and accurate enough to analyze patient samples. Misidentification or false positive results in clinical laboratories can also adversely affect patient care. The lack of reference standards reflects the difficulty not only in preliminary detection of the NPS but also of their appropriate target metabolites.

A mass spectrum of an unidentified drug may be achieved without a reference material, however, the spectrum cannot be properly identified without the aid of a reference standard. As new designer drugs are developed for illicit use, the reference material industry is usually one-step behind [19]. It is possible for mass spectrometer laboratories to purchase library software and libraries with spectrums of numerous drugs, however, this process still requires the library vendor to purchase the reference material, still availability-dependent.

The International Organization for Standardization (ISO) is responsible for ensuring a high degree of quality is maintained when producing reference materials. Vendors of reference materials must be compliant with ISO, otherwise the quality of the reference material may be suboptimal. The ISO has certifications for vendors' overall operations, quality management systems, medical devices, and technical competency. These certifications provide a level of confidence that the reference material was produced with a high level of quality. The challenge that reference material vendors face is producing quality reference materials in a rapidly changing, high demanding environment. Starting materials can be synthesized but this process usually requires control materials. Alternative sources for starting materials, such as pharmaceutical impurities or internet sourced products have been discussed as a means to supply reference materials for laboratories [20]. Drug standard availability is a major limitation that affects all clinical toxicology laboratories. The reference material industry has been disrupted by the demand to detect the latest illicit designer drugs, which changes rapidly and unpredictably.

It is important to consider the metabolites of the NPS parent drugs as well. Due to metabolism and excretion of the parent drug, metabolite(s) of a parent may be the only way to determine if a patient used an illicit substance.

Only after the parent NPS is identified and characterized can even more advanced techniques be used to determine proper metabolite targets in urine. Incubation with human liver microsomes and analysis by high-resolution mass spectrometric (HR-MS) identification is a common technique in detecting metabolites. These new metabolite targets must then be verified in authentic biological samples before synthesizing and marketing commercial reference standards. In one study, AM-2201 generated 13 different metabolites when examined *in vitro*, but only 3 were detected after in vivo administration of AM-2201 in rodents [3]. Additionally, some commercial reference standard manufactures synthesize theoretical urinary metabolites generated by *in silico* metabolomic models. These too must be verified in authentic urine samples by the laboratory if the manufacturer cannot provide sufficient evidence.

The metabolism of synthetic cathinones has been increasingly studied. Generally, synthetic cathinones undergo N-demethylation to basic amines and subsequent reduction of the ketone to 4-methylnorephedrine, followed by hydroxylation on the aromatic ring at the methyl substitution and oxidation to its carboxylic acid [21,22]. However, Majchrzak et al. describes, in a thorough literature review [21], many variations to this generalized metabolism for synthetic cathinones which makes development of contemporary and enduring targeted analytical assays more challenging.

Similarly, research into synthetic cannabinoid metabolism is trying to keep pace with the evolving illegal drug market. Many new synthetic cannabinoids are appearing at an increasing rate. Metabolites of a recently encountered synthetic cannabinoid, CUMYL-PEGACLONE, were identified by Mogler et al. [23]. In their analysis, over 22 individual metabolites were discovered and 2 of which were identified as sensitive and specific markers of CUMYL-PEGACLONE. Interestingly, in this study three different immunoassays were used to analyze the same urine samples and failed to detect the presence of this new synthetic cannabinoid due to insufficient cross-reactivity.

Several reference standard manufacturers have increased focus on providing standards for NPS. Cayman Chemical, Cerilliant, and Lipomed have been a reliable resources for quality reference standards in this area. Still, laboratories should verify, either through

peer-reviewed literature or in-house analysis, that the parent and/or metabolite is present in the biological fluid being tested. The pharmacokinetics of NPS are relatively unknown in most cases. One study examining matched urine and oral fluid samples acquired from the same individual and utilizing sensitive LC-MS/MS detection suggests poor concordance in synthetic cannabinoid drug disposition; in this study, PB-22, 5-Fluoro-PB-22, UR-144 5-pentanoic acid, and UR-144 4-hydroxypentyl were detected in urine and not the oral fluid [17]. In the same study, AB-FUBINACA was detected in five oral fluid samples and was matched with only one urine sample.

Furthermore, even targeted mass spectrometry drug assays may struggle with accuracy. In general, validated, targeted mass spectrometer-based assays contain the reference standard, but may not contain the analogous internal standard [19]. The lack of an analogous internal standard makes identifying and quantifying analytes difficult due to detector signal enhancement and suppression from the biological matrix and co-eluting compounds. Each sample, control, and calibrator is prepared with internal standards. Internal standards are used to aid in the quantification of a substance based on its area response compared to the area response of the substance (area ratio), which is then compared to the area ratios of a calibration curve [24]. Additionally, the retention time of the analogous internal standard can be used to identify the analyte of interest since they are very similar substances [24]. When analogous internal standards are not available, other closely-related internal standards can be utilized for quantitation and their relative retention times can be utilized to help identify a positive result, however, this is not the optimal approach. Due to subtle differences, the use of a non-analogous internal standard can have a drastic effect on the overall quality of the assay and may result in erroneous results [25].

The detection and structural characterization of synthetic cannabinoids and cathinones is best performed using a time-of-flight (TOF) mass spectrometer with a library database. Laboratories with these capabilities have the greatest ability to produce accurate results. In cases where a laboratory's libraries are not up-to-date, or do not possess a reference standard for the parent drug and/or metabolite, the TOF mass spectrometer can provide a detailed characterization and elucidation of the unknown substance and initiate further investigation about the unknown substance [25].

Assay sensitivity

Assuming that the NPS is identified, the optimal metabolite is elucidated, and the compound targeted is stable in the biological sample, the analytical assay must be sensitive enough to detect the NPS. Analyzing blood or oral fluid is a potential solution to the delay at which urinary metabolites are made commercially available [3]. Parent drugs may be more predominately present in blood and oral fluids. However, once parent drug standards are available, other factors that may affect the detection of NPS include analyte stability and drug concentrations in body fluids.

As technology progresses, laboratory instruments have become more sensitive over time. This has opened a new dimension into laboratory medicine as a whole. Scientists are now able to accurately measure in concentration ranges lower than ever before. If history is a good indicator for what the future holds, then we are sure to see even further advances in sensitivity.

Lower detection abilities significantly reduce the likelihood of reporting a false negative. If an instrument has low detection abilities, then it will detect small amounts of a substance. Simply stated, as sensitivity increases, detection abilities also increase. Therefore, in the clinical toxicology laboratory, it is highly advantageous to use the most sensitive instruments to assure detection. In terms of instrumentation-specific sensitivity, immunoassays tend to be very sensitive, sometimes even comparable to a mass spectrometer. The major drawback of immunoassays, as previously mentioned, is their lack of specificity. Recent literature recommends the use of qualitative definitive mass spectrometry tests over immunoassays due to the superior sensitivity and specificity benefits [26]. With regard to new psychoactive substances, immunoassay screening methods are not useful and are a source of false negatives in these drug classes. The antibodies designed for a certain target analyte will likely not be able to detect the newer versions of such drugs as the drugs evolve [27].

Sensitivity especially becomes paramount when trying to detect drugs which can be taken in small doses and still achieve the desired affect. Drugs such as sufentanil and carfentanil have extreme potency, even at low concentration levels (<1 mg), and thus, can be abused in small doses to achieve the desired effect. Measuring highly abused, potent substances can be challenging, even at a qualitative level, if an instrument is not sensitive enough [28].

As described previously, NPS may be extremely potent thus doses tend to be very low. As a result, detection must take pace at low nanogram or picogram per milliliter concentration ranges in biological samples. This challenge is exaggerated by the lack of in vivo blood, urine and oral fluid concentration data. For example, MDMA is present in urine anywhere from low hundred to several thousand ng/mL [22,29]. In contrast, JWH-018 (detected as its metabolite JWH-018 pentanoic acid in urine), has been detected at <5 ng/mL just 12 h after inhalation [30]. Due to excretion levels and dilution of oral fluid in collection buffer, drug levels

in oral fluids can be several orders of magnitude lower as compared to urine [31]. This limits oral fluid testing to clinical laboratories with higher-end sensitive mass spectrometers or expensive sample concentration procedures. It is imperative that detection methods be sensitive enough to detect low concentration ranges, even in urine matrix, to avoid false negatives [27]. Furthermore, labor-intensive drug extraction methods such as liquid-liquid extraction (LLE) and solid phase extraction (SPE) may be required first to concentrate the drug in order to ensure detection.

Highly sensitive methods certainly have their advantages although there are some drawbacks to be noted about high sensitivity methods. Users may eliminate a significant amount of false negatives while using a high sensitivity method, however, the risk now becomes reporting false positives. False positives can arise due to improper quantitation (or identification) of signal noise or a native interferring substance in the matrix. It is very important to analyze blank samples to establish a negative baseline before proceeding with testing. Additionally, it is important to reduce as many matrix effects as possible while developing high sensitivity methods to ensure that even a low concentration matrix effect does not interfere with the assay.

Mass spectrometer methods tend to be very sensitive and specific compared to immunoassays, and mass spectrometry methods also offer an advantage of using a qualifier ion to confirm the presence of a substance. The practice of using a qualifier ion (sometimes referred to as a secondary ion) is common to all types of clinical mass spectrometry. As previously mentioned, internal standards also help to identify substances, however, they are not always available and they are not exactly the same structure as the substance of interest. Qualifying ions originate from the substance of interest, but fragment slightly different than the ion that is used for the quantitation [25]. This difference in fragmentation is usually responsible for a slightly lower signal intensity, which may decrease its ability to serve as a quantifying ion, but it has sufficient signal to be detected [25].

Stability also plays an important role in the ability to accurately detect NPS. Some NPS have been shown to have a very short half-life in blood [32]. Without proper metabolite studies to identify urine targets and subsequent stability studies of these metabolites, the NPS may still fail to be detected. The same stability and detection time limits exist for oral fluid and urine sample types.

Glucuronidation, hydrolysis, and metabolites

During the metabolism phase of pharmacokinetics, a number of events take place with the parent drug. Parent drugs typically have a primary metabolic pathway resulting in multiple metabolites that too are subsequently metabolized. There are enzymes in one's body that have evolved to metabolize foreign substances. The majority of drug metabolism takes place in the liver, particularly metabolism involving drug elimination [66]. The liver will chemically attach a polar substance to the drug to aid in its elimination from the body [33]. This is commonly seen as glucuronidation, where a glucuronide molecule is added to the drug to increase its water solubility [33].

The presence of parent drug metabolites (and subsequent metabolites) provides information that the patient ingested a particular drug. In the absence of a parent drug, metabolites serve as positive indicators that the parent drug was used [34]. It is therefore imperative that clinical toxicology laboratories include metabolite testing in their test methods. Failure to analyze common, and/or specific drug metabolites increases the chance of reporting a false negative. Many immunoassay drug screening tests are capable of detecting the parent drug, but do not contain detection antibodies specific for metabolites [35]. It is possible that a parent drug could be completely metabolized and/or eliminated at the time of urine collection, and therefore, would test negative using an immunoassay. This same sample may contain only drug metabolites and be considered positive when testing with mass spectrometry. In the latter case where an inconsistent negative screening immunoassay was reflexed to mass spectrometry, the correct, positive result would be reported. In this example, which unfortunately many clinical toxicology workflows follow, the protocol requires an immunoassay screening first, and then only the positive samples reflex to definitive testing. This results in a negative report when the patient actually ingested the drug. The use of metabolites can also indicate whether a patient is compliant or not. Buprenorphine is an opioid drug that is used to treat opioid addiction [36]. Its major metabolite is norbuprenorphine [36]. Laboratory scientists expect to detect both buprenorphine and norbuprenorphine or only norbuprenorphine in samples of compliant users. Detection of very high amounts of only buprenorphine usually are indicative of a noncompliant patient spiking the sample with a crushed buprenorphine tablet to try to pass the drug test. The noncompliant patient may intend to induce a false negative; however, with the use of metabolite testing, the laboratory scientist can conclude that the patient was noncompliant rather than truly negative.

As mentioned, during metabolism, drugs may become modified by the addition of polar substances such a glucuronide moiety to increase elimination through the urine [33]. These modifications, however, change the chemical makeup of the drug and can lead to false negatives if not considered. Hydrolysis

techniques are employed in the clinical toxicology laboratory to ensure that the glucuronide molecule is cleaved and that the non-glucuronide version of the molecule is regenerated. Immunoassay screening methods may not be designed with antibodies to detect glucuronides, and many targeted mass spectrometry methods are not developed to detect glucuronides, therefore, it is necessary to perform hydrolysis prior to analyzing patient samples [35]. Hydrolysis sample preparation methods typically involve a glusulase enzyme that contains both beta-glucuronidase and beta-glucuronide sulfatase activities for hydrolysis of drug conjugates, a 50–60 °C incubation, and about 30–60 min of time. Recent advances in enzyme hydrolysis sample preparations have been shown to decrease incubation time to 15–30 min and not require heat [37]. An alternative to enzymatic hydrolysis is acid hydrolysis. The combination of acid and heat over a defined time period cleaves the glucuronide [37]. The caustic conditions of an acid hydrolysis can be dangerous to personnel and can be a source of false negatives. Certain analytes such as the heroin metabolite 6-monoacetyl morphine (6-MAM) can be rapidly converted to morphine in the presence of high temperatures [38]. Successful hydrolysis significantly reduces the risk of reporting a false negative. To ensure that the components of the hydrolysis function properly during sample preparation, many laboratories will incorporate a hydrolysis control to run along with their other assay controls. Hydrolysis controls generally consist of the target drug in its conjugated form dissolved in the proper matrix.

Due to the general increase in time and expenses involving hydrolysis, it is also possible to directly measure both the hydrolyzed and non-hydrolyzed species of drug. This can be done with relative ease using a mass spectrometer. The resulting report will display concentrations of parent drug, metabolite, parent drug glucuronide, and metabolite glucuronide without requiring the extra time and reagents for a hydrolysis procedure. The limitation to directly measuring the glucuronide is the availability of a reference standard for the glucuronide. Common drugs will likely have a commercially available glucuronide conjugate, however, as previously mentioned, many drugs, particularly synthetic cannabinoids and cathinones, do not have a reference standard available. There is a low likelihood that the glucuronide conjugate would also be available.

It has been shown that some synthetic cannabinoid analogs may share common metabolites. This lack of specificity can certainly cause confusion, especially if the parent synthetic cannabinoid cannot be found in the sample [39]. Due to ethical reasons, much research on new psychoactive substance metabolism has been done using human hepatocyte cultures. This practice effectively simulates the liver's response to an encounter with a new psychoactive substance [39]. This challenge is best resolved with high resolution mass spectrometry. High resolution mass spectrometry using a TOF mass spectrometer provides the user with robust data acquisition and processing capabilities in an effort to produce quality metabolite profiles [40]. To achieve accurate metabolite identification, mass spectrometry information-dependent acquisition data is used to trigger advanced-level scanning. Users must define settings such as signal intensity and a mass inclusion list in order to appropriately select candidate metabolites [39]. This technology, along with recent research in metabolite identification of new psychoactive substances, empowers clinical toxicology laboratories to target specific, confidently identified, metabolites of such drugs. Furthermore, the data surround the discovery of such metabolites can be used to generate certified reference material, leading to the development of targeted parent-metabolite panels.

NPS IN VARIOUS BIOLOGICAL MATRIX

In this section analysis of NPS in urine, hair, blood and oral fluid are addressed.

Urine

Urine is the most commonly tested matrix in the clinical toxicology laboratory. There is debate as to whether urine is the most practical matrix for novel psychoactive substances testing. Urine is typically collected in a private setting, leaving the sample vulnerable to adulteration by the patient. Since urine is a waste product, metabolites of drugs are commonly found in the urine, with or without their corresponding parent drug. Identifying these metabolites involves sophisticated instrumentation, software, and advanced users, which can be time consuming and not cost-effective for laboratories [3]. A study by Spiller et al. highlighted that laboratories that used GC/MS methods to identify and quantify urine samples from patients displaying agitation, combative behavior, tachycardia, hallucinations, paranoia, confusion, chest pain, myoclonus, hypertension, mydriasis, CPK elevations, hypokalemia, and blurred vision produced false negatives and inevitably led to misdiagnosing patients [41]. In some cases, no drugs were detected, but patients had admitted to taking a NPS substance, thus, highlighting the false negative problems associated with novel psychoactive substances in urine and technical methodology.

In case reports by Tang et al. urine samples were collected from emergency department patients that presented with histories of drug abuse, showing symptoms such as agitation, confusion, and self-muttering [42].

Sample preparation was performed by subjecting the urine samples to beta-glucuronidase followed by a solid phase extraction. LC-MS/MS analysis was performed using a traditional liquid chromatography approach coupled to a linear ion trap mass spectrometer. Two patients exhibited similar results. One revealed a peak at a retention time that did not have a peak when the control samples were run. The unknown peak was subjected to an enhanced product ion scan, which revealed an information-rich spectrum that was then library matched for identification. Another patient's sample followed a similar workflow. The identification of the unknown substance in both patients was 25B-NBOMe, an *N*-methoxybenzyl derivative. These classes of drugs are 5-HT receptor agonists, classified as NPS, which display hallucinogenic properties consistent with the patients' clinical presentation [42].

Cases like the ones mentioned are becoming more commonplace in the clinical toxicology laboratory. It is not unreasonable for laboratories to expect the urine specimen workflow dynamics to change from traditional targeted triple quadrupole LC-MS/MS methods to using enhanced product ion scans to match the unknown's spectrum to a library. Additionally, urinary sample workflows revolving around NPS may evolve to high-resolution TOF mass spectrometry to significantly reduce false negatives. Scheidweiler et al. used TOF mass spectrometry to analyze metabolites of specific parent compounds by first subjecting them to liver hepatocytes to create metabolites, then analyzing the metabolites via TOF mass spectrometry to produce a spectrum that can be saved in a library and used for analysis of unknowns [27]. The authors state that a high resolution methods provide accurate mass information on molecular and fragment ions which enable greater sensitivity and simplified data interpretation as compared to linear ion trap methods. Analysis via TOF mass spectrometry can also analyze data retrospectively. Unknown compounds' spectra can be saved at the time of analysis and can then be retrospectively identified at a later point in time [27]. Most clinical toxicology laboratories would be interested in not only identifying unknowns, but also quantifying them. Quantitation can also be performed with this instrumentation after identifying a positive result in a sample.

Hair

Hair testing, although not routinely performed in the clinical toxicology laboratory, can provide valuable information over a long period of time depending on the length of the hair. Additionally, hair can be easily collected without the risk of adulteration. An important advantage of hair testing is that the parent drugs can be detected in hair samples, therefore, difficult and time-consuming metabolite analysis can be avoided altogether when using hair samples. This can be a major advantage to avoiding false negatives; however, false positive may be reported if the patient has been exposed to the drug in the surrounding environment [36]. Furthermore, natural hair has a varying degree of melanin which effects a drug's ability to bind to it, thus results may be skewed depending on hair color and ethnic group tested [43].

Hair is to be collected close to the scalp using clean scissors and stored in tinfoil. If scalp hair is too short, other body hair source can be used instead. In a publication by Palamar et al. [43], a study was performed using ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) to analyze novel psychoactive substances in hair samples using a targeted method. Their positive identification acceptance criteria included comparison with an analogous internal standard (if available), and detection of a primary and secondary transition with a correct ion abundance ratio between the two ions. Their results showed that many ecstasy (MDMA) users also test positive for a variety of novel psychoactive substances. This may be due to intentional or unintentional use [43]. Due to the variability of hair growth rate, it was believed that exposures to novel psychoactive substances in the study could have been from exposures up to one year before the analysis date, thus exposing a limitation of hair testing [43]. The authors agree that other novel psychoactive substances may have been undetected by using a targeted method [43]. In a similar study by Rust et al. the authors utilized the enhanced product ion scan feature found in a linear ion trap to properly identify unknown peaks found in hair samples [44].

Moreover, analysis using TOF mass spectrometry provides the ideal technology for identifying the presence of additional novel psychoactive substances present in the sample. A TOF analyzer performs full-scan acquisitions with high sensitivity and high mass accuracy, allowing for the detection of chemical substances without the need for pre-defining masses. A study by Gottardo et al. utilized TOF-MS to determine synthetic cannabinoids in hair samples [45]. Using TOF mass spectrometry, Gottardo et al. was able to detect a wide variety of synthetic cannabinoids down to 10 pg/mg of hair at a signal to noise ratio of ≥ 10. The TOF mass spectrometry method can be applied to other classes of novel psychoactive substances in hair samples and can be used to identify the emerging novel psychoactive substances that rapidly enter the market [45].

Blood

Blood samples are obtainable by venipuncture and are not easily adulterated. Concentrations of drugs in

the blood matrix are usually significantly less than concentrations seen in the urine. Paillet-Loilier et al. reported that some novel psychoactive substances in biological fluids have detection limits of 100 ng/mL using gas chromatography mass spectrometry (GC–MS), but by using the same method on blood samples, 100 ng/mL sensitivity was not achievable [46].

Vaiano et al. used a targeted LC-MS/MS method to identify and quantify 64 different novel psychoactive substances across different classes of NPS in blood [47]. Blood samples were treated with 200 μL of acetonitrile, centrifuged, and then injected into the LC system. Their reported limits of quantitation ranged from 0.1 to 0.5 ng/mL and the assay was linear up to 100 ng/mL.

Dried blood spots are an alternative to serum/plasma and whole blood analysis. A finger stick can be used to collect the sample and a drop of blood can then be applied to filter paper for analysis. This requires low blood volume and specimens can be easily shipped to other laboratories in the mail. Approximately 10 μL of blood is applied to a piece of filter paper and allowed to dry. After extraction with methanol, samples can then be dried and reconstituted with water and 0.1% formic acid and then injected on to a mass spectrometer with a linear ion trap. The limits of detection for 64 targeted novel psychoactive substances using this method ranged from 1 to 10 ng/mL and showed no interfering substances. Total run time for one sample was 10 min [48].

Oral fluid

Oral fluid is collected from the oral cavity and can be done under supervision to prevent sample adulteration. Due to its nature, it can be collected in the field without use of invasive equipment. Similar to hair, parent drugs are found in the oral fluid matrix, therefore, complex and time consuming metabolite identification can be avoided by utilizing oral fluid [3].

Ares et al. used an ultra-high-performance liquid chromatography (UHPLC)-MS/MS method to detect synthetic cathinones, opiates, and cocaine in under 3 min using oral fluid samples [49]. Sample preparation was performed using micro-extraction by packed sorbent (MEPS), a technique that is completed within 15 min. The authors reported a linear range of 0.5–500 ng/mL with a correlation coefficient of 0.99. Extraction recovery ranged from 75% to 125% using this technique. A similar study to detect 31 novel psychoactive substances was performed by Rocchi et al. using protein precipitation, followed by MEPS and UHPLC-MS/MS on oral fluid samples [50]. Rocchi et al. reported that linearity ranged over 3–4 orders of magnitude and accuracy ranged from 85.9% to 107%. The limits of detection for the various drugs ranged from 0.005–0.850 ng/mL and limits of quantitation ranged from 0.015 to 2.600 ng/mL.

LIMITATIONS OF NPS IMMUNOASSAYS

Commercial immunoassay kits continue to be a prevalent testing methodology in clinical laboratories due to their high-throughput, cost and ease of implementation. However, they currently fall short of being a truly reliable testing methodology for NPS; most notably due to insufficient cross-reactivity and time to market from recognition of new substances.

Immunoassays rely on the reaction of an antigen (analyte(s) of interest) and an antibody combined with some type of indicator, usually colorimetric, fluorimetric, or chemiluminescence in nature [67]. These antibodies are designed to react with a portion of the molecular structure of the targeted antigen. As a result, different compounds with that similar portion of structure can react with the antibody as well. This is always referred to as the assay's cross-reactivity.

Cross-reactivity is well characterized and understood in validated clinical immunoassays. In the case of NPS, cross reactivity is a benefit and hindrance to accurate reporting. Should an emerging NPS react with an antibody targeted for a similar analogue, the immunoassay might function as an effective screening tool and perhaps alert the laboratory to a new-NPS. However, immunoassays are not suitable for definitive reporting and the laboratory, without a definitive-type mass spectroscopy method, would likely report a false-negative. It is often even more likely that an emerging NPS will be modified in such a way that the immunoassay is completely non-reactive, thus presenting another false-negative scenario.

Poly and monoclonal antibodies are expensive to develop and time consuming to optimize for commercial kits. In a retrospective study ($n = 549$) comparing a LC-MS/MS methodology (targeting 45 synthetic metabolites) to immunoassay kits for JWH-018 and UR-144, the immunoassay failed to identify any of the synthetic cannabinoids identified by the LC-MS/MS method [51]. In 2014, one attempt to validate a Randox Biochip assay (DOA-V) showed a large and unacceptable negative bias in mephedrone and MDPV results for the performing scientists [52]. Since this study, two new immunoassay chips have been released in attempts to keep pace with NPS. However, at the time of this publication no peer-reviewed third-party validations of these newest platforms have been published.

Progress continues in development of poly and monoclonal antibodies for NPS. Development of antibodies that could identify whole classes of NPS based on a prevailing moiety and not be negatively impacted

by small structural modifications would provide an excellent presumptive screening workflow. The laboratory could then have a longer lasting screening tool to use in conjunction with definitive LC-MS/MS analysis. In this scenario, positive immunoassay and negative LC-MS/MS confirmation would hypothetically alert the laboratory to a potential false negative and initiate further analysis by HR-MS to detect new unknown synthetic compounds/metabolites of the class [51]. Methodologies utilizing HR-MS techniques will be discussed later in this chapter.

CONFIRMATION OF NPS

High throughput, comprehensive and sensitive mass spectrometry methods remain the only suitable detection technology for responsible reporting of NPS in patient samples. The majority of definitive drug testing performed in clinical toxicology laboratories is done on a targeted basis using a triple quadrupole mass spectrometer (LC-MS/MS). This strategy is often successful for determining the concentrations of specific drugs within a class of drugs [25]. For example, many laboratories will develop a single targeted assay for multiple drugs within the benzodiazepines class. Since many of these drugs have similar chemical characteristics, the drugs within this class will extract and separate under common conditions, making it easy to detect them. Targeted panels have become standard in definitive testing, however, there are some drawbacks with targeted assays that could lead to false negatives.

As mentioned previously, targeted testing using triple quadrupole mass spectrometry will only detect the analytes for which a multiple reaction monitoring (MRM) scan has been developed, unless an isobaric interference is present. The presence of other drugs, even within a specific class, is unlikely to be detected when using an MRM scan if there is no MRM method developed for said drugs.

Extensive chromatographic separation is necessary to confirm the presence of specific compounds as many of the synthetic compounds are closely related and coelute, thus creating the potential for interferences and, possibly, false positive reporting. Many synthetic drugs have the same parent mass as another drug. Their chemical structures differ, but the parent mass remains the same. These are known as isobaric substances [53]. The advantage of using a tandem triple quadrupole mass spectrometer is that it provides fragment ions, or product ions, which could be unique for different isobaric substances [25]. The unique fragments can help distinguish the two isobaric substances, however, it is not guaranteed that a unique fragment will be produced. Older instrumentation, such as GC−MS, does not offer a fragmentation step, therefore, the tandem ability is not available with these instruments [53]. In such cases, chromatography can be used to separate isobaric substances. Recently, chromatography chemistries have greatly improved, even to the point where even some of the most similar classes of isomers can be chromatographically resolved. The use of high-quality instrumentation, high quality chromatography methods, and robust extraction techniques greatly decreases the chances of miss-reporting results.

Ambroziak et al. details an up-to-date (as of October 2017) LC-MS/MS screening assay for 72 synthetic cannabinoids in whole blood [54]. This assay was developed to provide a "fast and simple liquid chromatography−tandem mass spectrometry screening procedure for detection and identification of SCs [synthetic cannabinoids] in whole blood" [54]. However, simple is a relative term in this case with 217 transitions and a 16-minute run time. Of note, only one internal standard was used in this method, JWH-018-d9. As mentioned earlier, the lack of availability of internal standards also provides challenges for the development of accurate and precise NPS assays free from interferences and matrix effects. The same aforementioned assay reported matrix effect values ranging from 38.5% suppression to >1000% enhancement for some analytes. For perspective, Cerilliant, one of the largest providers of NPS reference standards currently (as of August 2018) only markets 14 synthetic cannabinoid internal standards.

Recent technological advances have been made commercially available from mass spectrometry vendors to enhance the specificity of the drug-testing assay. A differential mobility spectrometry (DMS) attachment can be inserted at the front end of the mass spectrometer, just after the ion source, to provide an alternate means of separation. With this technology, ions are separated on the basis of the difference in their migration rates under high versus low electrical fields. Separation occurs due to unique interactions between the ions of interest and neutral gas phase molecules [55]. This technology greatly increases the chances of detecting a drug that would otherwise be missed or falsely characterized as a closely related drug.

Another advanced MS technique to increase specificity can be performed with the use of the linear ion. A second fragmentation event is carried out on the first product ion of the precursor. This creates a second generation product ion [68]. This is often referred to as MS-MS-MS or MS^3. In situations where chromatography and the MRM cannot separate isobaric substances, MS^3 can be employed to further fragment the product ion to produce other fragments which may be unique to each of the isobaric substances [56]. This practice greatly increases the specificity of the assay, but usually decreases the sensitivity.

Currently, definitive qualitative analytical assays for NPS are more relevant than quantitative assays. The clinical laboratory should be more concerned with identifying all NPS present in the biological sample rather than their absolute concentration. Limited research has been compiled on clinically significant concentrations of NPS in the body. However, all NPS, schedule or unscheduled, are generally regarded harmful and presence of any is a significant finding.

Mass spectrometers and library searching

The ultimate instrumentation for the identification of unknown substances in a sample is a time of flight (TOF) mass spectrometer. TOF instruments measure the time it takes for a specific substance to travel through a flight tube to the detector [57]. Tandem TOF (TOF/TOF) mass spectrometers enhance specificity by isolating precursor ions in the first TOF based on their velocity and then subjects the ions to a collision cell. The second TOF then analyzes the fragment ions [58]. The utilization of TOF methodology is commonplace in fields such as pharmaceuticals, food & beverage, drug discovery, proteomics and others. A quadrupole is often used to filter the parent mass before the ion is subjected to the flight tube. The flight time is related to the substance's mass and an accurate mass, up to four or even five decimal places, can be obtained. The spectrum obtained can then be matched to a spectrum stored in a library database to identify the unknown substance in a sample. The major drawback is that these instruments can be expensive and, thus, are not often found in routine clinical laboratories. TOF instruments such as matrix-assisted laser desorption (MALDI) TOF instruments are increasingly gaining popularity among clinical microbiology laboratories for identification of bacterial and fungal strains [59]. The lack of TOF instrumentation in the clinical toxicology laboratory increases the probability that drugs in a sample may go undetected due to the use of simpler targeted drug testing methods.

For many years now, the use of GC−MS in the clinical toxicology lab has played a prominent role in identifying drugs in samples. Operators would prepare the sample using various sample preparation techniques and then inject the sample into the instrument to obtain a spectrum that was compared to a library. This workflow was effective but lacked specificity and has lately been surpassed by the use of more sensitive and more specific LC-MS/MS workflows in the clinical laboratory [60].

The LC-MS/MS platform brings targeted, multiple reaction monitoring (MRM) workflows to the toxicology laboratory and provides clinicians with quantitative results. Using LC-MS/MS, the chances of not identifying a drug present in the sample are high if the targeted method does not include the MRM for any given drug. Coincidently, laboratories containing mass spectrometers with linear ion traps provide operators with spectrum scanning ability and library searching, similar to the way that drug testing was performed on GC−MS instruments.

If the spectrum of a drug is known, it can be uploaded into a library. The library will store detailed spectral information such as mass to charge ratio and can be called upon to identify drugs in a sample. The spectrum in the library that best matches the drug from the sample is reported and results that match generally greater than 75% can be reported with confidence as the identified drug. For this to take place, standards of potential drugs must be purchased from a vendor then scanned by the linear ion trap to obtain their unique spectrum, and then the information must be uploaded and stored into a library. This process is the limiting factor to prevent false negatives. Spectrums that are not obtained cannot be uploaded and stored into a library, and thus, will not aide in the identification of a particular drug in the sample. However, as mentioned previously, retrospective data analysis on the unknown's spectrum can be done once a verified analytical standard is available.

Library searching using a linear ion trap is a feasible approach for laboratories. Many triple quadrupole mass spectrometers are available with a linear ion trap in the Q3 region. It is common for laboratories with these specialized instruments to employ them for targeted analysis using the MRM scan and to employ them for qualitative screening and identification using the linear ion trap in conjunction with a library. This type of workflow often requires specialized software to process the analytes of interest, but fortunately newly improved metabolite identification softwares are available from leading mass spectrometer vendors. Based on the spectrum obtained from the instrumentation, be it TOF/TOF or linear ion trap, the software is able to provide a chemical structure of the untargeted substance and report a likely identification for that substance. As new, novel psychoactive substances are being introduced into society, it will be critical for the high-performance instruments to be paired with descriptive and reliable software in order to determine the identity of such compounds. This combination of instrument and software will significantly reduce the time between the release of the new psychoactive substances to society and the identification in the laboratory, providing laboratories with an unprecedented tool to combat false negatives.

Recent published literature has utilized metabolite identification software to effectively identify new psychoactive substances' metabolites. Users can customize their metabolite identification method by modifying algorithms with the software. By using common product ion scanning, neutral loss scanning, mass defect

filtering, and generic peak finding, along with adjusting the mass spectrometer acquisition settings, users are able to confidently identify metabolites of the ever-changing classes of new psychoactive substances [61].

CONCLUSIONS

Novel psychoactive substances that are previsoly unidentified continue to proliferate our society. Inadequate laboratory instrumentation and the lack of proper reference material for identification and quantitation can increase the likelihood of not reporting a drug present in the sample. It is important for laboratories to equip themselves with the proper technology and test the proper matrix. This practice can facilitate the reduction of false negatives.

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CHAPTER

17

Ethanol determination using automated analyzers: limitations and pitfalls

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INTRODUCTION

Alcohol is a general term for an organic compound that contains one or more hydroxyl groups attached to a carbon. In common usage, however, alcohol is often used synonymously for ethanol-a two-carbon compound containing a single hydroxyl group. Due in part to its wide spread availability, ethanol is one of the most commonly abused drugs in the world. The World Health Organization (WHO) Global Information System on Alcohol and Health (GISAH) maintains data on global ethanol use and resulting impact on human and population well-being [1]. Data for the United States of America (USA) specifically is available from the National Institute on Alcohol Abuse and Alcoholism (NIAAA) [2] and Centers for Disease Control and Prevention (CDC) [3].

Ethanol use remains highly prevalent worldwide with 38% of individuals older than 15 years of age having consumed ethanol in the past year. The prevalence of ethanol use in a country generally correlates with the economic wealth of the country such that wealthier countries show higher rates of ethanol use/lower rates of abstainers. The 2015 National Survey on Drug Use and Health (NSDUH) showed that in the USA 86% of individuals over the age of 18 had consumed ethanol in their lifetime with 56% reporting having consumed ethanol within the past month [4]. Ethanol consumption is typically reported as a number of standard drinks- 12 ounces of beer (5% alcohol by volume (ABV)), 5 ounces of wine (12% ABV) or 1.5 ounces of distilled spirits (40% ABV).

The adverse effects of ethanol are substantial and contribute to a range of diseases and injuries. As with most drugs, it is the concentration of ethanol that determines the impact to health. Table 17.1 outlines some common drinking levels and the quantity of ethanol consumption that defines them. Moderate ethanol consumption has actually been shown to have some beneficial health effects including an increase in overall mortality and decreased risk for cardiovascular disease [5]. When ethanol consumption exceeds moderate levels, adverse health effects occur. Globally, GISAH has estimated 3.3 million deaths annually due to ethanol consumption. In the USA, over 88,000 deaths annually were reported [6]. Of these deaths, 39,000 related to chronic health conditions (primarily liver disease) while 49,000 were related to acute causes such as motor vehicle crashes, fall injuries, suicide, etc. The economic cost of ethanol consumption in the USA was estimated at $249 billion in 2010 [7]. This cost was primarily due to lost productivity but also included $28 billion in healthcare costs. These healthcare costs include those associated with both long-term care of chronic conditions and acute patient management primarily in the emergency department (ED). Recent data have shown that rates of ED visits related to ethanol consumption, both chronic and acute, have been steadily rising with a 62% increase between 2006 and 2014 [8].

It is important to have accurate knowledge of a patient's alcohol status, because of its potential to interact with co-ingested substances, increasing their potency and/or confounding or masking their symptoms. Laboratory practice guidelines for use of drug tests in patients treated in the ED have been prepared by expert panels of clinical toxicologists and ED physicians. The three most recent are those of the US National Academy of Clinical Biochemistry [9], the UK National Poisons Information Service and Association of Clinical Biochemists [10], and the Alberta Medical Association in Canada [11]. All endorse the provision of 24/7 availability of STAT (urgent) quantitative ethanol tests.

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TABLE 17.1 Quantity of ethanol consumed for various drinking levels.

Drinking level	Quantity of ethanol consumed
Moderate	Men: up to two drinks per day
	Women: up to one drink per day
Low risk for AUD	Men: up to four drinks per day and fourteen drinks per week
	Women: up to three drinks per day and seven drinks per week
Binge (NIAAA)	BAC levels to 0.08 g/dL or more
Binge (SAMSHA)	Men: up to five drinks on one occasion
	Women: up to four drinks on one occasion
Heavy	Binge drinking five or more days in the past month.

Abbreviations: *AUD*, alcohol use disorder; *NIAAA*, National Institute on Alcohol Abuse and Alcoholism; *SAMHSA*, Substance Abuse and Mental Health Services Administration.

Since there is no concentration that defines clinical intoxication, as opposed to the legal setting, the laboratory or ED has choices regarding specimens and testing methods that best meet its need. For practical reasons, this means clinical laboratories almost always perform testing on serum/plasma and/or urine. It is important to note that use of such specimens and clinical testing methods carries caveats if an attempt is made to apply the results to non-clinical purposes.

This chapter will focus on the use and performance of automated ethanol testing in the clinical laboratory. As can be helpful with many drugs, a short review of the pharmacodynamics and pharmacokinetics is initially presented. Following that, there is a thorough discussion of clinical ethanol testing methods and detailed discussion of the performance and limitations of most frequently used automated testing. Both pre- and post-analytical aspects of ethanol testing are then discussed. A quick review of markers of ethanol ingestion is included toward the end, though interested readers are referred to discussions that are more extensive should they be interested [12]. The chapter finishes with an overview of toxic alcohol testing and summation of ethanol testing.

PHARMACODYNAMICS OF ETHANOL

Ethanol is a psychoactive substance, which primarily affects the central nervous system (CNS) where it acts as a depressant. While the CNS affects are most prominent, ethanol also affects the digestive, endocrine, immune and cardiovascular systems. The ultimate effect of ethanol will depend on a range of factors including the individual, concentration of ethanol, tolerance of the individual and even the social setting in which consumption happens. Interestingly, ethanol also demonstrates a form of acute tolerance called the "Mellanby Effect" in which different effects are noted between a blood alcohol concentration (BAC) in which the BAC is rising and the same BAC in which it is falling [13].

Ethanol's primary effect on the CNS is to act as a general depressant [14]. It exerts this effect through interaction with a wide range of target proteins that include neurotransmitter receptors, ion channels and intracellular signaling proteins [15]. The observed effects on the CNS will depend on the BAC and thus amount of ethanol consumed. Table 17.2 illustrates the normal progression of symptoms with relation to quantity of ethanol consumed. Initially, ethanol affects emotion and decision-making. In the appropriate social setting with moderate ethanol consumption, this often entails

TABLE 17.2 Relationship of serum ethanol concentration and clinical effect in children and non-habituated adults.

Serum ethanol, mg/dL	Typical clinical picture
<25	Feeling of warmth and wellbeing
	Talkativeness and mild dis-inhibition
25–50	Euphoria
	Poor judgment
	Diminished fine motor coordination
50–100	Decreased sensorium
	Impaired coordination, balance and gait
	Slowed reflexes and increased reaction time
	Emotional lability
100–250	CNS dysfunction - ataxia, diplopia, slurred speech, visual impairment, nystagmus
	Confusion, stupor, difficulty sitting upright without assistance
	Nausea, vomiting
250–400	Stupor, coma, little response to stimuli
	Incontinence
	Respiratory depression
>400	Respiratory paralysis
	Loss of gag and other protective reflexes
	Hypothermia
	Death

increased relaxation, mood elevation and decreased self-consciousness. It should be noted though that the social setting, quantity and rate of ethanol consumption can alter this and a clear association between ethanol consumption and violent behavior has been found [16]. Cognition and memory are also affected at this stage and become increasingly impaired as BAC increases. With rising BAC, muscular control becomes more noticeably impaired causing delayed reaction time and loss of coordination and balance. Many of the acute ethanol deaths are attributable to these pharmacodynamics actions of ethanol, with impairment in decision-making and impaired coordination and reflexes leading to the high number of motor vehicle deaths. For this reason, most countries have established clear BACs at which impairment is assumed. The level varies from country to country. Some countries have set their limit at zero, meaning no detectable alcohol is allowed in individuals operating motor vehicles. Others have yet to establish limits that define impairment. Common upper limits range from 0.05 to 0.08% BAC.

Tolerance is an important consideration when thinking about the pharmacodynamics and pharmacokinetics of ethanol. Habitual users will show less impairment at higher BAC levels than naïve users. Prolonged exposure to ethanol leads to adaptive responses within the CNS that partially explain the altered impact of ethanol in such users. Consequently, if such individuals return to a non-drinking state they can experience a symptomatic withdrawal as their bodies attempt to readjust to the new, non-ethanol normal. Symptoms includes seizures and hallucinations. A small percentage of patients undergoing ethanol withdrawal will experience delirium tremens (DT), which is characterized by hallucinations, tachycardia, hypertension, hyperthermia, and diaphoresis [17]. Since few medical centers allow regular ethanol ingestion, any patient with alcohol dependency who is admitted is at substantial risk of undergoing such symptoms during their hospitalization.

Apart from the CNS, ethanol exerts a range of negative effects on other organ systems. With regards to the endocrine system, ethanol induces increased antidiuretic hormone secretion which results in increased urination (diuresis). In cases of alcohol dependence, increased adrenocorticotrophic hormone (ACTH) production can occur leading to alcohol-induced pseudo-Cushing syndrome [18]. Sexual dysfunction can also be observed in men due to decreased circulating testosterone, which can lead to impotence and testicular atrophy [19].

Ethanol's impact on the gastrointestinal system contributes to the overall mortality from chronic ethanol use. The negative impact to the gastrointestinal system affects the stomach, the liver and the pancreas. Through a wide variety of mechanisms, ethanol damages the gastrointestinal tract leading to ulcers and increased risk for upper gastrointestinal bleeding events [20]. Though the impact of ethanol on liver dysfunction is fairly well known, ethanol abuse is also the most common cause of chronic pancreatitis; ethanol metabolites are thought to lead to direct damage of the acinar cells in the pancreas [21]. Finally, ethanol has serious negative impacts on the liver. In 2010, almost half of all liver cirrhosis deaths were caused by alcohol-induced liver cirrhosis [22]. Through a number of complex mechanisms, ethanol induces accumulation of fat in the liver ("fatty liver"), which leads to alcoholic fatty liver disease [23]. Finally, ethanol displays an immunosuppressive effect and adverse effects on hematopoiesis [24]. There is a clear association between ethanol consumption and cancer risk and while the mechanism is still unclear, it is possible that decreased immune surveillance plays a role [25,26].

Though ethanol can have some positive effects on adults, it is a well-established teratogen (substance that causes malformation of an embryo) and *in utero* exposure can have substantial negative effects on the child [27]. Prenatal ethanol exposure leads to a continuum of disabilities known as fetal alcohol spectrum disorders (FASDs). FASDs are the leading cause of preventable developmental disabilities in the world [28]. Recognizing and properly categorizing FASD starts with appropriate assessment of maternal ethanol consumption. Since admission of prenatal ethanol use is uncommon, laboratory testing for ethanol and ethanol markers can be helpful in determining this.

PHARMACOKINETICS OF ETHANOL

Ethanol pharmacokinetics (absorption, distribution, metabolism and elimination) have been extensively studied. Joseph Widmark conducted pioneering studies attempting to apply mathematical models to predict blood alcohol levels as far back as the 1920s [29]. Since that time our understanding of ethanol pharmacokinetics and ability to predict levels have evolved substantially. Ethanol is primarily absorbed orally in the small intestine and distributes into a range of bodily tissues and fluids based primarily on their water content. There is substantial first pass metabolism and the majority of ethanol is eliminated via enzymatic metabolism-primarily alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) with contributions from cytochrome P450 2E1 (CYP2E1) and catalase [30]. Only a small portion is excreted in the urine unchanged or conjugated (ethyl glucuronide or ethyl sulfate).

Ethanol is primarily "dosed" orally, with absorption occurring throughout the gastrointestinal tract. While its bioavailability ranges, it generally achieves peak

blood concentrations 60 min after ingestion. The duodenum and jejunum absorb ethanol more rapidly than the stomach and as such, the rate of gastric emptying affects the rate of alcohol absorption [31]. In addition, the concentration of ethanol in the consumed beverage affects the rate of absorption with higher the alcohol content generally leading to faster absorption. However, too high an alcohol content will delay gastric emptying and actually slow absorption. First pass metabolism can be completely by-passed if alcohol is absorbed through the rectum. The decrease in first pass metabolism and increased rate of absorption have led to a lethal phenomenon known as alcohol enemas, whereby alcohol is administered rectally [32].

Ethanol has both hydrophobic and hydrophilic attributes, which explain its distribution in the body. The hydrophobic nature of alcohol means that it readily crosses cellular membranes and can distribute throughout tissues in the body. However, the hydrophilic nature of ethanol means that it is highly soluble in the bloodstream and its volume of distribution is directly related to an individual's total body water. Since women generally have more adipose tissue, they will exhibit a smaller volume of distribution and thus higher blood alcohol levels for equivalent dosages [33]. Likewise, the ultimate concentration of ethanol in various bodily fluids and tissues is based on their water content with a higher water content yielding higher levels of ethanol distribution; there is no plasma protein binding of ethanol. Table 17.3 outlines the range of bodily fluids available for ethanol testing with indications of the fluid: whole blood ethanol ratios. Clinical laboratories will most often be analyzing serum or plasma samples collected pre-mortem while forensic laboratory preferentially analyze femoral blood, urine and vitreous fluid. From a legal standpoint, breath, blood and urine are acceptable for deciding impairment. When attempting to reconcile results between different fluids the following caveats should be noted:

- the effect of shock on metabolism and excretion of ethanol
- how blood loss affects the dynamics of distribution
- administration of blood product, fluids or colloid

The validity of measuring ethanol in breath is based on the relationship of ethanol in end-expired (alveolar) air and that in the alveolar blood supply described by Henry's Law. Thus 1 mL of blood contains the same ethanol as 2100 mL of breath at 34 °C, the temperature of end-exhaled air (see Chapter 31).

Before alcohol undergoes distribution throughout the body, it undergoes extensive and variable first pass metabolism. The concentration of ethanol ingested substantially affects the degree of first pass metabolism; the bioavailability of ethanol ranges from <10% at 10 mg/dL to 90% at 100 mg/dL due to saturable first pass metabolism [34]. The majority of first pass and post-absorptive ethanol metabolism occurs in the liver at a rate dependent on the activity of hepatic alcohol dehydrogenase (ADH). Women generally show lower rates of gastric ADH expression leading to a lower rate of metabolism [35]. As shown in Fig. 17.1, ADH catalyzes the oxidation of ethanol into acetaldehyde with the concomitant reduction of nicotinamide adenine

TABLE 17.3 Select bodily fluids amenable to ethanol determination in humans pre- and postmortem.

Premortem	Postmortem
Serum/plasma [1.16]	Blood (femoral)
Urine [1.3]	Urine (bladder)
Venous blood	Vitreous humor [1.2]
Breath [0.0005]	Blood (heart)
Sweat	Blood (clot)
Saliva	Cerebrospinal fluid
Capillary blood	Bile [1.0]
Cerebrospinal fluid [1.1]	Synovial fluid

The [numbers] indicate the fluid:whole blood ratio for those fluid types.
Adapted from Kugelberg FC, Jones AW. Interpreting results of ethanol analysis in postmortem specimens: a review of the literature. Forensic Sci Int 2007;165:10–29.

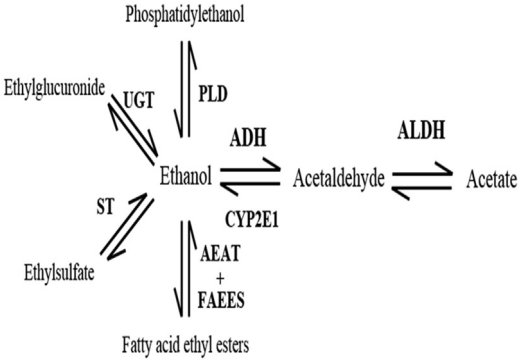


FIG. 17.1 Metabolism of ethanol in the human body. The primarily metabolism of ethanol occurs via alcohol dehydrogenase (ADH) in the cytosol of hepatocytes and requires reduction of nicotinamide adenine dinucleotide (NAD^+) to NADH. Ethanol can also be metabolized to acetaldehyde through cytochrome P450 2E1 (CYP2E1) in a reaction that converts nicotinamide adenine dinucleotide phosphate (NADPH) to NADPH with the concurrent consumption of molecular oxygen. Metabolism of acetaldehyde to acetate occurs in the mitochondria where it is catalyzed by aldehyde dehydrogenase (ALDH) and entails reduction of NAD^+ to NADH. Minor metabolic pathways: phase two metabolism to generate ethyl glucuronide via UDP-glucuronosyltransferase (UGT) and ethyl sulfate via sulfotransferase (ST) as well as formation of phosphatidylethanol (PEth) via phospholipase D (PLD) and fatty acid ethyl esters (FAEEs) via fatty acid ethyl ester synthase (FAEES) and acyl-CoA:ethanol O-acyltransferase (AEAT).

dinucleotide (NAD^+) to NADH. This reaction results in consumption of NAD^+ and contributes to the generation of a highly reduced cytosolic environment. Combined with the formation of acetaldehyde, a highly reactive compound, this contributes to a great deal of the cellular damage caused by ethanol. Damage due to acetaldehyde primarily relates to its ability to form adducts with cellular components-deoxyribonucleic acid (DNA), proteins and lipids. Acetaldehyde degradation is rapid through mitochondrial aldehyde dehydrogenase (ALDH) to produce acetate [36].

Human ADH is a heterodimeric protein, encoded by at least seven genes, producing >20 dimers all with different rates of ethanol metabolism [36,37]. There are five distinct classes of ADH in humans with class I and II being the primary forms in the liver. Human ADH (in contrast to the yeast enzyme) is substrate-saturated at low ethanol concentrations (km is only 5–10 mg/dL), and metabolism proceeds by zero order kinetics when serum ethanol is >25 mg/dL. The rate is usually expressed in terms of a decline in serum ethanol, and a range between 13 and 25 mg/dL/hour is often quoted - hence the mean "twenty per hour" rule, although this is sometimes wider in ED patients [38,39]. Although hepatic function is severely compromised in cirrhosis there is no compelling evidence that the rate of ethanol metabolism is altered [40,41]. CYP2E1 also oxidizes ethanol to acetaldehyde once the blood concentration exceeds 30 mg/dL (its km 60–80 mg/dL) (Fig. 17.1). In contrast to ADH, this enzyme is inducible by ethanol, and alcoholics may route up to 30% of the dose via this pathway [42].

Both ADH and ALDH in humans have important isoenzymes and polymorphisms. A wide number of polymorphic forms of class I ADH exist and these forms show distinct racial prevalence. *ADH1B*2*, which metabolizes ethanol to acetaldehyde very efficiently, is common in people of Chinese, Japanese, and Korean descent [43]. The rapid build-up of acetaldehyde leads to facial flushing, nausea and an overall unpleasant feeling when ethanol is consumed. Build-up of acetaldehyde can also occur due to reduced activity of ALDH. *ALDH2*2* encodes a ^{487}Glu→Lys polymorphism associated with almost complete loss of function; this polymorphism is carried by some 40% of Asians [44].

The majority of ethanol is eliminated through metabolism, with a small percent (5%) eliminated unchanged through sweat, breath and urine. In addition, a small percentage of ethanol is eliminated via phase two metabolism as ethyl glucuronide and ethyl sulfate (Fig. 17.1). Ethyl glucuronide is generated via mitochondrial membrane-bound UDP-glucuronosyltransferase while ethyl sulfate is generated through the action of sulfotransferase. These metabolites are excreted in the urine and have gained interest given their ability to provide a larger window of detection for ethanol consumption than serum ethanol levels. Testing for these metabolites in the clinical laboratory has become more common.

ALCOHOL MEASUREMENT METHODS

Because of the widespread availability and toxicity of ethanol, and its relatively large concentration in body fluids, it naturally became a target for early analytical toxicologists. Just as today, their methods were based on the reducing properties of ethanol: addition of a strong oxidizing agent (potassium dichromate in concentrated sulfuric acid) induced a color change (from yellow/orange to blue/green chromic sulfate) that was easily monitored. Widmark introduced a distillation step to vaporize the ethanol from the biological fluid prior to the reaction [45], and the ethanol vapor captured by reagent impregnated into filter paper placed above the boiling solution. Later, a micro-diffusion well was used for the same purpose. These methods are now considered obsolete because of their lack of specificity (methanol and iso-propanol are both strong enough reducing agents to produce a positive reaction), their tediousness, and inapplicability to automation.

Volatile alcohols are extremely well suited to analysis by gas chromatography; mixtures could be separated by molecular weight and volatility on a column packed with nothing more than activated charcoal. The relatively high concentrations ensured adequate sensitivity with even the most basic flame ionization detector introduced in the 1960s [46]. With the advent of capillary columns and programmable ovens, quantitative analysis of alcohols by gas chromatography is one of the easiest and most robust tests available in the clinical laboratory, and soon became established as the gold standard for both alcohols and glycols [47]. Despite its obvious advantages in specificity, and the ability to identify and quantitate several toxic alcohols simultaneously, Gas Chromatography is not widely employed in clinical laboratories, especially those that do not serve a major trauma or poison treatment center. The equipment is relatively expensive (approx. $30–40,000), and its use requires specialized training. The need to test large numbers of samples for ethanol necessarily requires a method that can be adapted to an automated chemistry platform.

To this end, enzymatic assays were investigated based either on alcohol oxidase (AOD) or alcohol dehydrogenase (ADH). Again, both these tests are based on the ability of ethanol to act as a reducing agent, and produce acetaldehyde plus a reduced product. Of these enzymatic methods, ADH proved to be more easily automated because of its good stability in solution,

and methods for the most part use spectrophotometric detection of the NADH (nicotinamide adenine dinucleotide reduced form) product as the detection mechanism. Point of Care methods for ethanol, however, mostly use AOD since although the enzyme is highly unstable in solution it has good stability when formulated onto a dry dip stick pad or solid support. AOD methods are currently of interest since their oxygen consumption and peroxide formation makes them more amenable to conversion to electrical signals that can be measured with an electrode sensor. Alternatively, the hydrogen peroxide product can be used to make a visible color reaction using added peroxidase. A wide variety of test kits are available for use with urine, saliva and breast milk, but these are discrete single-use tests, are not suitable for automated analyzers. The reaction mechanisms for both AOD and ADH assays will be discussed in more detail as the different methods are evaluated later in this chapter.

Testing methodologies: alcohol oxidase (AOD)

Even though there are no automated methods at present using AOD, brief description is appropriate since technological advances may shortly result in the successful automation of AOD methods. The left portion of Fig. 17.1 shows generic AOD methods. These are amenable to electrochemical detection methods and are available on the Analox GM series of bench top instruments (Analox Instruments, London, UK). They offer the potential for adaptation onto handheld devices such as iSTAT (Abbott Diagnostics, Abbott Park, IL), or blood gas analyzers. The main drawback of AOD methods is that the enzyme is not terribly specific for ethanol: all the common toxic alcohols are substrates for this enzyme, producing a signal that cannot be distinguished from that produced by ethanol. However, substrate preferences (as indicated by different Km values) very much depends on the biological origin of the AOD.

Testing methodologies: alcohol dehydrogenase (ADH)

Direct measurement of ADH activity is the most straightforward and commonly utilized of the enzymatic methods: a single enzymatic reaction is required and one of the products is measured directly. Both the oxidized cofactor NAD^+ and its reduced product NADH strongly absorb ultraviolet light attributable to the adenine moiety. The peak absorption of NAD^+ is at a 259 nm, with a molar extinction coefficient of 16,900 $M^{-1}\,cm^{-1}$. Many automated clinical chemistry methods take advantage of the fact that NADH (but not NAD^+) also absorbs at higher wavelengths, with a second peak in UV absorption at 339 nm with an extinction coefficient of 6,220 $M^{-1}\,cm^{-1}$ [48]. The reaction obeys Beer's Law, and the concentration of ethanol in a specimen can be inferred directly from the increase in absorbance at 340 nm as NADH is produced.

The Vitros application is a variant of this method. Patient sample applied to the slide is evenly distributed by the spreading layer (containing $BaSO_4$ and NAD^+), onto the underlying reagent layer (containing ADH and Tris, at pH 8.75). The Tris buffer (tris (hydroxymethyl) aminomethane) in the reaction layer traps the acetaldehyde produced by the reaction of ethanol with ADH, and drives it to completion. The reflection density of the reduced NADH is measured and is proportional to the concentration of ethanol in the sample. Serendipitously, the filtering of the specimen through the spreading layer reduces potential interference from myeloma proteins in many assays, and also reduces the interference from elevated concentrations of lactate dehydrogenase. Other trapping reagents for acetaldehyde have been used in other applications, such as semicarbazide.

A less frequently encountered formulation of an ADH assay was the ADH with Radiative Energy Attenuation (REA). In this assay ethanol substrate is used in a coupled catalytic reaction with ADH and a second enzyme Diaphorase to generate a color change in a dye. The method was originally designed for the Abbott TDx/FLx platforms, and is currently available on the Abbott AxSYM. Radiative Energy Attenuation (REA) measures the degree of inhibition of the fluorescence of fluorescein dye resulting from the production of a colored product. In the original version of the assay [49,50], the ADH reaction with ethanol and NAD^+ was coupled to a second reaction between the NADH produced and the chromogen iodonitrotetrazolium violet dye (INT). This diaphorase catalyzed reaction results in re-oxidation of NADH to NAD^+, along with the generation of a red chromophore formazan-INT. This product has an absorbance peak at 492 nm, which overlaps the excitation and emission spectral profile of fluorescein which is included in the reaction mixture. Subsequent reformulation of the kit eliminated the need for a time consuming probe wash by replacing the INT with thiazoyl blue (Monotetrazolium, Methylthiotetrazolium, MTT). The detection strategy is unchanged with the reduced MTT (Formazan-MT) yielding a purple color with an absorbance maximum at 565 nm. The logarithm of the fluorescent light emitted is inversely proportional to the chromophore present, the production of which is directly linked to the ethanol concentration in the sample.

PERFORMANCE EVALUATION OF ENZYMATIC ALCOHOL ASSAYS

The many different strategies for measurement of ethanol by enzymatic assays are summarized in Fig. 17.1. All the major clinical chemistry automated instrument manufacturers currently market alcohol test kits. Several diagnostic reagent manufacturers sell kits suitable for use on a number of different automated chemistry platforms. All are based on the enzymatic oxidization of ethanol to acetaldehyde, using the enzyme ADH (EC 1.1.1.1., Ethanol: NAD^+ oxidoreductase). However, the specific application of the test may differ between manufacturers, and it is naive to assume that all ADH-based ethanol tests are created equal, will have the same performance characteristics, and be subject to the same limitations and interferences. Variability between the different tests arises from differences in the:

- Species source of ADH
- Reagent concentration of substrate, cofactors, enzyme
- Source contamination of NAD^+ (Nicotinamide Adenine Dinucleotide)
- Use of NAD^+ or an NAD^+-analog
- Use of a trapping reagent to drive the reaction to completion
- Sample and reagent addition volumes, incubation timings, and blanking (pre-incubation)
- Nature of any coupled indicator reaction, and its reagent formulation
- Detection and blanking spectrophotometer wavelength
- Measurement timings and mode (end-point or kinetic)

Most assays are calibrated with a two-point calibration curve, using a 0 mg/dL and a 100 mg/dL ethanol calibrator (or 300 mg/dL Siemens Dimension); Vitros uses three calibrators at 2, 100 and 300 mg/dL, and Abbott REA uses multiple levels (0, 25, 50, 100, 200 and 300 mg/dL) because the concentration-response relationship is non-linear. In contrast to peptide assays there should be, and is, excellent gravimetric agreement between different sources of ethanol used for calibration. When the information is included in the package insert, there is generally little or no interference by hemolysis, icterus or lipemia (except if this exceeds the absorbance maximum of the individual instrument photometer). The specimen pH does not affect the reaction over the range 3–11, an important consideration for urine testing.

Most vendors of assay kits include calibration materials in their kits, or as separately purchased items. These are often matrix matched to give optimal performance on the specific platform. Calibration materials can also be obtained from independent vendors such as Cerilliant Corporation, Round Rock TX; UTAK Laboratories Inc., Valencia, CA. These can also be used for chromatographic methods, although many laboratories performing ethanol analysis by GC prefer to make their own multi-component calibrators, and verify them with independent controls. All vendors of commercial test kits provide quality control materials for assessing method performance prior to testing of patient specimens. Automated test methods are usually designated as applicable to more than one body fluid, which typically include both serum/plasma and urine. To fulfill regulatory requirements for using matrix-matched control materials, commercially available controls in blood, serum, saliva and urine are widely available (UTAK, Bio-Rad Laboratories, Hercules, CA, Microgenics Corporation, Freemont, CA). In addition to these internal controls, a laboratory's performance of all clinically important analytes is monitored by their participation in an external proficiency testing program, such as that offered by the College of American pathologists (CAP). In the US, CAP is required to submit proficiency test results to CMS (Centers for Medicare and Medicaid Services) for all laboratories that have provided a CLIA identification number.

CAP interprets the CLIA '88 (Clinical Laboratories Improvement Act) regulations (42 CFR Part 493, section 493.937) for acceptable performance as obtaining a value within ± 25% of the all-method mean for ethanol, and as a value between 0 and 9 mg/dL for alcohol-free samples. Results are issued for each sample provided at least 80% of participating laboratories have returned results. If the result is in doubt, there is concern over the quality of the specimen, or there is no clear consensus, it is ungraded. Returns are evaluated by each of the four major analytical method groups: Gas Chromatography (GC), ADH with UV/Visible detection, ADH with REA, and the Vitros method.

In 2017, CAP distributed survey sets to over 3400 laboratories participated in the CAP alcohol survey (AL2), and the vast majority of these (approximately 98%) used automated methods for measurement. Of the participants, 82% used ADH UV/Vis methods, 15% Vitros, 2% Gas Chromatography (84 labs), and well under 1% Abbott ADH with REA. Accuracy was assessed by comparison of the returned results to the gold standard GC results. While ADH UV Vis and ADH REA methods compared very favorably with GC (within 2% and 0%, respectively), the Vitros method consistently underestimated the true ethanol content by about 9%. This bias is believed to be explained by a matrix effect, because the Vitros method compares favorably with other methods when testing authentic patient materials. Method imprecision (as defined by the coefficient of

variation) for all four method groups was similar, ranging usually from 3 to 5% across the full range of ethanol concentrations tested. Both GC and the enzymatic methods performed equally in this regard.

Shortcomings of existing automated testing methods

Assays based on the measurement of enzyme activity offer many advantages over gas chromatography methods because of their amenability to automation and consequent speed and widespread use, and have undoubtedly contributed to saving many lives. However, they are prone to inaccuracy on two fronts: cross-reactivity of the enzyme with alternate substrates such as toxic alcohols other than ethanol; and interference from elevated plasma concentrations of substrates and/or enzymes that could also generate NADH, the indicator compound used to monitor the reaction. Although industrial property often prevents the end user from knowing the precise ingredients of the kit, appreciation of enzyme reaction principles at least gives the operator some clues as to the appropriate starting point in troubleshooting wayward results.

At the outset of this discussion it is important that the reader appreciate that the ADH present in human liver and other tissues has quite different characteristics to the *Saccharomyces cerevisiae* (yeast) enzyme present in all of the ADH-based ethanol kits [51]. Active yeast ADH is a homo-tetramer, with a molecular mass of approximately 145 kDa. The active site of each subunit contains a zinc atom, two reactive sulfhydryl groups and a histidine residue. Its pH optimum is alkaline, 8.6–9.0. Inhibitors are compounds that either react with free sulfhydryl, chelate zinc, or are alternate substrates such as NAD-analogs, purine and pyrimidine derivatives, and halogenated alcohols [52]. Yeast ADH is most reactive with ethanol, its preferred substrate, and less so with methanol, and then its activity decreases with increasing size of the alcohol substrate molecule. Branch-chain and secondary alcohols (e.g. iso-propanol) have very low reactivity [53].

Enzyme reactions are characterized in terms of two parameters: the velocity (rate of change of concentration of substrate concentration with time) and the Michaelis constant, Km (the substrate concentration that produces half the maximum velocity). A typical enzyme rate curve is shown in Fig. 17.1. Velocity is typically expressed in amount (moles or mg) of substrate turned over to product in a given time, and is sometimes factored to the amount of enzyme present. The maximum reaction velocity possible, Vmax, is a function of the reactivity of the enzyme itself, including any polymorphic forms, as well as the prevailing reaction conditions (amount of enzyme in the reaction mixture, temperature, co-factor concentration, pH etc.). First Order reaction kinetics is followed when the exclusive determinant of the enzyme rate is the substrate concentration available (see notation on Fig. 17.1). Therefore, during this time, the reaction rate can be used to calculate the substrate concentration, and this is the basis for a large number of clinical enzymatic assays for substrates (glucose, urea, creatinine, etc). At higher substrate concentrations, the reaction kinetics might conform more closely to Zero Order (see notation on Fig. 17.1). Here, the enzyme is substrate-saturated (all available substrate binding sites on the enzyme are occupied) and the reaction proceeds at a fixed rate (Vmax) that cannot be exceeded regardless of any increase in substrate concentration that might occur. While ADH kinetics is zero order at relatively low ethanol concentrations in man, yeast ADH has a much higher Km, and first order kinetics prevail under the operating conditions of most test methods.

All enzyme reactions are reversible, and ADH is no exception. The reaction is as follows, with ethanol being oxidized to acetaldehyde, and the reaction monitored spectrophotometrically at 340 nm by the production of NADH.

$$\text{Ethanol} + \text{NAD}^+ + \text{H}^+ \overset{\text{ADH}}{\rightleftarrows} \text{Acetaldehyde} + \text{NADH}$$

The position of the equilibrium of an enzymatic reaction cannot be changed by altering the amount of enzyme present in the reaction mixture. For this reaction, the equilibrium position lies far to the left, and the time required for the assay to reach equilibrium may be from 10 to 60 min depending on prevailing conditions of temperature, substrate or cofactor concentration etc. Since equilibrium (or end-point) methods are relatively insensitive to small changes in the reaction conditions, this approach was initially preferred, but lost favor once technological advances made it possible to finely control instrument conditions. The reaction can however, be driven toward the right, primarily by increasing the reaction pH, or by adding a trapping reagent to siphon off the acetaldehyde product, such as semicarbazide or Tris (THAM, tris(hydroxymethyl) aminomethane). Coupling one of the ADH reaction products to a second enzymatic reaction also accomplishes the same end, but this strategy is usually employed for the purpose of modifying the measurement wavelength to avoid potential interferences and is a more expensive option since other substrates/cofactors and enzyme must be added to the reagent kit.

The analysis time can also be shortened by using a kinetic measurement to monitor the reaction rather than the equilibrium end-point. However, this requires that the substrate concentration ([S]) be kept sufficiently

low ([S] ≪< Michaelis Constant, Km) to ensure that first order reaction conditions prevail, so that the amount of product formed is dependent only on the amount of substrate present. This originally necessitated predilution of the sample since ethanol concentrations are relatively large, but modern automated pipettes can now dispense specimen volumes of 2−5 mL with excellent precision (<2%). Addition of extra ADH enzyme could also be used to increase the amount of substrate consumed, but this can be expensive, so an alternative strategy might be to add a competitive enzyme inhibitor such as pyrazole [54]. Once the Km is increased by the competitive inhibitor, the assay response is linear over the required range of ethanol concentration to cover most clinical eventualities.

Automated ADH assays are liable to two potential sources of interference that can lead to inaccuracy in some clinical settings. The first is having a degree of cross-reactivity with other toxic alcohols, and the second is being subject to positive bias introduced by elevated concentrations of Lactate and/or Lactate Dehydrogenase (LDH). Interestingly, toxic alcohol interference is better known, but occurs less frequently, while the Lactate/LDH interference is less predictable and may be more prevalent, but is less well recognized.

Shortcomings of existing automated testing methods: cross reactivity with other alcohols

Most enzymes can use a number of different substrates, albeit with different preferences. Substrates that are metabolized most quickly or selectively have low values for Km, the substrate concentration at which the reaction proceeds at half its maximum velocity − see Fig. 17.1. ADH is no exception, and a number of different alcohols can be converted into their respective aldehydes with the concomitant use of NAD^+ and production of NADH. Thus NADH production, which is usually monitored to specifically determine the ethanol concentration, is not specific and gives no indication of which substrate has been utilized. A large concentration of an alcohol such as methanol with a high Km value produces NADH at a relatively low rate, and this might lead the technologist to believe there is a small amount of ethanol present in the sample.

However, when ethanol is present *in addition* to another alcohol, the situation is more complicated because both alcohols can be metabolized by ADH. The term competitive inhibitor refers to a compound that directly competes with ethanol for substrate binding site on the enzyme and occupies it, thus reducing the amount of ethanol that can bind and be converted to acetaldehyde. The magnitude of the effect is determined by the relative proportions of ethanol to inhibitor that are present, and once the inhibitor has been metabolized or excreted, the effect is dissipated. Some competitive inhibitors are also alternate substrates for the enzyme, and their binding to the active site results in their metabolism into products (and thus NADH production). Most toxic alcohols are alternate substrates for ADH and can therefore inhibit the metabolism of other alcohols that have higher Km values. Indeed, this very principle, preventing the metabolism of methanol or ethylene glycol by addition of ethanol, has been taken advantage of for many years in the treatment of poisoning from toxic alcohols with intravenous or oral ethanol until it was replaced by 4-methyl pyrazole (Fomepizole, Antizol), another competitive inhibitor of ADH. However, for the yeast ADH used in these automated assays the Km for ethanol, the preferred substrate, is only 2.1 mM; while that for methanol is very much larger at 130 mM, and for iso-propanol 140 mM [53]. The potential for interference is therefore more theoretical than practical since a serum methanol of 130 mg/dL gives an apparent ethanol of only 2 mg/dL. Likewise, to obtain a positive ethanol result (10 mg/dL) the methanol concentration must be 650 mg/dL, which requires almost heroic ingestion.

In 2010, the potential for ethanol assays to cross-react with other toxic alcohols was evaluated in three proficiency testing samples sent out by CAP. These samples contained only toxic alcohols but no ethanol. The toxic alcohols evaluated included: methanol (48 mg/dL), Acetone (30 mg/dL), isopropanol (72 mg/dL) and ethylene glycol (152 mg/dL). The data, with the method means for each method being under 1 mg/dL, clearly showed that there is no interference in ethanol measurements at these values. More recent CAP surveys support this finding as no significant difference is noted between enzymatic method means and GC means for samples that do or do not contain other toxic alcohols. These studies show that the presence of a toxic alcohol, even at large concentrations, is unlikely to produce a false positive ethanol result by any of the automated ADH methods in current usage. The downside of this however is that toxic alcohol ingestions are not going to be detected by automated ADH ethanol tests, and other strategies must be employed to avoid missed diagnoses and missed treatment opportunities.

Nearly all kit manufacturers include interference information for methanol, acetone, iso-propanol and ethylene glycol in their package inserts. Manufacturers' interference data can sometimes be misleading, and induce a false sense of security. Authentic patients' specimens represent a complex mix of endogenous and drug-related metabolites that can produce a quite different pattern of interference than when alcohols are spiked into clean samples *in-vitro*. Data on interferants other than toxic alcohols are not always available and sometimes only come to light in case reports. Alcohol Oxidase ethanol methods are much more susceptible

to false positive interference in samples containing other toxic alcohols. This coupled with the instability of AOD in solution led to the overwhelming preference for the use of ADH in automated ethanol test kits.

Shortcomings of existing automated testing methods: elevated lactate and LDH

As outlined in the section above, the production of NADH by ADH is not specific for the presence of ethanol, but the mere presence of large concentrations of toxic alcohols is unlikely to produce a false positive ethanol result. However, there may be other enzymes and/or substrates in the patient specimen that could result in the generation of NADH from the reduction of NAD^+. It has been known for almost 20 years that high concentrations of LDH and/or lactate interfere with ADH-based ethanol assays, giving false positives results [55], and has been observed in the glycerol dehydrogenase-based ethylene glycol assays as well [56,57]. This appears in some quarters to have been forgotten with the passage of time, or sidelined in the belief that this will only be applicable to post-mortem tests and not relevant to clinical testing [58,59].

The ADH and Lactate Dehydrogenase (LDH, E.C. 1.1.1.27 L-lactate: NAD + oxidoreductase) reactions are outlined below. When both enzymes and their substrates are present in a specimen, there are clearly two sources of NADH production.

$$\text{Ethanol} + \text{NAD}^+ + \text{H}^+ \overset{\text{ADH}}{\rightleftarrows} \text{Acetaldehyde} + \text{NADH}$$

$$\text{Lactate} + \text{NAD}^+ + \text{H}^+ \overset{\text{LDH}}{\rightleftarrows} \text{Pyruvate} + \text{NADH}$$

Both the ADH and LDH reactions are equilibrium reactions, and if both reactions are occurring within a single specimen on the analyzer, the enzymes not only compete for NAD^+, but also produce NADH at rates consistent with their concentrations and the appropriate substrate concentrations in a kinetic assay. Thus, an apparently large amount of ethanol might be determined to be present in the sample, which may in fact be completely untrue. Furthermore, since there is no mechanism in the test reaction mixture (as opposed to *in-vivo*) to re-generate NAD^+ from NADH, NAD^+ may become the limiting factor in the ADH and/or the LDH reactions.

In late 2010 the CAP Toxicology Resource Committee sponsored a pilot study which demonstrated continued occurrence of false positives in automated ADH ethanol test kits. In this mini-survey, 50 laboratories agreed to test a serum specimen containing 120 mM/L Sodium plus 25 mM/L Sodium Lactate, and 5000 U/L human LDH (mixed isoenzymes not characterized). Summarized results for the seven different test systems targeted are shown in Table 17.4. Using a cut-off of 10 mg/dL for a positive ethanol result, the Dade Dimension, Abbott REA and Vitros platforms gave the correct negative ethanol result for the sample. The Roche platform assay results were positive but close to 30 mg/dL, and the other three assays (Beckmann, Abbott Aeroset and Microgenics DRI) gave ethanol results between 70 and 106 mg/dL, representing significant inebriation. In the manufacturer package inserts, elevated Lactate/LDH is mentioned in five: the Abbott Aeroset, Roche and Microgenics DRI methods give a cautionary note about lactate and LDH in post mortem samples, but do not give specific details, while Abbott ADH REA, Randox and Vitros give specific data only for lactate (tested at 25, 90 and 20 mM/L respectively) producing <10% interference, which is in line with the CAP experimental data. Reference ranges are somewhat method dependent, but for serum lactate is typically below 2 mM/L, and for LDH <300 U/L.

TABLE 17.4 Lactate and LDH interference in ethanol assays.

Test method	Number of labs	Mean measured ethanol, mg/dL
Abbott Aeroset	6	102.5
Abbott AxSYM REA	2	9
Beckman (LX & DX)	14	73.2
Microgenics DRI	2	106
Ortho Vitros	7	9.8
Roche Platforms	7	29.3
Siemens Dade Dimension	10	4.2

Source data: CAP Toxicology Resource Committee 2010 Pilot Survey, reported with 2011 AL2-C.

The above results beg the question "what makes these seemingly similar assays perform so differently?" Manufacturers' interference data, while informative, can also be misleading. Authentic patient specimens represent a complex component mix comprised of endogenous metabolites and drug components, while manufacturers' data are generated by spiking good quality specimens with purified pharmaceuticals and chemicals. While having a high serum LDH activity *per se* is usually not sufficient for a significant false positive to occur in the ADH-based ethanol assay, a high substrate (lactate) concentration is also required. Blood (plasma or serum) is therefore more likely to be affected than urine, since although there may be plenty of lactate in urine, it is unlikely to be any LDH.

It is difficult to identify a precise LDH activity at which interference occurs in each ethanol assay kit for several reasons. Most LDH assays (and hence the value for LDH attributed to the offending sample) employ the IFCC (International Federation of Clinical Chemistry)

recommended Lactate-to-Pyruvate (L→P) reaction direction, since it is less dependent on the NAD^+ and lactate concentrations, and there is less contamination of NAD^+ with inhibiting products during manufacture. The reference range is up to 225 U/L for L→P assays, but P→L methods give rather higher results (normal <450 U/L). More importantly, the LDH present in human serum comprises 5 isoenzymes (LD1 through 5), composed of hetero-tetramers of H and M type subunits (4H = LD1 through to 4 M = LD5). The typical percentage composition in a healthy person's serum is approximately 22% LD1, 35% LD2, 23% LD3, and 10% each of LD4 and LD5. The conditions favorable for the P→L reaction direction are a more acidic pH (7.0 optimum), and a high concentration of pyruvate, a high ratio of NADH to NAD^+, and a preponderance of LD 5 and LD4. Converse conditions are favorable for the L→P together with a preponderance of LD1 and LD2.

LDH is highly specific for the L(+)-isomer of lactate that is produced metabolically in man. Although ß-hydroxybutyrate (produced in excess in ketosis) is considered a poor substrate for LDH, the isomers do not behave equally in this regard. ß-hydroxybutyrate is in fact the preferred substrate for LD1 (resulting in LD1 being known as Hydroxybutyrate Dehydrogenase; HBD), the serum activity of which can be measured, and the ratio of HBD to LDH used to indicate the tissue-specific source of LDH in a more efficient manner than electrophoretic separation of LDH isomers.

It would be tempting to think that by contrast, the determination of serum lactate would be straightforward. Most assays for lactate use the enzyme L-Lactate Oxidase (EC 1.13.12.4; (S)-lactate: oxygen oxidoreductase) to oxidize L-lactate to pyruvate with the concomitant production of hydrogen peroxide. Whole blood analyzers (such as blood gas analyzers) measure the hydrogen peroxide amperometrically. These methods show significant interference from glycolic acid, the toxic metabolite of ethylene glycol [60], and may give values falsely elevated by up to 60 mM [61]. In most plasma lactate methods on chemistry analyzers, the hydrogen peroxide is consumed in a second enzymatic reaction with peroxidase and a chromogenic substrate to form a colored product which is measured spectrophotometrically. Typically, these are less subject to glycolate interference [62] but may return an apparent lactate at approximately twice the glycolate concentration. This can sometimes result in a high absorbance flag and a suppressed lactate result. The Roche methods do not use Lactate Oxidase, but use LDH to measure lactate concentration, and are subject to lesser (20%) glycolate interference [63]. These are therefore more reliable in ethylene glycol intoxication.

Gharapetian et al. [64], presented three patients with virtually identical apparent serum ethanol values (33, 33 and 34 mmol/L (152−156 mg/dL)) measured using the Siemens ADH-based ethanol assay on a Bayer Advia 1650 analyzer. Following calculation of very abnormal negative osmolal gaps (−23, −42 and −44), the samples were re-tested for ethanol on the Siemens (Dade Behring) Dimension RXL Flex reagent and also by Gas Chromatography and found to be negative (<2 mmol/L, or <9.2 mg/dL). Interestingly, all three patients had marked elevations in serum LDH (between 5000 and 10,000 U/L), caused by acetaminophen hepatotoxicity, but only cases 1 and 3 had elevated serum lactate concentrations (method of measurement not given) at 22.5 and 5.1 mmol/L respectively; patient 2 had a lactate of only 1.2 mmol/L. However, Siemens reported to the authors that the LDH would need to be >100,000 U/L (by L→P assay), with a concomitantly elevated lactate to produce an apparent ethanol concentration of 17.4 mmol/L. The patients' damaged hepatocytes will undoubtedly have spilled a number of substrates and other enzymes besides LDH into their serum, resulting in very high circulating levels of transaminases, other DHs, amino acids, ammonia, etc. This highlights the importance of disassociating manufacturer's spiked interference data from clinical samples, since clearly additional factors are at play here in producing the erroneous ethanol results. The Siemens Advia assay uses a NAD^+-analog with high oxidizing potential rather than NAD^+ as the enzyme cofactor.

In most instances, the amount of either lactate or LDH present is not sufficient to make a significant contribution to the measured ethanol result. In fact, retrospective assessment of laboratory data has shown that elevated LDH and lactate do not yield false positive enzymatic ethanol results in live patients presenting to the emergency department [65]. Still, the degree of cross-reactivity will depend on the degree of LDH and lactate so clinical laboratorians should be aware of this possibility if the LDG or lactate reach excessive levels.

ELIMINATING INTERFERENCES IN ALCOHOL ASSAYS

When endogenous interference in an enzymatic assay is problematic, blanking and pre-incubation can help reduce interference. This additional step increases the cost and analytical time. Unless a large number of samples are affected, this modification is not justified. Instrument parameters for FDA-approved reagent kits are pre-defined within the instrument application, and not adjustable by the end user. However, these can sometimes be altered if analyzer has "open channel" or "user defined" applications. Hopefully the recent re-awareness of this problem and the legal ramifications of an unreliable result will prompt the manufacturers to

reformulate the poorly performing kits. Further research is required to pursue NAD^+ analogs that react almost exclusively with ADH and not LDH, or do so at vastly differing speeds so that the two reactions can be isolated from each other. Alternate strategies could focus on side-reactions that make colorless or non-interfering products. Coupling of the acetaldehyde product to another reaction (peroxidase to hydrogen peroxide) with judicious choice of chromogen offers the promise of a visible color reaction that might be distinguished from the LDH assay.

LDH can be removed from serum with PEG (Polyethylene glycol) 3000, which osmotically sequesters water from the sample so the proteins precipitate out. After centrifugation, the clear supernatant is analyzed. This technique is used extensively in clinical chemistry to remove interference from immunoglobulin-bound "macro-proteins" such as prolactin, creatine kinase, amylase etc [66,67]. Care should be taken to establish that added chemicals do not interfere with a subsequent reaction, and that any specimen dilution is accounted for in the final result calculation. Alternatively, a protein-free filtrate of the sample can be prepared by centrifugation in a centrifugal filter device to obtain a protein free ultra-filtrate [68]; a 30 kDa cut-off is appropriate for LDH. Such specimen manipulation must be carefully controlled by the processing of quality control samples in parallel to the patient sample. Clearly it is not practical to process all samples by this extended assay, and identification of problem samples would require simultaneous testing for LDH, which again is not practical. Specimen filtration also does not remove excess lactate from the sample, but once the LDH is removed, lactate is probably no longer a significant problem.

Pre-analytical considerations

Pre-analytic variables can lead to erroneously increased or decreased levels of ethanol; increases in ethanol primarily occur via microbial production or sample contamination while decreases are largely related to evaporation (vapor pressure of ethanol at 25 °C is about 55 mm Hg). It is important to remember though that most clinical laboratories are concerned with ethanol levels accurate enough for appropriate patient management. Thus, pre-analytic variables that might cause inaccurate ethanol levels from a legal standpoint (i.e. questions of impairment) might not cause issues from a clinical management standpoint. In addition, most clinical laboratory perform emergent ethanol testing meaning there is less time for microbial ethanol production or evaporation during prolonged storage. Still, inaccurate ethanol levels can be obtained due to a variety of pre-analytic variables including collection tube, sample contamination, evaporation and unintentional exposure of the patient to ethanol.

Ideally, specimens for ethanol testing (and lactate) should be collected into tubes containing sodium fluoride to inhibit glycolysis and prevent generation of ethanol by certain bacteria or yeasts within the specimen. Microbial contamination could be endogenous if the patient is septic or has a urine tract infection, or exogenous if it is introduced by aseptic collection techniques. The Clinical & Laboratory Standards Institute (CLSI) recommends collecting samples for blood alcohol analysis in gray rubber stoppered tubes containing sodium fluoride plus K3EDTA as additives to prevent fermentation and coagulation respectively [69]. The literature, however, has shown storage of samples for up to ten days with or without fluoride has no effect on ethanol levels [70]. In the interest of preventing therapeutic anemia from drawing unnecessary tubes and since fluoride oxalate is unsuitable for many clinical tests, ethanol tests are usually performed on serum or plasma. This has little impact on the clinical utility of the measurements as samples are almost always processed emergently Microbial production of ethanol in urine is also a concern, though the amount of required substrates in urine for microbial ethanol production from healthy donors is usually too low to be of importance. However, if the patient has a ketosis, whether diabetic (hyperglycemic) or alcoholic (hypoglycemic), and the correct microbes are present then ethanol production may be sufficient to suggest severe toxicity [71]. Indeed, a small amount of fermentation may actually take place within the bladder if there is urinary stasis with a urinary tract infection such as *Candida albicans*.

Concerns over the correct vacutainer selection go beyond the question of additive and to finer points such as the presence or absence of gel and glass versus plastic. The thixotropic polymeric gels used in cell separator tubes offer numerous advantages in the clinical laboratory-improved stability of several analytes, easier sampling from the primary tube, reduced/easier aliquoting and higher yields of useable serum or plasma [72]. Unfortunately, these gels are composed of an array of chemicals (viscous liquids, organic and inorganic fillers, tackifiers) that can interfere with subsequent sample analysis. Early reports showed that use of such gel containing tubes could result in falsely decreased levels of certain hydrophobic compounds [73]. This has been used as evidence to support requiring non-gel tubes for ethanol but there is no evidence in the literature that gel tubes are unacceptable. Studies have shown that the use of glass or the more favorable plastic vacutainers has no impact on the stability of samples for ethanol analysis [74].

If microbial production of ethanol is the major pre-analytic concern for falsely increased levels, evaporative

loss is the major concern for falsely decreased levels. It must be noted that evaporative loss of ethanol from an uncapped vacutainer tube will vary substantially based on the temperature (higher temperatures lead to greater losses), concentration of ethanol in the solution (higher concentrations lead to greater absolute losses) and volume-to-surface-area of the container (smaller opening vacutainer tubes will show slower losses than wider mouth secondary containers sometimes used). Clinical laboratorians should be aware of these factors. Studies have shown that while statistically significant differences are observed between stoppered and unstoppered samples within 30 min at room temperature, the differences did not exceed the desired analytic imprecision (<9%) till 2 h after unstoppering [75].

As with other analytes, the potential for false elevations due to sample contamination is always a possibility; any specimen with an ethanol content >15,000 mg/dL is almost certainly contaminated regardless of the symptomatology. While considered a pre-analytical error, this error can certainly happen in the laboratory as illustrated by a case presented by Foley [76] in which a specimen submitted for drug testing from a heroin abuser was determined to have an ethanol content of 12,500 mg/dL by headspace GC. A concomitant blood ethanol was negative, and it was determined that the ethanol had been added to the urine by a laboratory technician who misread the ethanol order for an instruction to add ethanol as a fixative for cytology testing.

Adulteration of urine samples submitted for drug testing has been a problem since testing first began. A number of adulterant tests are used in legal testing but these are not commonly applied in the clinical setting. Since testing only proceeds to confirmation if the primary screen is positive, adulteration aims to make the screen test negative by denaturing the antibody or preventing it binding to its target drug. Ethanol or other alcohols are sometimes used for this purpose, but adulterers often are rather generous with the amount of additive, and the contamination is often obvious as soon as the top is taken off the tube. This is illustrated in the following case. A 46-year-old patient with a long psychiatric history was receiving treatment for overuse of prescription pain medications. On her first visit, her urine screened positive for opiates, oxycodone and acetaminophen, and acetone was detected by gas chromatography at >400 mg/dL, but with no trace of isopropanol (<10 mg/dL) or evidence of ketone bodies. The unusual alcohol result was commented in the patient's EMR as suggestive of contamination rather than ingestion. Returning two weeks later, her urine screened positive for oxycodone, benzodiazepines and salicylates, and was found to contain iso-propanol at 6500 mg/dL; again a comment on suspected contamination was entered in the chart. On the third visit the drugs of abuse panel was negative, but the urine appeared very dilute with a measured ethanol of 98,050 mg/dL (creatinine <0.02 mg/dL, BUN <1 mg/dL). After the physician was specifically alerted, the patient was challenged and elected to seek medical care elsewhere.

If the serum or urine ethanol result is a valid one, there are a number of alternative explanations for its presence other than ingestion of beverage alcohol. These include but are not limited to direct or indirect contamination. "Auto-brewery syndrome" has been invoked as a legal defense against DUI, but this *in vivo* generation of ethanol by the GI tract has rarely been proven, and then not outside of Japan. It is believed to be the result of a combination of low mucosal and hepatic ADH activity, high intake of rice and concomitant yeast infection [77].

Non-beverage ethanol is present in many common household items: perfumes and aftershaves are 60–95% ethanol, hand sanitizer 65%, food essences 35%, mouthwash 15–35%, and NyQuil 10% ethanol. There is recent trend for young teens to abuse ethanol in products such as "Four" beverages with caffeine, in canned whipped cream, ethanol-laced jello and drunken gummy bears, and even ethanol soaked tampons.

Dermal absorption of alcohols is generally insignificant [78], but once the epidermis is damaged, absorption may be increased 1000-fold. Bandages soaked with ethanol and applied for preoperative preparation for plastic surgery in a 2-year-old girl resulted in significant toxicity - 12 h later the child was comatose with serum ethanol 80 mg/dL and glucose 15 mg/dL [79]. Immature skin is also highly alcohol-permeable, and there are case reports of premature infants with systemic toxicity following application of surgical spirit to cleanse skin prior to surgical procedures. For example, a serum ethanol of 259 mg/dL was reported at 18 h post procedure in a 27-weeks neonate, with a methanol of 26 mg/dL [80]. Surgical spirit (95% ethanol and 5% methanol) is the equivalent of rubbing alcohol, which in the US contains iso-propanol. Reports of ethanol positivity due to use of ethanol-based hand sanitizer have been studied. Use of ethanol-based hand sanitizer every 5 min for 10 h on three consecutive days did show some positive markers of ethanol ingestion but no positive ethanol levels [81].

Post-analytical considerations

Once accurate ethanol levels are obtained, the challenge becomes interpreting these levels concerning the specific question being asking (post-analytical). Before interpreting ethanol levels, it is essential to ensure proper units and unit conversions are used. Most international toxicology publications use g/L as the standard unit of measure for ethanol, while clinical chemists prefer mmol/L. In the US mg/dL is commonplace, but

to add further confusion, ethanol in the legal arena is expressed simply as %, and clinicians often drop the units completely. Good laboratory practice mandates a reference range be appended to any result, reducing the potential for misunderstanding. The most common inter-conversion between these different units of ethanol measurement are shown below:

- To convert g/L into mg/dL multiply result by 100
- To convert mg/dL into mmol/L multiply result by 0.217
- To convert mmol/L into mg/dL multiply by 4.6
- To convert % into mg/dL multiply by 1000

Even when correct units are in use, the calculated concentrations must be adjusted for the sample type analyzed. Since ethanol primarily associates with total body water, it has a higher concentration in serum compared to whole blood. Thorough analysis has shown that serum levels are an average of 11% higher (4–16%) compared to whole blood [70]. While plasma has a higher protein content than serum, studies have shown this has a negligible effect on ethanol levels (average ratio of 1.0).

Estimating dosages for exposures is notoriously difficult. It is tempting to envisage the worst possible scenario, in children especially, and surmise that if the bottle is now half full, then the patient must have ingested the other half. Small children do not often ingest sufficient to seriously harm themselves, and since they have no better aim with tablets or alcohol than they have with food, the scene often looks far more dramatic than its reality.

This is a relatively simple calculation (Widmark's Formula) which can be stated as:

$$\text{Dose} = \text{Vd} \times \text{BW} \times \text{Cp} \quad \text{or} \quad \text{Cp} = \frac{\text{Dose}}{\text{Vd} \times \text{BW}} \qquad (17.1)$$

where the dose of pure ethanol is estimated in grams; Vd, the distribution volume of ethanol is 0.55 L/Kg in males and 0.68 L/Kg in males; BW is the body weight in Kg; and Cp is the blood ethanol concentration in g/L.

It is then only a short step to calculate the volume of beverage consumed by multiplying by the ethanol content of the formulation, remembering that "proof" is twice the percentage composition (approximate values are: beer 3 g/100 mL; wine 8 g/100 mL; spirits 35 g/100 mL). As a rule of thumb, one "unit" of ethanol is about 12 g.

If the ethanol concentration is not appropriate for the ingested dose, either the patient history is in error, the ethanol result itself may not be correct, or there may have been an alternate source of ethanol, such as those described in the next section.

Occasionally, a falsely low serum ethanol concentration is discovered. This most often occurs when a severely traumatized patient has been resuscitated emergently with large amounts of transfused (ethanol negative) blood, RBCs or colloid at the time the samples are being drawn for analysis. Dilution of the sample directly from the line fluid by poor phlebotomy technique is also possible, and this is usually discovered because the hematocrit is very low, glucose is exceptionally high, or the electrolytes are out of balance [82].

With the exception of the first 90 min after ingestion, urine ethanol always exceeds that of the serum, with a ratio typically of 1.2–1.3 urine/serum in the early post-absorptive phase increasing to 1.4–1.5 later on. In the terminal stages after exposure, the ratio increases appreciably as ethanol is present in bladder urine for some hours after the serum alcohol is no longer detectable [83]. However, during the absorption phase, the plasma is higher than the urine, although it takes only 10 min for ingested ethanol to appear in "primary" (i.e. ureter) urine [84]. Thus the ratio of the ethanol content of the two fluids might be used not just to determine the historical time-course of events, but also to check the validity of results. For example, a serum ethanol measured at 350 mg/dL with a simultaneous urine ethanol of only 20 mg/dL must either indicate that the patient downed a large amount of ethanol in the minutes prior to sampling, or that the serum ethanol was grossly overestimated due to an interfering substance, or that the blood was contaminated during phlebotomy. Even though the urine may have been measured using the same assay as the serum, active LDH will not be present in the urine and so will not produce a false positive ethanol result in the way that the serum might.

MARKERS OF ETHANOL INGESTION

Markers of ingestion may be aimed at detecting acute (osmole gap), recent (ethyl glucuronide, ethyl sulfate, phosphatidylethanol (PEth), fatty acid ethyl esters (FAEEs)) or chronic exposure to ethanol (hepatic enzymes, hematological markers, carbohydrate deficient transferrin). The use of these markers will vary based on the clinical indication for testing. More acute assessment of ethanol ingestion can be helpful for emergent treatment. In contrast, markers of recent exposure are commonly used to ascertain abstinence in patients undergoing substance abuse treatment or being managed on chronic opioid therapy (for which a patient might not be allowed to consume alcohol). Chronic markers can be useful in identifing alcohol abusers, especially since some studies have shown physicians may otherwise recognize only 20–50% of patients with alcoholism [85]. This recognition is challenging since chronic alcoholics develop tolerance and may demonstrate mild symptoms relative to the amount of ethanol ingested.

Markers of chronic alcoholism have highly variable sensitivities and specificities. It is important to remember that no single laboratory test is reliable enough on its own to support or refute a diagnosis of alcoholism [86,87].

Markers of ethanol ingestion: osmole gap

Osmolality is the total number of dissolved particles (osmoles) per kilogram of solvent. In a healthy individual, four major osmoles regulate serum osmolality: sodium, chloride, glucose and blood urea nitrogen (BUN). Ethanol, as well as a range of other alcohols (methanol, ethylene glycol, etc), readily dissolves in serum and thus contributes to the overall serum osmolality. A high serum osmolality could theoretically suggest the presence of ethanol. In practice, the absolute serum osmolality is rarely considered. Instead, an osmole gap (OG) is calculated to detect the presence of osmotically active substances that are not otherwise accounted for by the four major osmoles.

The OG is a simple calculation that involves subtracting the calculated osmolality (OSMc) from the measured and true osmolality (OSM) as shown in Eq. (17.2).

$$\mathrm{OG} = \mathrm{OSM} - \mathrm{OSMc} \tag{17.2}$$

The OSM can be measured directly by osmometry using one of two approaches: freezing point depression (FPD) or vapor pressure dew point depression (VPD). The OSMc is determined by measuring the major osmotically active substances (sodium, chloride, glucose and BUN). These values are then substituted into a formula to obtain the OSMc. In practice, only the sodium is typically measured and this value is multiplied by two to account for the associated chloride ions. (Eq. 17.3). Eq. (17.4) shows the more commonly used OSMc variation in the USA where the traditional units are converted to mmol/L.

$$\mathrm{OSMc} = 2(\mathrm{sodium}) + \mathrm{BUN} + \mathrm{glucose} \tag{17.3}$$

$$\begin{aligned}\mathrm{OSMc} = {} & 2(\mathrm{sodium}) + (\mathrm{BUN\,mg/dL} \div 2.8) \\ & + (\mathrm{glucose\,mg/dL} \div 18)\end{aligned} \tag{17.4}$$

Since the presence of ethanol is so common, a number of formulae have been derived based on studies of healthy individuals, or patients with known exposure to ethanol alone. The one most commonly used in medicine is that of Smithline and Gardner [88], modified to include ethanol by Purssell et al. [89], noting that all measurements are made in mmol/L (Eq. 17.5). The variation used with the traditional units used in the USA is given in Eq. (17.6).

$$\mathrm{OSMc} = 2(\mathrm{sodium}) + \mathrm{BUN} + \mathrm{glucose} + 1.25(\mathrm{ethanol}) \tag{17.5}$$

$$\begin{aligned}\mathrm{OSMc} = {} & 2(\mathrm{sodium}) + (\mathrm{BUN\,mg/dL} \div 2.8) \\ & + (\mathrm{glucose\,mg/dL} \div 18) \\ & + (\mathrm{ethanol\,mg/dL} \div 3.7)\end{aligned} \tag{17.6}$$

Using the 1.25 multiplier (3.7 traditional unit divisors) as opposed to the theoretical 1.0 (Eq. 17.5), in part accounts for the body's response to the osmotic effect of ethanol by releasing cellular osmolytes, and metabolic derangements such as elevated serum amino acids that result from the effects of ethanol on metabolism [90]. Additionally, ethanol alters the dissociation behavior of other analytes within the serum thus distorting the osmolality [91].

The method by which serum osmolality is measured significantly affects the reliability of the result in the presence of volatile components. FPD yields the most reliable measure of altered osmolality in the presence of toxic alcohols including ethanol. Because of the frequency with which ethanol is responsible for hyperosmolar samples, osmolality must be measured by FPD rather than by VPD. Since there is a linear relationship between the ethanol concentration and ΔOSM (difference between FPD and VPD osmometry results) this has been used as a tool for estimating serum ethanol [92]. In clinical practice, it would be unusual to have both types of osmometry available since FPD is the technique of choice. It must be pointed out that other toxic alcohols are also falsely represented by the OG when OSM is measured by FPD. Acetone, ethanol, isopropanol, and methanol when added to serum at relevant concentrations, produce the expected linear increase in FPD osmolality, while VPD osmolality is unaffected: only ethylene glycol produces a linear increase in both VPD and FPD osmolality [91]. Walker et al. [93], describe a patient in whom methanol toxicity was strongly suspected based on history, visual impairment, and severe AG metabolic acidosis. No OG was present, but the patient was dialyzed and treated with ethanol infusion with good result. Later, the serum methanol was reported >100 mg/dL. Investigation revealed that osmolality had been measured using VPD, and subsequent *in-vitro* testing confirmed the insensitivity of VPD to toxic values of both methanol and ethanol. Thus it is important to be cognizant of the technique used by the laboratory to avoid missing a potentially life-threatening but treatable intoxication.

There is considerable interest in using the rearranged form of the OG equation (Eq. 17.6) to calculate the ethanol concentration present [89] (Eq. 17.7):

$$\mathrm{Ethanol\ conc.mg/dL} = \mathrm{OG} \times 3.7 \tag{17.7}$$

However, actual ethanol concentrations could not be predicted with sufficient accuracy when testing patient specimens [91]. This is partly because the wide range for a normal OG makes it difficult to decide how much of the OG to attribute to the ethanol; at higher ethanol concentrations investigators consistently report overestimation of the ethanol concentration by the OG, sometimes by as much as 30% [94]. Taking this notion one step further, even in the presence of ethanol, the concentration of another toxic alcohol could theoretically be estimated from its molecular weight. The osmotic activity attributed to 1 mg/dL of each is: Methanol = 0.34, Isopropanol = 0.17, Ethylene glycol = 0.16, and Propylene glycol = 0.13 mOsm/Kg. OGs in children are even more problematic to interpret as a normal OG may reach 22 mOsm/kg, which is unfortunate since small children are one of the patient populations most at risk of an unknown ingestion who would benefit from an early diagnosis [95].

One practical use of the OG from the laboratory's perspective is to ensure that a measured ethanol value is consistent with the measured osmolality. In the three cases reported by Gharapetian et al. [64], described earlier in this chapter in the interference section, the OSMs were 306, 286 and 278 mOsm/kg respectively. The calculated OG if ethanol was absent were relatively normal for such sick patients at 18, −1 and −1 mOsm/kg respectively, but if ethanol was indeed positive at the concentrations measured the OGs re-calculated to −23, −42 and −44 mOsm/kg respectively. Such grossly abnormal results should immediately raise suspicion that one of the parameters measured is in error.

From the physician's perspective, the clinical value of the OG is to determine whether other pathological substances are in the patient's serum contributing to OSM that are not accounted for in the OSMc value. Specifically, in practical terms, deducing the presence of other toxic alcohols, such as methanol, iso-propanol, or ethylene and propylene glycols, especially in situations where direct measurements of these are not available in a time-sensitive manner. This highlights the need for accurate ethanol concentrations. If the ethanol measurement is inaccurate, then an OG may be attributed erroneously to the presence of a toxic alcohol, or conversely one may be assumed not to be present when in fact it is, thus missing potential treatment options. Readers should also note that normal gap values can vary considerably with instrumentation, so what constitutes normal must be taken in context of each laboratory or patient group. For example, in one study [96], OGs in healthy patients increased by some 12 mOsm/kg over the period 1996 to 2004. Despite these caveats, the OG still remains a particularly useful *screening* test for toxic alcohols in asymptomatic patients, as the latency period to symptoms can be long since it is the production of metabolites which is responsible for the majority of the toxicity.

Markers of ethanol ingestion: ethyl glucuronide, ethyl sulfate, phosphatidylethanol (PEth) and fatty acid ethyl esters (FAEEs)

Markers of ethanol use can be broadly divided into two categories: indirect and direct. Indirect markers represent cellular alterations that arise from the presence of ethanol in the human body; these include biochemical abnormalities and will be discussed in more detail in the next section. Direct markers are measurable analytes that arise directly from exposure to ethanol; these include ethanol itself and any of the metabolite which arise from the presence of ethanol in the human body. Four major direct markers of ethanol use have gained in popularity in recent years: ethyl glucuronide, ethyl sulfate, phosphatidylethanol (PEth) and fatty acid ethyl esters (FAEEs). Formation of these direct markers occurs as shown in Fig. 17.1.

Though accounting for a small percentage of ethanol elimination, phase two metabolism does occur and yields ethyl glucuronide and ethyl sulfate which are primarily eliminated in the urine. In contrast to ethanol, ethyl glucuronide and ethyl sulfate are non-volatile, water soluble, and stable to storage - properties that give them significant advantages as routine tests for ethanol use [97]. Testing for these metabolites is widely used but typically performed via tandem mass spectrometry. There are, however, enzyme immunoassay kits available for ethyl glucuronide for some automated platforms [98]. This screening test has the potential to provide a rapid cross-check on a serum ethanol that is inconsistent with the history or symptoms, and could be performed in the routine chemistry lab on the same automated analyzer used for the original ethanol test [99]. While ethyl glucuronide accounts for less than 0.1% of the dose, its presence is highly specific for ethanol, and it can be found in urine, serum and oral fluid for three to five days after a single 50 g dose of ethanol. This is a favorable detection span compared to ethanol, which is typically 10−24 h in body fluids.

While ethyl glucuronide and ethyl sulfate can extend the detection window of ethanol use from hours to days, PEth in blood and FAEEs in hair have been shown to potentially extend this to weeks [100]. Analytic methods for these markers are limited to mass spectrometry-based laboratory developed tests and use of these markers clinically is limited. However, substantial and growing data has shown that these markers correlate with ethanol consumption, can be used to accurately determine abstinence and, in the case of PEth, can offer some insight into the patterns of drinking [101]. These markers have shown increased sensitivity and

specificity compared to indirect markers, such as liver enzymes, which will be discussed in the next section.

Markers of ethanol ingestion: biochemical abnormalities

Long-term alcohol abuse can give rise to a number of biochemical abnormalities that can be used to suggest chronic abuse (termed indirect markers). Rather than testing for the toxic substance itself, testing is directed at expected consequences of exposure to the substance. These biochemical abnormalities have a long history of use clinically and when used properly can still offer insight into the patient's use, or misuse, of ethanol.

As discussed in the section on ethanol pharmacokinetics, the liver serves as the primary site for ethanol metabolism and generation of toxic metabolites. Not surprisingly, indicators of liver damage are thus common markers of ethanol use/abuse. Assays for the transaminases, aspartate amino transferase (AST) and alanine amino transferase (ALT), are universally available on automated chemistry analyzers, inexpensive to perform and routinely assessed if there is concern for chronic ethanol misuse. Both enzymes are elevated in chronic liver disease, but usually <1000 U/L; higher values reflect acute hepatic disturbance. To add further insight, a ratio of the AST over ALT activity can be calculated (known as the De Ritis ratio) [102]. If the ratio lies between 1.5 and 2, alcoholic hepatitis is to be suspected. While there are several caveats and shortcomings to this ratio, the availability of transaminase measurements makes it widely available and one easy to use marker in assessing a patient.

Additional liver enzymes and hematologic abnormalities are also observed in chronic alcoholics. The activity of GGT (γ-glutamyl transferase) and the RBC Mean Corpuscular Volume (MCV) have been the traditional markers of chronic ethanol use: serum GGT >500 U/L with an MCV >100 fL in the absence of another condition should raise suspicion of ethanol abuse [103,104]. Both tests are readily available in most laboratories. Care should be taken to rule out megaloblastic anemia from B12 and/or folate deficiency as the cause of an increased MCV. GGT lacks specificity since numerous disorders and drugs can elevate GGT, including diabetes, hyperthyroidism, cardiac and renal disease, smoking, barbiturates, phenytoin, carbamazepine and rifampicin [105,106].

In habitual drinkers the proportion of transferrin with none, one, or two sialic acid chains (collectively termed carbohydrate deficient transferrins; CDT) is >6%, whereas healthy individuals with no or low alcohol consumption will have a CDT <2%. CDT analysis is somewhat specialized (HPLC, capillary electrophoresis, IEF) [107,108] but an immunoassay is recently available on automated chemistry platforms [109]. However, genetic transferrin variants, and some non-alcoholic hepatic diseases such as hepatocellular carcinoma, chronic hepatitis C, and primary biliary cirrhosis give false positives, and the main strength of the test lies in detection of relapse in chronic alcoholics [110,111].

TOXIC ALCOHOLS

No discussion of laboratory testing for ethanol is complete without mentioning toxic alcohols. Toxic alcohols are in common usage and often taken in overdose, either deliberately or unintentionally, particularly in children. It is important therefore to have a method which can distinguish between these and identify them correctly both in the presence or absence of ethanol. The salient laboratory tests and distinguishing clinical features of exposure to toxic alcohols are presented in Table 17.5, which is intended to be illustrative rather than exhaustive.

To date, enzymatic procedures for toxic alcohols can only be used on automated analyzers that have "open" applications, and are not FDA-approved for this purpose. None of these is widely adapted or well controlled, characterized or standardized. For example, methanol is converted to formaldehyde by AOD and then to formic acid by formaldehyde DH, following the reaction by NADH production [112]. Since the latter reaction is highly specific for formate, the entity which correlates directly with methanol toxicity, it may be more appropriate to measure formate directly with FDH [113]. Another simple assay utilizes periodate to oxidize ethylene glycol to formaldehyde, which is then reacted with a chromogen. This commercially available veterinary product is reported to show good performance with human samples [114]. A similar test generates a blue chromophore, which also shows positive for propylene glycol, but has some cross-reactivity with ethanol (>50 mg/dL) [115]. Serum oxalate may also be measured by converting it to formate with oxalate decarboxylase, then analyzing the formate as described above [116].

Failing the provision of such tests, an alternate strategy is to use the "gap" between the results of two non-ideal tests in the hope of providing a good result. Despite its shortcomings, the OG remains a useful *screening* test for toxic alcohols in asymptomatic patients, since the metabolites are primarily responsible for the toxicity. Often ethanol (with the OG) is measured as a means of determining if another alcohol is present. Performance of the OG for such use was investigated by Lynd et al. using rigorous Standards for Reporting Diagnostic Accuracy (STARD) Criteria [117]. Samples from 20 patients with analytically proven exposure to ethylene

TABLE 17.5 Uses, toxic metabolites, and clinical and laboratory features of toxic alcohols.

Compound (common name)	Sources	Toxic metabolite(s)	Distinctive clinical & lab features
Ethanol (sugar slcohol)	Surgical spirit Perfumes Beverages Mouthwash Hand sanitizer Food flavors Cough syrups	Acetaldehyde	Increased OSM and OG Anion Gap Metabolic acidosis Hypoglycemia Ketonuria if chronic Tests for chronic use Inebriation, CNS depression Vomiting
Methanol (wood alcohol)	Windshield wash fluid Gasoline antifreeze Paint remover Photocopying fluid Surgical spirit Moonshine	Formaldehyde Formate	Increased OSM and OG Profound Anion Gap metabolic acidosis Lactic acidemia Hypoglycemia Transient inebriation Nausea/vomiting Blindness
Iso-propanol (rubbing alcohol)	Rubbing alcohol Windshield wash fluid Glue remover Cleaners & De-greasers Skin lotions	Acetone (non-toxic)	Increased OSM and OG Normal Anion Gap and pH Normoglycemia Kentonuia, no glucose Profound inebriation and CNS depression Tachycardia, hypertension, vasodilation Gastritis, hematemesis
Ethylene glycol (antifreeze)	Radiator fluid Anti-freeze	Glycolate Oxalate	Increased OSM and OG Anion Gap Metabolic acidosis Lactic Acidemia (may be falsely increased) Hypoglycemia Hypocalcemia Muscle dysfunction, seizures Acute renal failure Oxalate crystaluria Transient inebriation
Propylene glycol	I.V. drug preparations	D-Lactate	Increased OSM and OG Anion Gap Metabolic acidosis Lactic acidemia (maybe falsely low) Acute renal failure Hippuric aciduria

glycol (10) or methanol (9) or both (1) were included and using a diagnostic threshold of OG ≥10 mOsm/kg for patients requiring antidotal therapy resulted in sensitivity of 0.9 and specificity of 0.5; sensitivity was 1.0 when identifying patients requiring dialysis.

Other gaps have been attempted for identification of toxic alcohols. Ethylene glycol might be inferred from the "triglyceride gap" between the results of two different automated analyzer kits, one containing ethylene glycol-reactive glycerol dehydrogenase and the other non-reactive glycerol dehydrogenase. This again showed some cross reactivity with propylene glycol (<15%) [118]. As described in the section on lactate determination, the lactate gap (amperometric value minus a glycolate-unaffected assay value) can be used as a surrogate to identify EG exposure, and thus to estimate the glycolate concentration [119]. A strategy comparing the sample response between a semi-quantitative AOD dipstick and an ADH-based "specific" ethanol assay could be used to gauge the presence of methanol which reacts strongly with AOD but not ADH [120].

Efforts should be devoted to ensuring that clinical laboratories can provide direct measurements for methanol and ethylene glycol in serum or plasma since toxicity can occur without clinical signs of inebriation so surrogates could be used" [9]. Gas Chromatography (GC) could be available for this purpose, but only rarely is. The limitations of the methodology and need for 24/7 availability limit its use. The NACB places the onus on diagnostic reagent manufacturers to provide reliable tests for these toxic alcohols and glycols, rather than have the laboratory make a "diagnosis by exclusion" or rely on surrogate testing methods.

CONCLUSIONS

Ethanol misuse remains a substantial problem worldwide with significant associated costs in terms of both lives lost and financial impact. This widespread misuse of ethanol has spurred the development of automated ethanol tests for the clinical laboratory. Today, automated ethanol testing is widely available in clinical laboratories throughout the world. This testing, based primarily on spectrophotometric detection of ADH activity, has demonstrated excellent performance in terms of both accuracy (compared to GC) and precision. In spite of the excellent analytic performance of the testing, pre- and post-analytic challenges remain which can result in inaccurate, or inaccurately interpreted, ethanol results. As with any laboratory result, it is helpful to evaluate the results in the context of the patient. Toward this end, a range of direct and indirect markers of ethanol ingestion are available. Unfortunately, widely available automated tests for non-ethanol alcohols are still lacking. Hopefully future work will focus on addressing this shortcoming and making such testing more widely available.

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CHAPTER

18

Effects of herbal supplements on clinical laboratory test results

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INTRODUCTION

The popularity of using herbal supplements is steadily increasing among the general population in the United States where it has been estimate that roughly 20,000 herbal products are commercially available. However, the ten most commonly used herbal supplements are echinacea, ginseng, ginkgo biloba, garlic, St. John's wort, peppermint, ginger, soy, chamomile and kava [1]. Population surveys also indicates that one-third to one-half of Americans take dietary supplements more or less on a regular basis. Users are more likely to be women, non-Hispanic whites, and more financially secure than non-users. The annual sale of herbal supplements has increased from $9.6 billion in 1994 to $36.7 billion in 2014 [2]. The Natural Medicine Comprehensive Database include a list of 54,000 dietary supplements but 12% of such supplements have safety concerns. In the United States an estimated 23,000 emergency department visits annually are caused by significant toxicity related to use of herbal supplements [3].

According to the World Health Organization (WHO), approximately 80% of the world population relies on herbal medicines. In addition, many patients take herbal medicines concurrently with conventional drugs, which may be problematic due to many clinically significant interactions of herbal supplements with drugs that may either cause treatment failure or drug toxicity. In the United States, it has been estimated that 20–30% patients may concurrently take both herbals supplements and conventional drugs. In Taiwan, 94% patients and in Korea 83% patients simultaneously use prescribed drugs and herbal supplements [4]. Although the general population considers herbal medicines safe, scientific studies have shown significant toxicity and even fatality associated with certain herbal medicines.

Herbal remedies are sold in the United States according to 1994 Dietary Supplement Health and Education Act and are classified as food supplements. Manufacturers of herbal remedies are not allowed by the law to claim any medical benefit derived from using such products but at the same time these products are not under the FDA (Federal Drug Administration) surveillance. Therefore, dietary supplements can be manufactured and sold without demonstrating safety and efficacy to the FDA as required for drugs. Unlike drugs which are highly regulated by the FDA, for herbal supplements, the FDA cannot take steps against a toxic supplement unless patients have already been harmed. Furthermore, no government agency has the authority to force the manufacturer to provide proof of efficacy of any herbal supplement [5].

Herbal supplements are more regulated in European countries. For example, in Germany, German Commission E has some control over marketing of herbal supplements as the commission publishes monographs prepared by an interdisciplinary committee using historical information; chemical, pharmacological, clinical, and toxicological study findings; case reports; epidemiological data; and unpublished manufacturers' data. If an herbal supplement has an approved monograph, it can be marketed. The European Directive 2004/24/EC released in 2004 by the European Parliament and also by the Council of Europe now provides the basis of regulation of herbal supplements in European market. This directive requires that herbal medicines require authorization by the national regulatory authorities of each European country and that these products must

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be safe before they can be marketed to the general population. The safety of a supplement will be established based on published scientific literature and when safety is not sufficient, it will be communicated to consumers. In Europe, there are two kinds of herbal supplements; (A) herbal supplements with well-established safety and efficacy, (B) traditional herbal supplements, which do not have a recognized level of efficacy but are relatively safe [6,7]. The Australian Government also created a Complementary Medicine Evaluation Committee in 1997 to address regulatory issues regarding herbal remedies. In Canada, the Federal Government implemented a policy on January 1st, 2004 to regulate natural health products but naturopaths, traditional Chinese medicine practitioners, homeopaths and Western herbalists are concerned that this policy may eventually affect their access to the products they need to practice effectively [8].

ISSUES WITH VARIABLE ACTIVE INGREDIENTS AND POOR MANUFACTURING PRACTICE OF HERBAL SUPPLEMENTS

Herbal supplements are crude extract of plant products. Therefore, due to seasonal variation in active ingredients in these plants, significant variations in active components of herbal supplements are expected. As a result, two different preparations of the same herbal supplement may have different efficacies. Moreover, if that particular herbal product has a known interaction with a drug or known effect on a laboratory test, such effect may also vary significantly between two different preparations of the supplement. Shah et al. performed content analysis of hypericin in six commercially available St. John's wort preparations. All products stated to contain 0.3% hypericin but actual analysis revealed that total hypericin content varied from 7.725 to 38.57% of the labeled claim indicating that no product contained 0.3% hypericin as claimed in the package label [9].

Increased clearance of midazolam due to induction of the CYP3A4 enzyme by St. John's wort is well documented but the expected magnitude of such midazolam-St. John's wort interaction depends on the hyperforin content of the preparation. Muller et al. determined midazolam pharmacokinetics the day before initiation of St. John's wort intake and on day 14 of St. John's wort intake in 42 male healthy volunteers. The authors observed a 79.4 % reduction in the area under the curve at 0−12 h (AUC_{0-12}) when volunteers consumed St. John's wort extract (hyperforin intake: 41 mg/day) but AUC reduced only by 57.9% when volunteers consumed St. John's wort powder (hyperforin intake: 12 mg/day) [10]. In another study, the authors observed no clinically significant interaction between midazolam and St. John's wort when 20 health male volunteers consumed St. John's wort powder containing low amounts of hyperforin [11].

Another serious problem associated with use of herbal supplements is poor manufacturing practices. In 2008, FDA initiated inspecting manufacturing process of herbal supplements in the United States. In general, approximately 50% of manufacturing facilities inspected between 2008 and 2012 by the FDA violated good manufacturing practices. The violations included lack of recipes for the product and unsanitary conditions. Moreover, testing was not performed to verify the identity of active herbal ingredients. In a study of commercial herbal products, DNA was recovered from 91% of 44 herbal supplements manufactured by 12 companies. DNA barcoding analyses revealed that only 2 out of 12 companies provided products that contained DNA of the plant listed as active ingredient. Moreover, 59% of herbals contained plant species not listed on the product labels and 33% contained DNA that matched rice, soybeans and wheat [12].

FDA WARNINGS TO TOXIC HERBS

Although the FDA does not have authority to force manufacturers of herbal supplements to withdraw a product due to known toxicity, the FDA issues public warning regarding toxicity of herbal supplements thus encouraging the general population to avoid such toxic herbal products. In 2001 the FDA issued a warning regarding liver toxicity from use of comfrey, in 2001 liver toxicity associated with use of Lipokinetix (weight loss product), in 2002 liver toxicity due to use of kava, in 2009 liver failure due to use of weight loss product hydroxycut, in 2011 warned against use of uprizing because it contained anabolic steroid methyl drostanolone, and in 2013 acute liver failure due to use of weight loss product OxyElite Pro [13].

Lipokinetix was promoted for weight loss by mimicking exercise because this product increased metabolic rate. This product contained norephedrine, caffeine, yohimbine, and diiodothyronine as well as sodium usniate. Usnic acid was believed to be

associated with liver toxicity. Hydroxycut was introduced in the market as a weight loss product (fat burner) in 2002. The original formulation contained ma huang (ephedra) which was banned by the FDA in 2004. Although manufacturer removed ephedra from the formula, further reports of liver toxicity from using this product continued. Although the exact hepatotoxic compound in this formulation has not been identified, the suspected herbal is Camellia sinensis. After the FDA warning, hydroxycut was reformulated and re-introduced in the market as hydroxycut, SX-7 clean sensory but a new case of liver toxicity from using the reformulated product has been reported. Following the FDA warning, OxyElite Pro was reformulated but the FDA informed the manufacturer that one of the ingredient aegeline which is N-[2-hydroxy-2(4-methoxyphenyl) ethyl]-3-phenyl-2-propenamide, a natural product from bael tree (*Aegle marmelos*, a tree native to India) may be responsible for toxicity [14]. Hepatotoxicity due to use of kava is discussed later in the chapter.

MECHANISMS BY WHICH HERBAL SUPPLEMENTS AFFECT LABORATORY TESTS

Use of herbal supplements can affect laboratory test results by various mechanisms:

A. Physiological effects: When an herbal remedy alters normal physiological functions of the body or causes organ damage, unexpected laboratory tests results may be the first indication of toxicity of the herb. Example, abnormal liver function tests due to use of kava.

B. Drug-herb interaction may lead to unexpected levels of a therapeutic drug causing either treatment failure or drug toxicity. Therapeutic drug monitoring is very useful in identifying such clinically important drug herb interactions, for example, an unexpectedly low level of cyclosporine in a patient who showed therapeutic levels before, due to an interaction of the herbal antidepressant St. John's wort with cyclosporine.

C. Component of an herbal remedy may cross-react with an assay antibody causing erroneous results, for example falsely elevated serum digoxin values due to interference of bufalin, active ingredient of Chinese medicine Chan Su, with serum digoxin immunoassays.

Commonly observed abnormal laboratory test results due to use of herbal supplements are summarized in Table 18.1.

TABLE 18.1 Abnormal laboratory test results due to use of herbal supplements.

Abnormal laboratory test results	Affected organ	Commonly known herb causing such toxicity
Liver function test (elevated Liver enzymes and bilirubin	Liver	Kava, Chaparral, Comfrey, Germander, Coltsfoot, Mistletoe, Pennyroyal, Lipokinetix, Skullcap, Ma Huang, Green tea extract (but not green tea), Black cohosh etc
Elevated creatinine, blood urea nitrogen (BUN)	Kidney	Aristolochic acid containing Chinese herbs, Wormwood oil, Sassafras, Horse Chestnut, Calamus, Bladder-Wrack, White Sandalwood oil, Juniper berry, Yohimbine, Willow Bark, Chaparral
Hypoglycemia	Not Applicable	Ginsengs (Asian, Korean, American), Coccinia indica, Bitter Melon (Momordica charantia), Fenugreek seeds, Gymnema sylvestre, Prickly pear cactus (nopal), Fig leaf, Milk Thistle, Holy basil (Ocimum sanctum), garlic, ginger, cinnamon, Chromium
Hypokalemia	Not applicable	Licorice
Thyroid function test	Thyroid	Kelp
Falsely elevated serum digoxin		Interference due to the presence of bufalin (Chinese medicine Chan Su, Lu-Shen- Wan, oleandrin (oleander containing herb) and convallatoxin (herbs containing lily of the valley extract)

HERBAL SUPPLEMENTS AND ABNORMAL LIVER FUNCTION TESTS

Liver is the largest organ of the body responsible for many important physiological functions including metabolism of drugs and toxins for elimination from the body. Many potentially toxic compounds enter the body through the gastrointestinal track and are immediately transported via the portal vein to the liver. Therefore, the liver sustains the highest exposure to some toxins and is susceptible to damage. Liver function tests are used for diagnosis of adequate liver function as well as to identify any potential injury. The liver function tests include enzymes, bilirubin, proteins, and coagulation factors. Some are chosen because they are readily released in large quantities following cellular injury. Measurements of the serum or plasma activities of the enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transferase (GGT), and alkaline phosphatase (ALP) are routinely

performed to detect liver injury. These intracellular enzymes are released when hepatocytes are damaged, but none of these are specific to liver. GGT, along with alkaline phosphatase (ALP) also reflects injury to biliary cells. Total bilirubin and its conjugated and unconjugated forms are useful in differentiating the causes of jaundice. Various herbal products such as kava, chaparral, comfrey, pennyroyal, black cohosh, mistletoe and green tea extract may cause liver damage. Despite known toxicity and warning from authorities, these herbal supplements are still available in health food stores.

Kava

Kava is the most commonly cited herb related to liver toxicity. Kava, an herbal sedative with antianxiety or calming effects, is prepared by extracting the rhizomes of the *Piper methysticum*, a South Pacific plant. The active ingredients of the plant, known as kavalactones, are found mostly in the rhizomes [15]. Early scientific research indicated that kava was as efficacious as antidepressant drugs and tranquilizers in treating anxiety disorders [16]. In fact, kava extracts were considered safe alternatives to drug therapy for treating anxiety disorders before 1998. However, by 2003, 11 cases of hepatic failure were reported related to use of kava including seven patients requiring liver transplantation and four deaths. In January 2003, kava was banned in the European Union and Canada; and the FDA issued another warning. By 2009, more than 100 cases of hepatotoxicity have been linked to kava exposure. Many have followed co-ingestion with alcohol which appears to potentiate the hepatoxicity [17].

There are six major kavalactones; methysticin, 7,8-dihydromethysticin, kavain, 7,8-dihydrokavin, yangonin and desmethoxyyagonin. These six kavalactones account for approximately 96% of all kavalactones present in kava. Commercial products are usually ethanol or acetone extracts whereas traditional extracts used by native people in South Pacific are aqueous extract [18]. It has been previously proposed that kava hepatotoxicity is not observed after consumption of traditional aqueous extract of kava as consumed by native people of Pacific Islands during ceremonies but hepatotoxicity is observed after consumption of commercially prepared kava extracts where ethanol or acetone is used for extraction. However, cases studies and published reports analyzed by experts of the World Health Organization (WHO) revealed that traditional aqueous extracts used in New Caledonia, Australia, United States, and Germany may also cause hepatotoxicity [19]. Pipermethysticine, a toxic alkaloid present in kava leaves and stem may contaminate kava products during production if quality control is poor. Flavokavin, another toxic alkaloid present in kava root may also cause toxicity. Hepatocellular toxicity results from mitogen-activated protein kinase signaling leading to oxidative stress and apoptosis. The chemical content of kava products and therefore potential for toxicity vary according to plant age, parts use, cultivating condition, geographic location and growth condition of the plant [20].

Hepatotoxicity associated with kava consumption is usually reflected by increases in the serum activities of ALT, AST and GGT. A 50-year-old male who exceeded the maximum recommended dose of 3 kava capsules daily for two months was found to have AST and ALT increased 60–70 fold above the reference ranges. All hepatitis serology for this patient was negative and kava use was determined to be the cause of the liver damage. This patient eventually received a liver transplant [21]. Ingestion of kava may cause temporary yellowing of the skin, hair and nails. A 70-year old man, who took kava products for anxiety for two to three weeks, experienced itching several hours after sun exposure and developed plaques on his face, chest and back. A similar case involved a 52-year-old woman who presented with papules and plaques on her face, arms, back and chest after taking kava products for three weeks. In both cases, a skin biopsy revealed lymphocytic infiltration of the dermis with destruction of the sebaceous glands [22]. Although liver damage is the most widely documented toxicity of kava, several cases involving central nervous system (CNS) depression have been reported when the herbal is combined with other sedatives and hypnotics. One such case report described a 54 year old male who became comatose after three days of kava ingestion while taking alprazolam, cimetidine and terazosin. It was thought that the adverse reaction was related to a pharmacodynamic interaction between kava and alprazolam [23].

Case Report: A 42 year old healthy white male presented with weakness, loss of appetite and jaundice. He admitted alcohol consumption of not more than one drink a day but denied use of any medication or abuse of any drug. However, he reported that he came back from honeymoon three weeks ago and during honeymoon he and his wife stayed 20 days on the Samoan Island. The patient showed signs of scleral and skin jaundice and also had abnormal liver function tests including AST of 1602 U/L, ALT of 2841 U/L, GGT of 121 U/L, ALP of 285 U/L and LDH of 460 U/L. His total bilirubin was 9.3 mg/dL. Serological tests for hepatitis, cytomegalovirus and Epstein-Barr virus were negative. Abdominal ultrasound showed a hyperechoic liver structure with normal biliary ducts. Because the available information did not provide any satisfactory cause of his disease, the patient as interviewed again and he admitted that during his trip to Samoa Island he had repeatedly participated in a kava ceremony and consumed a total two to three liters of traditional kava extract during his stay at Samoa. The patient was discharged 19 days after admission and after 36 days he completely recovered based on repeated ultrasound scan and laboratory test results [24].

Case Report: A 52 year old woman presented to her local doctor with a two week history of fatigue, nausea and increasing jaundice. For last three months she took an herbal supplement (Kava 1800 Plus, Eagle Pharmaceuticals, Castle Hill, Australia) prescribed by a naturopath for anxiety. She took one tablet thrice daily labeled as containing 60 mg kavalactones, 50 mg Passiflora incarnata and 100 mg Scutellaria lateriflora (blue skullcap). She was then referred to authors' hospital and on admission her serum ALP was 190 U/L, ALT 4539 U/L, serum albumin 3.4 gm/dL and bilirubin 12.2 mg/dL. All tests for hepatitis A, B, C, Epstein Barr virus and cytomegalovirus were negative. Over subsequent weeks her condition deteriorated and on day 17 of admission to the hospital she underwent liver transplant. Unfortunately the procedure was complicated by excessive bleeding and the patient died. It was determined that her liver failure was due to use of kava [25].

Comfrey and coltsfoot

Comfrey (*Symphytum officinale*) is a perennial plant whose leaves and roots are used traditionally for wound healing, repairing broken bones, and in the treatment of arthritis, gout and psoriasis. However, there is no scientific evidence to support these claims. Comfrey contains pyrrolizidine alkaloids which are well-known hepatotoxins and Russian comfrey is even more toxic than the European and Asian comfrey due to higher content of toxic alkaloids. Yeong et al. described a case involving a 23-year old male presenting with severe veno-occlusive disease and hypertension. He subsequently died from the liver failure. The patient was a vegetarian who had used comfrey leaves as a dietary supplement [26]. Fatal cardiac glycoside poisoning has been reported when a woman consumed "comfrey tea" but mistakenly used foxglove (*Digitalis purpurea*) for preparing the tea [27].

Pyrrolizidine alkaloids and their N-oxides are found in comfrey as well as in a variety of related herbs. Coltsfoot (*Tussilago farfara*) is another herb closely related to comfrey that also contains a pyrrolizidine alkaloid (senecionine). Veno-occlusive disease has been reported in a newborn infant of a woman who ingested coltsfoot containing tea.

Case Report: A female infant was referred to the neonatal intensive care unit five days after birth due to jaundice, massive hepatomegaly and ascites. The baby was delivered by cesarean section at 36 weeks of gestation due to an unconfirmed suspicion of premature separation of placenta. Initial biochemical tests showed AST 3725 U/L, ALT 760 U/L, and bilirubin 9.6 mg/dL but all tests for hepatitis, Epstein-Barr virus, and cytomegalovirus were negative. When no cause of liver failure in the infant was found, the mother admitted that she had used an herbal tea daily throughout her entire pregnancy. Toxicological analysis of the herbal tea using thin layer chromatography revealed the presence of senecionine, a pyrrolizidine alkaloid. The herbal tea was found to contain 10 different plants of which Coltsfoot (Tussilago farfara) was 9% of the preparation by weight. Although the mother did not experience any adverse effect, the immature liver of the fetus was unable to detoxify the alkaloids leading to the development of the veno-occlusive disease. Unfortunately the infant died 38 days after birth [28].

Germander

Germander, *Teucrium chamaedrys*, is an aromatic plant in the "mint family" belonging to the genus Teucrium. Its blossoms are used as a folk medicine to treat dyspepsia, diabetes and gout. However, chronic use of germander may cause hepatotoxicity. In general, the toxic effects of germander are first seen within 9 weeks of use and are manifested by jaundice and elevated liver enzymes; ALT and AST. After discontinuation of the herbal, recovery may take 6 weeks to 6 months. The mechanism of toxicity is thought to be related to di-terpenoid compounds that are metabolized to more potent toxins within the liver [29]. The major hepatotoxic diterpene found in germander is teucrin A which is bio-activated by the cytochrome P-450 enzymes in the liver [30]. In addition to teucrin A, another hepatotoxic ingredient of germander is teuchmaedryn A. More than 45 cases of hepatotoxicity following germander use have been reported in France including one fatality [31].

Two Case Reports: In the first case, a 55-year-old woman became ill after taking 1600 mg per day of germander for six months in an effort to reduce her cholesterol. Her laboratory finding included: total bilirubin 13.9 mg/dL direct bilirubin 8.4 mg/dL, AST 1180 U/L, ALT 1500 U/L, and ALP 164 U/L but all serological tests for hepatitis (A, B and C) were negative. Her hepatitis resolved within two months of discontinuing the germander. In the second case a 45-year-old woman took germander (260 mg/day) for weight loss. Her laboratory studies also suggested hepatotoxicity: total bilirubin 3.5 mg/dL, direct bilirubin 2.2 mg/dL AST 417 U/L, ALT 451 U/L, and ALP 79 U/L. She discontinued the herbal and her health condition improved. After 4 months she felt much better and resumed the germander. Within the week, she again developed indications of liver toxicity: total bilirubin 17.0 mg/dL, direct bilirubin 13.2 mg/dL. AST 1245 U/L, ALT 784 U/L and ALP 89 U/L. Hepatitis test were again negative. The patient stopped using germander again and her liver function tests improved over three months [32].

Chaparral

Chaparral is a plant that grows in Southwestern US and Northern Mexico and leaves are used as an herbal therapy in the treatment of a wide variety of symptoms from cold sores to muscle pain.

Unfortunately, chaparral has been associated with severe hepatotoxicity as documented by several reports of chaparral-associated hepatitis. Sheikh et al. reviewed 18 cases of suspected chaparral toxicity reported to the FDA and confirmed 13 cases of hepatotoxicity related to the herbal. Clinical presentation included significant elevations in liver enzyme activities and other biochemical markers of hepatic injury 3–52 weeks after ingestion of chaparral. In most cases the liver enzymes returned to the reference range after cessation of use, but at least in two cases fulminate hepatitis was observed suggesting irreversible liver injury [33].

Pennyroyal

Pennyroyal, *Mentha pulegium*, is a plant in the mint genus whose leaves release a spearmint-like fragrance when crushed. Because of the strong fragrance, it is often found as an additive to bath products and aromatherapy. Traditionally, pennyroyal has been brewed as a tea to be ingested in small amounts as an abortifacient and emmenagogue. The plant and oil contain several components including pulegone (major compound). Pulegone is metabolized by the liver to a more toxic compound, menthofuran, and is known to deplete the hepatic glutathione stores (similar to that induced by overdoses of acetaminophen). Anderson et al. reported four cases of pennyroyal ingestion where one patient died. In the fatal case, postmortem analysis of serum showed pulegone concentration of 18 ng/mL and menthofuran concentration of 1 ng/mL. Interestingly, N-acetylcysteine, an antidote for treating acetaminophen (paracetamol) poisoning is also effective in treating poisoning due to ingestion of pennyroyal. The authors successfully treated another patient with N-acetylcysteine. The two other patients were not treated with N-acetylcysteine because they ingested minimal amounts. In addition, authors also reviewed 18 cases of toxicity related to pennyroyal ingestion and concluded that ingestion of as little as 10 mL of pennyroyal oil may cause severe toxicity [34]. Gordon and Khojasteh commented that most of the reported toxicity and fatality associated with pennyroyal ingestion occurred in women who used the herb as an abortifacient [35].

Green tea extract

Green tea (Camellia sinensis) contains beneficial bioactive polyphenolic compounds known as catechin (epicatechin, epigallocatechin, gallocatechin, epicatechin gallate and epigallocatechin 3-gallate). These compounds are superior antioxidants. However, epigallocatechin 3-gallate (EGCG), the major bioactive component of green tea may be a hepatotoxin at higher concentrations. In general, drinking green tea is good for health and there is no reported hepatotoxicity from consumption of 2–3 cups of green tea per day. However, concentrations of EGCG are much higher in weight loss products such as Slim Quick which contains green tea extract. Consuming such product on a regular basis may cause hepatotoxicity [36]. Interestingly drinking green tea on a regular basis may help in maintaining body weight or may even cause modest weight loss [37].

Consuming green tea extract supplements may increase liver enzymes due to hepatotoxicity. In a large trial involving 1,021 women (513 women consumed green tea extract for 12 months, 508 women in placebo group), the authors reported that among women who consumed green tea extract, alanine aminotransferase (ALT) was increased by 5.4 U/L and aspartate aminotransferase (AST) by 3.8 U/L, which were significantly higher than those women in the placebo group. Overall 26 women developed moderate to more severe abnormalities in liver function tests [38]. Dekant et al. commented that consumption of green tea, and green tea based beverages are safe but taking concentrated extract of green tea may cause liver damage. The author proposed that the tolerable upper intake of EGCG is 300 mg/day but usually no liver toxicity is observed for EGCG intake below 600 mg/day [39]. An average cup of green tea contains 112 mg of EGCG, 51 mg epigallocatechin and 15 mg epicatechin [40].

Other supplements associated with liver damage

The most dangerous Oriental weight-loss product is ma huang, which contains ephedra alkaloids. Ma huang may cause severe cardiotoxicity, hepatotoxicity and even death. Ephedrine found in ma huang and related weight loss products may be responsible for the toxic effects observed. Use of ephedra in herbal supplement is banned by the FDA. Oriental weight loss products Chaso and Onshido contain N-nitrosofenfluramine which is toxic to the liver. Other Oriental herbal supplements containing multiple herbs and several Kampo medicines (notably Dai-Saiko-to and Sho-Saiko-to) are known to cause liver injuries. Jin-Bu-Huan, a sedative analgesic, is also to known to cause liver damage. Shou-Wu-Pian, a proprietary Chinese medicine used for treating dizziness, back pain and constipation, may also cause hepatitis. Shen-Min, another Chinese medicine, has also been associated with drug induced hepatitis. In addition, use of Chinese medicines such

as Da Huang, Gan Cao, Huang Qin, Zexie, Zhen Chu Cao and others also may cause liver toxicity [14].

Liver toxicity associated with LipoKinetix, a weight loss product has been discussed earlier in the chapter. Sodium usniate found in LipoKinetics is derived from usnic acid which is a known liver toxin. Usnic acid is also present in Kombucha Tea (also known as Manchurian Mushroom or Manchurian Fungus tea), which is prepared by brewing Kombucha mushroom in sweet black tea. While Kombucha tea is considered a healthy elixir, the limited evidence currently available raises its safety concern, especially potential hepatotoxicity and possibility of life threatening lactic acidosis. A 22 year old male newly diagnosed with HIV experienced shortness of breath and fever up to 103 °F within 15 h of drinking Kombucha tea and was admitted to the hospital. His serum creatinine was 2.1 mg/dL and he experienced life-threatening lactic acidosis (lactate 1.9 mmol/L; 116.2 mg/dL, upper limit of normal: 19.8 mg/dL) [41].

There are many other herbs that may cause liver damage. Skullcap, for example, is used as a sedative, calming agent, and also recommended for nervous tension, epilepsy and hysteria. There are several cases of hepatotoxicity involving skullcap in combination with another herbal. One case involved skullcap in combination with valerian while another case involved herbals in combination with gingko biloba. Black cohosh may also cause liver toxicity [42]. Gotu kola (Sanskrit: Mandukaparni, *Centella asiatica*) has been used in Indian Ayurvedic medicine for a long time for the treatment of hypertension and wound healing. This preparation contains the pentacyclic triterpenic saponosides, asiaticoside and madecassoside, which may cause liver damage. In one study the authors presented three cases of women aged 61, 52 and 49 who presented with high ALT: 1193, 1694 and 324 U/L, ALP: 503, 472 and 484 U/L and bilirubin 4.23, 19.89 and 3.9 mg/dL, respectively. All patients improved after discontinuation of *C. asiatica* [43]. Common herbal supplements that may cause liver damage are listed in Table 18.2.

Case Report: A 55-year-old man with no significant medical history was admitted to a hospital with a 10 day history of mild fever, brown urine and epigastric pain. The liver function test showed elevated AST (958 U/L) and ALT (1000 U/L), which were further increased (AST: 1108U/L, ALT: 1528 U/L) after the patient was admitted to the hospital. His bilirubin was increased to 9.4 mg/dL on day 4. The patient was taking mistletoe extract for 1 month and kudzu extract for 10 days for general health. Therefore, a mistletoe and kudzu induced liver injury with a hepatocellular pattern was diagnosed. After discontinuation of herbal supplement his ALT and AST levels gradually decreased and the patient was discharged on day 8 with no other abnormal symptoms [44].

TABLE 18.2 Herbal supplements that may cause liver damage.

Herbal supplements associated with liver damage	Mechanism of liver toxicity
Kava	Kava if taken on a regular basis may cause liver damage due to presence of kava lactones.
Chaparral	Mechanism of liver injury is not fully understood.
Comfrey	Pyrrolizidine alkaloids present in comfrey are responsible for hepatotoxicity
Coltsfoot	Pyrrolizidine alkaloid (senecionine) is responsible for liver toxicity.
Germander	Teucrin A, and teuchmaedryn A are hepatotoxic components of germander.
Pennyroyal	Oxidation of pulegone by liver cytochrome P-450 enzymes produces a toxic metabolite menthofurn which depletes glutathione in the liver.
Skullcap	Mechanism of hepatotoxicity is unknown. However, sometimes skullcap is adulterated with hepatotoxic herb germander.
Mistletoe	Mistletoe lectins have hepatotoxic properties.
Green tea extract	Epigallocatechin 3-gallate (EGCG), the major bioactive component of green tea EGCG at very high concentration may damage liver.
LipoKinetix	Sodium usniate (usinic acid), a component of this weight loss product is probably responsible for liver toxicity.
OxyELITE Pro	Aegeline, component of this weight loss product may be responsible for hepatotoxicity.
Hydroxycut	The original formulation contained ma huang (ephedra) which was banned by the FDA in 2004. Although manufacturer removed ephedra from the formula report of liver toxicity from using this product continued.
Herbal Life	There are reports of hepatoxicity in some users of herbal life, weight loss product. It contains many herbs and mechanism of toxicity in some users is unknown.
Uprizing	Liver toxicity may be related to anabolic steroid methyl drostanolone
Ma Huang	Contains toxic ephedra alkaloids.

HERBAL SUPPLEMENTS ASSOCIATED WITH KIDNEY DAMAGE

In 1993, rapidly progressing kidney damage was reported in a group of young women who were taking pills containing Chinese herbal weight loss product while attending a weight loss clinic in Belgium. It was discovered that one prescription Chinese herb has

been replaced by another Chinese herb containing aristolochic acid, a known toxin to kidney [45]. This was the first comprehensive document of nephropathy associated with use of aristolochic acid which is now referred as "Chinese herbal nephropathy", which is characterized by progressive fibrosing interstitial nephritis leading to renal failure and severe anemia. Later there were many reports of kidney damage due to use of herbal supplements containing aristolochic. In most of the reported cases even when a patient discontinued the use of herbal supplement containing aristolochic acid, renal disease may still proceed to end stage renal disease [46]. Aristolochic acid is also a known carcinogen and despite the FDA warning still sold in the United States [47]. Kong et al. reported cases of a married couple where the man developed Fanconi's syndrome and his wife developed end stage renal failure and anemia after using Chinese herbs containing aristolochic acid. However, five children who never used the herbal product did not have any renal insufficiency indicating that the renal failure was not related to any genetic factor but was due to use of the herbal supplements containing aristolochic acid [48].

Use of other herbal supplements has also been associated with nephrotoxicity. Licorice although relatively safe if used as a flavoring agent in candies, when used chronically as a supplement may cause hypokalemia that can lead to rhabdomyolysis and eventually acute kidney injury. Yohimbine may cause lupus like syndrome and proteinuria. Willow bark contains salicin, which is metabolized to salicylate. Chronic use of willow bark products may cause nephrotoxicity. Bladder-wrack is a large brown alga which is a common food in Japan. Long term use of bladder-wrack tablets may cause chronic kidney disease [49]. The National Kidney Foundation stated that wormwood plant, sassafras, Chinese herbal medicine Tung Shueh and horse chestnut may be toxic to kidney. In addition, people suffering from chronic renal disease should not take alfalfa, aloe, bayberry, blue cohosh, broom, buckthorn, capsicum, cascara, coltsfoot, dandelion, ginger, ginseng, horsetail, licorice, mate, nettle, noni juice, rhubarb, senna and vervain. According to National Kidney Foundation herbals such as chaparral, comfrey, ephedra (ma huang), lobelia, mandrake, pennyroyal, pokeroot, sassafras, senna and yohimbe are unsafe to all people and these herbal products should be avoided (http://www.kidney.org/atoz/atozItem.cfm?id=123). There are several herbal supplements that are known to cause hematuria and loss of protein in urine (proteinuria). Examples of these herbs are aloe juice from leaf, kava, saffron etc. Many other herbs such as calamus, chaparral, horse chestnut seed, wormwood oil etc. may cause nephrotoxicity [50,51]. In addition, several herbs also have diuretic effects and may cause irritation to renal epithelial cells) These herbs, such as asparagus roots, lovage root, parsley herb and root, watercress and white sandalwood, should be avoided if you have any kidney problem [52].

Case Report: A 75-year-old man presented with a 2 month history of lethargy, poor appetite and nausea. He used Chinese herbal supplements (long dan xie gan wan, lei gong teng and ke yin wan) to treat his psoriasis for 3 years and observed significant improvement. Laboratory investigation revealed renal failure with a serum creatinine level of 965 μmol/L (10.9 mg/dL, upper limit of normal: 1.2 mg/dL), and urea nitrogen of 43.1 mmol/L (259.6 mg/dL, upper limit of normal: 18 mg/dL). Renal ultrasound demonstrated unobstructed, small kidneys with cortical thinning bilaterally. A renal biopsy showed severe tubulointerstitial fibrosis and atrophy. These finding were consistent with chronic exposure to a nephrotoxin. Chinese remedies taken by the patient were sent for further analysis using high performance liquid chromatography, which revealed the presence of aristolochic acid in the Chinese medicine long de xie gan wan. Hemodialysis was initiated and later converted into ambulatory peritoneal dialysis. The patient died 4 years after initial presentation following withdrawal from dialysis [53].

Case Report: A 41-year-old Chinese man with a history of urinary tract infection purchased four boxes each containing 40 small packets of Chinese herbal preparations "Fen Qing Wu Lin Wan". The preparations were taken twice daily (one small packet each time) and after two days he had loose stools four to five times a day and abdominal pain. On day 23, his abdominal pain was so severe that he went to the hospital. His serum creatinine was 484 μmol/L (5.5 mg/dL) and urea nitrogen was 16.8 mmol/L (101.2 mg/dL). He also had oliguria (200 mL urine per day) and died from multiple organ failure one and half month after initiation of taking herbal supplement. Pathological examination during autopsy revealed acute tubular necrosis with significant protein cast, acute hemorrhagic necrotic enteritis with hematoceles in the ileum, severe bilateral pulmonary hemorrhage with blood accumulation in the bronchi, mild cardiac hypertrophy, mild edema and congestion in the brain, mild fatty degeneration of the liver and anemic spleen. The product "Fen Qing Wu Lin Wan" contains Chinese her "GuanMu Tong", which is the dried stem of Aristolochia manshuriensis containing aristolochic acid. If such herb is consumed in excessive dosage or for a longer period, aristolochic acid may cause acute necrosis of the renal tubules as well as chronic interstitial nephritis [54].

HERBAL SUPPLEMENTS AND HYPOGLYCEMIA

In humans, glucose is the primary fuel for the brain and muscles and normal range of glucose is 70–99 mg/dL after overnight fasting while fasting glucose over 126 mg/dL measured in two different occasions may indicate diabetes mellitus. Glycosylated hemoglobin levels over 6.5% also indicate diabetes. Yeh et al. reviewed data from 108 clinical trials examining 36 herbs (single or combination) and 9 vitamin/mineral supplements and concluded that there is still insufficient data to draw definitive conclusions regarding the efficacy of herbal supplements in controlling diabetes, but the best evidence of efficacy for controlling blood glucose exists for *Coccinia indica* and American ginseng. Other supplements that show promises are *Gymnema sylvestre*, *Aloe vera*, vanadium, bitter melon (*Momordica charantia*) and nopal. Chromium was the most widely studied supplement for controlling blood glucose [55]. Recently, Ota and Ulrih reviewed herbal products and secondary metabolites used for management of type 2 diabetes and included most herbal supplements covered by Yeh et al. The authors commented that herbal supplements most commonly used for management of optimal blood sugar include bitter melon, fenugreek, gurmar (*G. sylvestre),* ivy ground, nopal (also known as prickly pear cactus), ginseng, Russian tarragon *(Artemisia dracunculus),* cinnamon, garlic, ginger and psyllium *(Plantago ovata).* In addition, resveratrol, which is found in grape skin and also in red wine, has antidiabetic activity because it improves insulin sensitivity. The authors also reviewed active ingredients of these herbal supplements responsible for antidiabetic effects. Flavonoids, which are found in abundance in fruits and vegetables, may also help in maintaining a healthy blood glucose level [56]. Active ingredients of common herbal supplements with antidiabetic activity are summarized in Table 18.3.

C. indica (ivy ground) is a creeping plant that grows wildly in many parts of India and was used in Ayurvedic medicine for treating diabetes. Many formulations containing *C. indica* are available in India and in the United States. Cinnamon-6 herbal formulation (Herbal Pride) contains this herb as one of its many components. However, the FDA has not evaluated the efficacy of this product in controlling Type 2 diabetes. Several different plant species are referred as ginseng, including Asian ginseng, Korean ginseng, Siberian ginseng, Japanese ginseng and American ginseng. Two long term trials administering American ginseng for 8 weeks reported decreases in fasting blood glucose in subjects. Glycosylated hemoglobin, another marker of long term glucose control was also decreased in subjects taking American ginseng. Various studies also indicate reduction in blood glucose after taking Korean or Asian ginseng. Use of garlic in lowering blood sugar is controversial although few studies showed positive effects. Fenugreek may also be effective in reducing blood sugars but studies reported in the literature were not always well designed [55]. Fig leaves (*Ficus carica*) in breakfast may be effective in lowering blood sugar [57]. Although not an herbal supplement, chromium, a trace element, is required for the maintenance of normal metabolism of glucose. Chromium deficiency may cause glucose intolerance although most diabetic patients are not chromium deficient. Chromium picolinate may be effective in lowering blood glucose although some studies showed no benefit of chromium in reducing blood sugar.

However, diabetic patients being treated with either oral hypoglycemic agent or insulin are at higher risk of hypoglycemic episode if they concurrently take herbal supplements that may lower blood glucose levels. A 78-year-old man with type 2 diabetes was brought to

TABLE 18.3 Commonly used herbal supplements with antidiabetic activity.

Herbal supplement with antidiabetic activity	Active ingredients
Bitter melon	Cucurbitane type triterpenoids charantin, karaviloside IX, momordicoside S, and its aglycone monordicosides
Fenugreek	Most active components are diosgenin, 4-hydroxyisoleucine and the soluble dietary fiber fraction of fenugreek seeds
Gurmar (means sugar destroyer in Hindi); *Gymnema sylvestre*	Gymnemic acids, gurmarin (a 35-amino acid peptide)
Coccinia indica (ivy ground)	Mechanism of glucose lowering effect is not understood
Nopal (prickly pear cactus)	Nopal is rich in highly soluble fiber and pectin which can affect intestinal glucose uptake
Ginsengs	Asian ginsengs contain ginsenosides which may have hypoglycemic properties.
Cinnamon	The procyanidin oligomers are probably responsible for antidiabetic properties.
Garlic	Antidiabetic properties are due to volatile sulfur compounds including alliin, allicin, diallyl disulfide, S-allyl cysteine, and allyl mercaptan.
Ginger	Gingerols, shogaols, paradols, zingiberene

the emergency room with a critically low blood sugar of 1.2 mmol/L (21.6 mg/dL). He was immediately treated with intramuscular glucagon. Next morning the patient was responsive and reported that he took his usual dose of 34 units of insulin and ate his breakfast. However, the patient admitted taking a herbal supplement Pro-Stavita (Medicine Beauty, S.A, Geneva, Switzerland) for improving his prostatic symptoms. The authors concluded that the life threatening hypoglycemic event in this patient may be related to ingestion of the herbal supplement [58].

Case Report: A 46-year-old woman was diagnosed with non-insulin dependent diabetes 10 months ago and her serum glucose was stabilized with diet, exercise and glipizide. She reported to the clinic with three day episodes of low glucose levels in serum ranging from 60 to 85 mg/dL using a home glucose monitoring device. In the clinic her non-fasting glucose using finger-stick testing was only 55 mg/dL. She was not taking any other medication and there was no change in her lifestyle. The doctor treated her with glucagon to increase her serum glucose level and when her serum glucose concentration reached 110 mg/dL, she felt much better. At that point she admitted taking several herbal supplement; ginseng for lowering blood glucose and cholesterol, garlic to prevent heart disease, astragalus for lowering blood glucose and juniper berry tea for aiding in digestion. Such herbal supplements were responsible for her abnormally low serum glucose and her symptoms [59].

Case Report: A 58-year-old male with type 2 diabetes mellitus was treated with metformin 1000 mg twice daily and extended release glipizide 10 mg daily. He showed good glycemic control as his fasting glucose ranged from 113 to 132 mg/dL and his two hemoglobin A (1c) values were 6.8% and 6.7% respectively obtained over a one-year period. He denied using any herbal supplements. One month later the patient reported four hypoglycemic events with glucose readings between 49 and 68 mg/dL. At that point glipizide was discontinued. One month later he reported no hypoglycemic event but during reconciliation he admitted consuming crude prickly pear cactus pads daily for two months for glucose control. Patients with type 2 diabetes mellitus receiving hypoglycemic agents should be routinely counseled about the use of herbal supplements in order to avoid hypoglycemic events [60].

Adulteration of herbal supplements with oral hypoglycemic agents

Adulteration of herbal antidiabetic products with pharmaceuticals is a significant problem. This is particularly associated with herbal supplements manufactured in Asian countries. One report from Hong Kong indicated that the authors found undisclosed pharmaceuticals in several herbal supplements including glibenclamide in 22 products, phenformin in 18 products, metformin in 6 products, rosiglitazone in 6 products, gliclazide in 2 products, glimepiride in 2 products, nateglinide in 1 product and repaglinide in one product. Other pharmaceuticals (non-hypoglycemic agents) were also detected in some products. Certain products also contained up to four pharmaceuticals. The authors concluded that patients taking such pharmaceuticals could be at risk of serious adverse events and even fatality [61].

Case Report: A 56-year-old Indonesian tourist from Jakarta presented to the emergency department of Royal Perth Hospital, Australia three day after his arrival to Australia with flu like symptoms. On arrival of the ambulance, his capillary blood glucose level was 2.1 mmol/L (37.8 mg/dL) and he received 1 mg of glucagon intramuscularly. During transfer to the hospital he was also given aspirin (300 mg) and isosorbide mononitrate (10 mg, sublingually). On arrival at the hospital his capillary blood glucose level was 4.3 mmol/L (77.4 mg/dL). On admission to the hospital he reported having diet-controlled type 2 diabetes mellitus, ischemic heart disease (with coronary artery bypass surgery 12 years ago), hypertension and hypercholesterolemia. He was taking amlodipine, aspirin and atorvastatin, but denied taking any medications for his diabetes. He was given a meal containing a sandwich but shortly his venous glucose concentration was reported by the laboratory to be 2.9 mmol/L (52.2 mg/dL), indicating persistent hypoglycemia despite being given sugary drinks, and he was given increasing dextrose infusions and intermittent boluses of dextrose. Finally, a 50 mL per hour infusion of 50% dextrose was required to prevent hypoglycemia. At that point the patient admitted that although he was not taking any hypoglycemic agents for diabetes he was taken a traditional Chinese medicine for diabetes called "ZhenQi" that he had purchased in Malaysia. He had been taking this medication for the last five years, initially taking five capsules per day, then having increased the dose to three capsules three times daily (with no dose alteration) for the last two years. The label on the bottle of this preparation listed the ingredients as ginseng, pearl, ram's horn, bark and "frog extract". He had started a new bottle of the preparation recently and felt lethargy, cold and tremor. Serum taken during the period of hypoglycemia (when his glucose level was 52.2 mg/dL) had elevated levels of C-peptide (3.80 nmol/L; normal range, 0.20–0.90 nmol/L) and insulin (50 mU/L; normal range, 3–26 mU/L). Analysis of the herbal medication capsules by gas chromatography and mass spectrometry revealed the presence of glibenclamide, a known hypoglycemic agent. Fortunately, the patient responded

to the treatment and was discharged from the hospital on day 3. This case report indicates episode of severe hypoglycemia due to contamination of a Chinese herbal formula with a pharmaceutical [62].

LICORICE AND HYPOKALEMIA

Licorice prepared from the root of *Glycyrrhiza glabra*, has a sweet taste and it is used in various candies and sometimes also in cough suppressant syrup. It is believed that licorice is effective in treating ulcers but licorice has glycyrrhizinic acid, which affects the endocrine system and may also cause hypertension. Individuals taking blood pressure medications should not use licorice. Even in normotensive people, excessive intake of licorice may cause hypertension, hypokalemia and related electrolyte imbalance requiring medical attention. Licorice should not be used by anyone taking digoxin because licorice induced electrolyte imbalances may cause severe digoxin toxicity especially in elderly. A 84 old year old man who was taking digoxin experienced severe toxicity from using licorice requiring hospitalization [63]. Long term licorice ingestion is a well-known cause of secondary hypertension and hypokalemia. Lin et al. reported a case of an Asian man who ingested tea flavored with 100 gm of natural licorice root containing 2.3% glycyrrhizic acid daily for three years and presented to the hospital with paralysis, metabolic alkalosis and severe hypokalemia (serum potassium: 1.8 mmol/L). His renal potassium wasting continued for two weeks after discontinuation of licorice and therapy with spironolactone and potassium chloride supplement [64]. Meltem et al. reported a case of a 21 year old man who ingested the powdered over-the-counter product "Tekumut" for two weeks to quit smoking and presented to the hospital with muscle weakness. The product contained licorice. The authors commented that this case report is the first report of hypocalcemia due to consumption of licorice although licorice usually induces hypokalemia that is mild in nature, but licorice use may cause paralysis, rhabdomyolysis or ventricular fibrillation leading to death if untreated [65].

Case Report: A 31-year-old, previously healthy Caucasian man was admitted to the hospital due to progressive weakness of the legs and muscle cramping developed over a few hours. His clinical examination revealed paraparesis without sensory loss but laboratory report revealed severe hypokalemia (1.5 mmol/L; normal range, 3.6−4.5 mmol/L), hypophosphatemia (0.42 mmol/L; normal range, 0.87−1.45 mmol/L) and mild hypomagnesemia (0.65 mmol/L; normal, 0.7−1.1 mmol/L). His creatine kinase (CK) level was elevated (3425 U/L; normal value, <190 U/L), indicating rhabdomyolysis. His creatinine was 1.2 mg/dL. The patient admitted consuming roughly one and a half packets of original Fisherman's Friend menthol eucalyptus lozenges (Lofthouse of Fleetwood Ltd., UK) daily for the past three months. These lozenges contained licorice and his consumption of glycyrrhizinic acid, the active ingredient of licorice, was approximately 60−90 mg/day. Despite discontinuation of these lozenges his potassium level remained low. The patient was initially treated with parenteral and enteral potassium, spironolactone. Within 24 h, his potassium level began to rise and his neurological symptoms disappeared. After three days, the patient was asymptomatic; at this point, his potassium level had increased to 2.9 mmol/L and the diagnosis was hypokalemia and secondary hyperaldosteronism suggestive of Gitelman syndrome. Although he was discharged from the hospital at that point he was later readmitted to the hospital for recurring symptoms despite treatment with aldosterone antagonist and supplement of potassium and magnesium. Trigger could be his consumption of alcohol, lemon juice and iced tea in excessive amounts. Finally vomiting and failure to replace salt lead to a serum potassium level of 1.0 mmol/L and the patient was put temporarily to mechanical ventilation. This case indicated serious consequence of ingesting excessive amount of licorice and dietary choices [66].

KELP AND ABNORMAL THYROID FUNCTION TESTS

Kelp (seaweed) is prepared from seaweed and contain very high amount of iodine. Seaweed or kelp is a part of regular diet in some Asian countries especially Japan. In addition, kelp is used as an ingredient in many Oriental medicines. Teas et al. studied iodine content in common available seaweeds available from commercial sources in the United States and observed that iodine content varied widely from 16 μg to over 8165 μg in one uncooked serving. The authors commented that it is possible that in some Asian seaweed dishes the iodine content may exceed 1100 μg per day, the upper tolerable iodine level in humans [67].

Eating kelp supplement especially kelp containing Oriental medicines may be problematic because excess iodine may cause thyroid malfunction especially in people taking thyroxin supplements. In healthy individuals, the thyroid gland has intrinsic autoregulatory mechanisms to handle excess iodine intake. The oxidation of iodine, which is essential for synthesis of thyroid hormone, is inhibited in the presence of high iodine concentration in blood and this acute inhibitory effect known as the "Wolff-Chaikoff" effect lasts

approximately 48 h followed by normalization of normal thyroid hormone synthesis. However, patients with underlying thyroid disorder may not be able to adopt excessive iodine in circulation. These patients may develop either hypothyroidism or hyperthyroidism. Individuals with elevated sensitivity such as patients with Grave's disease, chronic autoimmune thyroiditis, etc, may develop hypothyroidism after intake of excess iodine. In contrast, patients with low sensitivity such as patients with endemic goiter, iodine deficiency, etc, may develop hyperthyroidism [68]. A 72-year-old woman with normal thyroid gland developed hyperthyroidism after ingesting kelp tablets. The thyroid functions returned to normal six months after discontinuation of kelp [69]. In another case report a 32-year-old woman with goiter presented to the hospital with symptoms of hyperthyroidism and her problem developed four weeks after taking kelp containing herbal tea following recommendation of a Chinese herbal practitioner [68].

DRUG-HERB INTERACTIONS

Approximately 25% adults report concurrently taking a prescription medication with a dietary supplement but some of the supplements such as St. John's wort and goldenseal are known to cause clinically significant interactions with many drugs. Therefore, patients receiving pharmacotherapy should avoid taking St. Jon's wort and goldenseal. However, other supplements such as curcumin, echinacea, garlic, Asian ginseng, green tea extract, and kava may cause interactions with fewer medications. Other supplements such as black cohosh, cranberry, ginkgo, milk thistle, American ginseng, saw palmetto and valerian have a low likelihood of drug interaction and can be safely taken with most medications [70].

Sood et al. surveyed 1818 patients and identified 107 drug-herb interactions that have clinical significance. The five most common herbal supplements (St. John's wort, ginkgo, garlic, valerian and kava) accounted for 68% of such interactions and four different classes of prescription drugs (antidepressants, antidiabetic, sedatives and anticoagulation medications) accounted for 94% of all clinically significant interactions [71]. In one report, the authors identified 32 drugs that interact with herbal supplements [72]. Shi and Klotz commented that drug-herb interactions certainly increase the risk of therapy with drugs with narrow therapeutic range such as warfarin, cyclosporine and digoxin [73]. St. John's wort is the most commonly encountered supplement causing clinically significant drug-herb interactions. Therapeutic drug monitoring is useful in identifying certain clinically significant drug-herb interactions including interaction of St. John's wort with many drugs. Warfarin therapy is monitored using INR (international normalized ratio), which is very helpful in identifying clinically significant drug-warfarin as well as warfarin-herb interaction.

Interaction of St. John's wort with various drugs

St. John's wort, an herbal antidepressant, is sold in the United States as an alcoholic or dried extract of hypericum, a perennial aromatic shrub with bright yellow flowers that bloom from June to September. The flowers are believed to be most abundant and brightest around June 24th, the day traditionally believed to be the birthday of St. John the Baptist and hence the name St. John's wort. Hypericin, hyperforin and quercetin are major constituents in St. John's wort that mediate reduction of intestinal absorption as well as bioavailability of many drugs by induction of intestinal P-glycoprotein drug efflux pump (MDR1: multidrug resistant 1) expression, and induction of activities of both intestinal and liver cytochrome P-450 mixed function oxidase (CYP) enzymes responsible for metabolism of many drugs. Hyperforin appears to play major role in activating the pregnane X receptor leading to the transcriptional activation of genes that regulate activities of CYP3A4 and other cytochrome subtype enzymes responsible for drug metabolism. Therefore, St. John's wort preparations with low hyperforin (<1%) content may not cause any clinically significant interaction with any drugs [74]. In general, it is considered that St. John's wort induces CYP3A4, CYP2E1 and CYP2C19 with no effect on CYP1A2, CYP2D6 and CYP2C9. However, St. John's wort also induces clearance of drugs that are not metabolized via liver enzymes, for example digoxin, fexofenadine and talinolol, which are well known P-glycoprotein substrates [75]. Although most drug-herb interactions involving St. John's wort are pharmacokinetic in nature, pharmacodynamic interaction of certain antidepressants with St. John's wort have also been reported.

Most clinically important pharmacokinetic drug interactions with St. John's wort involve immunosuppressants, warfarin, and antiretrovirals because treatment failure may have serious consequences to these patients. Transplant recipient taking cyclosporine or tacrolimus may experience acute organ rejection due to self-medication with St. John's wort because St. John's wort induces the metabolism of both drugs thus reducing whole blood concentrations of these drugs by more than 50%. Ernst commented that St. John's wort can endanger the success of organ transplantation due its interaction with cyclosporine leading to several cases of organ rejection [76]. Alschner and Klotz reported a case study where a 57-year-old kidney transplant patient taking cyclosporine (125–150 mg/day) and

prednisolone (5 mg/day) showed cyclosporine trough level (100−130 ng/mL) over the past two years. This patient suddenly demonstrated a sub-therapeutic cyclosporine concentration of 70 ng/mL despite increasing the daily cyclosporine dose to 250 mg. The patient admitted taking an herbal tea mixture for depression, which contained St. John's wort. Five days after discontinuing the herbal tea his cyclosporine level was increased to 170 ng/mL. Then the cyclosporine dose was reduced to 175 mg per day to readjust his cyclosporine whole blood concentration and his trough cyclosporine level again returned to 130 ng/mL [77]. Mai et al. reported that the hyperforin content of St. John's wort determines the magnitude of interaction between St. John's wort and cyclosporine. Patients who received low-hyperforin-containing St. John's wort showed minimal changes in pharmacokinetic parameters requiring no dose adjustment. In contrast, the patients who received high-hyperforin-containing St. John's wort needed dose increases within three days in order to maintain trough therapeutic concentration of cyclosporine [78]. Significant reduction in the area under the curve (AUC) for tacrolimus was also observed in ten stable renal transplant patients receiving St. John's wort. The maximum concentration of tacrolimus was also reduced from a mean value of 29.0 ng/mL to 22.4 ng/mL following co-administration of St. John's wort [79]. Interestingly pharmacokinetic parameters of mycophenolic acid, another immunosuppressant, were not affected by co-administration of St. John's wort [80].

Warfarin (Coumadin) is an anticoagulant drug that is used in treating a variety of conditions including heart disease such as atrial fibrillation, preventing blood clots in lower extremities (deep vein thrombosis), stroke, pulmonary embolism etc. Warfarin therapy should be carefully controlled by measuring the clotting capacity of blood (INR monitoring). A patient attending a Coumadin clinic must avoid St. John's wort because of potential failure of warfarin therapy due to increased clearance of warfarin. Jiang et al. studied interaction of warfarin and St. John's wort using 12 healthy subjects and concluded that St. John's wort significantly induces clearance of both S and R-warfarin, which in turn results in a significant reduction in pharmacological effect of racemic warfarin [81].

Case Report: An 85-year-old male with a history of hypertension, previous anterior wall myocardial infarction and atrial fibrillation was receiving warfarin (5 mg daily) for one year. After watching a television program on herbal remedies he decided to start taking St. John's wort. One month later he presented himself to the emergency room with upper gastrointestinal bleeding. His hemoglobin was 7.9 g/dL, hematocrit, 23% and his INR was 6.2. He was treated with fresh frozen plasma and blood transfusion and his bleeding was controlled. The patient was instructed not to use any herbal supplement. Although St. John's wort reduces the efficacy of warfarin by increasing its hepatic metabolism, this rare case indicates the opposite effect where the patient developed bleeding episode due to interaction of St. John's wort with warfarin. The ingredients of St. John's wort may potentiate effect of warfarin in a sensitive individual instead of reducing its efficacy [82].

Patients suffering from AIDS and receiving HAART Therapy (highly active antiretroviral therapy) must not consume St. John's wort or other herbal supplements due to possibility of treatment failure from drug-herb interactions. Clinically significant interactions of antiretroviral agents with St. John's wort have been documented. Therefore, patients with AIDS taking amprenavir, atazanavir, zidovudine, efavirenz, indinavir, lopinavir, nelfinavir, nevirapine, ritonavir, and saquinavir must avoid concomitant use of St. John's wort [83]. St. John's wort was shown to reduce the area under the curve of the HIV-1 protease inhibitor indinavir by a mean of 57% [84]. Reduced concentration of nevirapine due to administration of St. John's wort has also been also been reported [85].

Interaction between St. John's Wort and digoxin is of clinical significance. Johne et al. reported that 10 days usage of St. John's wort resulted in a 33% decrease of peak and 26% decrease in trough serum digoxin concentrations. The mean peak digoxin concentration was 1.9 ng/mL in the placebo group and 1.4 ng/mL the group taking St. John's wort [86]. Clearance of imatinib mesylate, an anticancer drug is also increased due to administration of St. John's Wort resulting in reduced clinical efficacy of the drug. In one study involving ten healthy volunteers, two week treatment with St. John's wort significantly reduced the maximum plasma imatinib concentration by 29%, and the area under the curve (AUC) by 32%. The half-life of the drug was reduced by 21% [87]. St. John's wort also showed significant interaction with another anticancer drug irinotecan. In one study involving five patients, ingestion of St. John's wort (900 mg per day) for 18 days resulted in an average 42% reduction in the concentration of SN-38, the active metabolite of irinotecan. This reduction also caused decreased myelosuppression [88]. Many benzodiazepines are metabolized by CYP3A4 and as expected St. John's wort reduces plasma concentrations of alprazolam, midazolam and quazepam. St. John's wort also reduces plasma concentrations of the antiepileptic drug mephenytoin but did not interact with another antiepileptic drug, carbamazepine. Theophylline is metabolized by CYP1A2, CYP2E1, and CYP3A4. Reduced plasma concentrations of theophylline due to intake of St. John's wort have also been reported [75]. Fortunately, theophylline is subjected to routine therapeutic drug monitoring and such interaction can be suspected from observing reduced theophylline levels

in a patient who showed therapeutic theophylline levels previously. Reduced plasma levels of methadone were also observed in the presence of St. John's Wort. Long term treatment with St. John's wort (900 mg/day) for a median period of 31 days (Range 14–47 days) decreased the trough concentrations of methadone by an average of 47% in four patients. Two patients experienced withdrawal symptoms due to reduced plasma levels of methadone [89].

St. John's wort has significant interaction with oral contraceptives. Murphy et al. studied the interaction between St. John's wort and oral contraceptives by investigating pharmacokinetics of norethindrone and ethinyl estradiol using sixteen healthy women. Treatment with St. John's wort (300 mg three times a day for 28 days) resulted in a 13–15 % reduction in dose exposure from oral contraceptives. Breakthrough bleeding increased in treatment cycle as did evidence of follicle growth and probable ovulation. The authors concluded that St. John's wort increased the metabolism of norethindrone and ethinyl estradiol, reducing the effectiveness of contraception [90].

St. John's wort also induces both CYP3A4-catalyzed sulfoxidation and CYP2C19-dependent hydroxylation of omeprazole [91]. Tannergren et al. reported that repeated administration of St. John's wort significantly decreases the bioavailability of R and S-verapamil. This effect is caused by induction of first pass metabolism by CYP3A4 most likely in the gut [92]. Xu et al. reported that treatment with St. John's wort significantly increases the apparent clearance of gliclazide, an oral hypoglycemic agent [93]. Sugimoto et al. reported interactions of St. John's wort with cholesterol lowering drugs simvastatin and pravastatin. In a double blind cross over study using 16 healthy male volunteers, the authors demonstrated that use of St. John's wort (900 mg per day) for 14 days decreased peak serum concentration of simvastatin hydroxyl acid, the active metabolite of simvastatin, from an average of 2.3 ng/mL in the placebo group to 1.1 ng/mL in the group taking St. John's wort. On the other hand, St. John's Wort did not influence plasma pravastatin concentration [94].

Case Report: A 59-year-old black man with hyperlipidemia was initially treated with pravastatin but was later changed to rosuvastatin 10 mg per day and marked improvement was observed in his lipid profile. However, a lipid profile six months later showed markedly increased cholesterol (165 mg/dL in April 2007 but 237 mg/dL in October 2007) and low density lipoprotein (LDL) cholesterol (99 mg/dL on April 2007 but 162 mg/dL in October 2007). The patient admitted taking St. John's wort capsules (two capsules per day containing 300 mg St. John's wort, 80 mg rosemary and 40 mg Spirulina) every day for insomnia staring in June 2007. He was asked to stop taking St. John's wort and four months later showed significantly reduced cholesterol (163 mg/dL) and LDL-Cholesterol (95 mg/dL). Although simvastatin and atorvastatin are metabolized by CYP3A4 and as expected, St. John's wort reduces the efficacy of both drugs, rosuvastatin is primarily eliminated through biliary excretion. However, 10% of the drug is metabolized by CYP2C9 and CYP2C19 and most likely St. John's wort induced CYP2C9 and CYP2C19 mediated clearance of rosuvastatin. The authors speculated that it was unlikely that rosemary or Spirulina affected the metabolism of rosuvastatin [95].

Clozapine is an antipsychotic drug that is metabolized by the liver cytochrome P 450 enzymes. St. John's wort reduces plasma levels of clozapine and may cause treatment failure [96]. Ambrisentan is an oral medication for treating pulmonary hypertension. This drug is metabolized by CYP3A4. Based on a study using 20 healthy volunteers, Markert et al. demonstrated that St. John's wort reduced exposure with ambrisentan irrespective of CYP2C19 genotype [97].

Although pharmacokinetic interactions of drugs with St. John's wort are more common, there are also clinically significant pharmacodynamic interactions with of certain antidepressants. Therefore, if a patient takes antidepressant medication such as fluoxetine, sertraline, paroxetine and venlafaxine along with St. John's wort, the serotonin syndrome may occur [98]. Hoban et al. reported that adverse drug interaction between St. John's wort and fluoxetine were more commonly reported in women aged 26–60 years [99]. Taking St. John's wort along with buspirone may also cause the serotonin syndrome [100].

Case Report: A 50-year-old woman with asthma and chronic depression was taking paroxetine 40 mg per day. She was a non-drinker and did not use any tranquilizer. She stopped taking paroxetine 10 days prior to her clinic visit and was switched to St. John's wort 600 mg powder each day. The night after her clinic visit she slept poorly and then took 20 mg paroxetine along with St. John's wort in order to sleep better. About noon next day her sister visited her and found her incoherent, groggy, slow moving and almost unable to get out of her bed. When the author examined her that day at 2 p.m., her physical examination and laboratory reports were unremarkable but she was groggy and lethargic. The patient presented with a clinical syndrome resembling sedative/hypnotic intoxication after taking both St. John's wort and paroxetine [101].

In one study based on 20 healthy volunteers, St. John's wort decreased the renal clearance of metformin but did not affect the metformin pharmacokinetic parameters. St. John's wort improved glucose tolerance in these volunteers by enhancing insulin secretion independently of insulin sensitivity in healthy male subjects taking metformin [102]. Soleymani et al.

in their expert opinion stated that St. John's wort preparations have demonstrated clinically important interactions with several classes of conventional drugs such as immunosuppressants, anticancer agents, cardiovascular drugs, oral contraceptives and lipid lowering drugs that caused life-threatening events in several cases [103]. Davis et al. based on National Ambulatory Medical Care Survey between 1993 and 2003 commented that potentially dangerous St. John's wort-drug interactions involved drugs such as selective serotonin reuptake inhibitors, benzodiazepines, warfarin, statins, verapamil, digoxin and oral contraceptives [104]. Significant St. John's wort-drug interactions are listed in Table 18.4.

TABLE 18.4 Clinically significant drug interactions with St. John's wort.

Class of drug	Specific	Type of interaction	Clinical effect	Drug interaction detected by TDM?
Immunosuppressant	Cyclosporine Tacrolimus	Pharmacokinetic	Possibility of organ rejection due to 30–50% reduction in trough due to CYP3A4 induction. No interaction with mycophenolic acid.	Yes, both cyclosporine and tacrolimus are routinely monitored.
Protease Inhibitors	Atazanavir, Lopinavir, Indinavir	Pharmacokinetic	Significantly reduced level, for example, AUC of indinavir reduced by 57% due to induction of CYP3A4	No, protease inhibitors are not routinely monitored.
Non-nucleoside reverse transcription inhibitor (NNRTI)	Nevirapine	Pharmacokinetic	Reduced level due to induction of CYP3A4.	No, NNRTI drugs are not routinely monitored.
Anticoagulant	Warfarin (INR monitoring)	Pharmacokinetic	Reduced level due to induction of CYP2C9	Yes, warfarin is monitored through INR.
Cardioactive	Digoxin	Pharmacokinetic	Trough concentration reduced by 26% due to induction of P-glycoprotein	Yes, digoxin is routinely monitored.
Antianginal	Ivabradine	Pharmacokinetic	Reduced level due to induction of CYP2C9	No, this drug is not routinely monitored.
Anticonvulsants	Phenytoin, Phenobarbital	Pharmacokinetic	Reduced efficacy due to induction of CYP3A4.	Yes, these drugs are routinely monitored.
Anticonvulsant	Mephenytoin	Pharmacokinetic	Reduced efficacy due to induction of CY2C19.	No, mephenytoin is not routinely monitored.
Calcium Channel Blocker	Nifedipine, Verapamil	Pharmacokinetic	Reduced level due to induction of CYP3A4.	Nifedipine is not monitored but sometimes verapamil is monitored.
Antihistamine	Fexofenadine	Pharmacokinetic	Decreased level due to induction of P-glycoprotein	No, this drug is not routinely monitored.
Benzodiazepines	Alprazolam, Midazolam, Quazepam	Pharmacokinetic	Reduced blood level due to induction CYP3A4	These drugs are infrequently monitored.
Anticancer	Irinotecan, Imatinib	Pharmacokinetic	Reduced blood level due to induction CYP3A4	No, these drugs are not routinely monitored.
Tricyclic antidepressant	Amitriptyline	Pharmacokinetic	Reduced blood level due to induction CYP3A4	Yes, this drug is routinely monitored.
Antipsychotic	Clozapine	Pharmacokinetic		No, this drug is not routinely monitored
Statins	Atorvastatin, Simvastatin	Pharmacokinetic	Reduced blood level due to induction CYP3A4	No, these drugs are not routinely monitored.

Continued

TABLE 18.4 Clinically significant drug interactions with St. John's wort.—cont'd

Class of drug	Specific	Type of interaction	Clinical effect	Drug interaction detected by TDM?
Oral Contraceptives	Norethindrone Ethinyl Estradiol	Pharmacokinetic	Reduced blood level due to induction CYP3A4 and CYP1A2 causing contraception failure.	No, these drugs are not routinely monitored.
Antiasthmatic	Theophylline	Pharmacokinetic	Reduced blood level due to induction of CYP1A2	Yes, this drug is routinely monitored.
Proton Pump Inhibitor	Omeprazole	Pharmacokinetic	Reduced level due to induction of CYP2C19	No, this drug is not monitored but symptoms of hyperacidity may be an early indication of interaction.
Hypoglycemic agent	Gliclazide	Pharmacokinetic	Reduced level due to CYP2C9 induction	No, this drug is not monitored but hyperglycemia may indicate interaction with St. John's wort.
Hypoglycemic agent	Metformin	Possibly both pharmacokinetic and pharmacodynamic but exact mechanism is unclear.	Improved glucose tolerance in subjects taking both metformin and St. Joh's wort compared to subjects taking metformin alone.	No, this drug is not monitored, but improved glucose control may indicate beneficial interaction between metformin and St. John's wort
Opioid	Methadone	Pharmacokinetic	Trough concentration reduced by 47% due to induction of CYP3A4.	Yes, this drug is monitored.
Opioid	Oxycodone	Pharmacokinetic	AUC reduced by 50% and half-life shortened from 3.8 h to 3 h.	Yes, this drug is monitored.
Endothelin-receptor antagonist	Ambrisentan	Pharmacokinetic	Reduced effect due to increased clearance.	No, this drug is not monitored.
SSRI (Selective serotonin reuptake inhibitor)	Fluoxetine, Sertraline Paroxetine	Pharmacodynamic	Possibility of serotonin syndrome due to synergistic effect	No
SNRI (Serotonin and norepinephrine reuptake inhibitor)	Venlafaxine	Pharmacodynamic	Possibility of serotonin syndrome due to synergistic effect	No
Other Antidepressant	Bupropion, Buspirone	Pharmacodynamic	Possibility of serotonin syndrome due to synergistic effect	No
Anti-migraine agent	Eletriptan	Pharmacodynamic	Possibility of serotonin syndrome due to synergistic effect	No

TDM, therapeutic drug monitoring.

Interactions of warfarin with herbal supplements

Warfarin therapy must be critically monitored by measuring INR because warfarin is known to interact with many Western drugs and herbal supplements. Although potential interaction between a Western drug and warfarin can be easily avoided because clinicians are well aware of such interactions, interaction of warfarin with an herbal supplement can be dangerous because a patient may take herbal supplements without realizing that combination of that supplement with warfarin may cause potential harm.

Warfarin acts by antagonizing the cofactor function of vitamin K. Although clinical efficacy of warfarin varies with intake of vitamin K and genetic polymorphisms that modulate expression of CYP2C9, the isoform responsible for clearance of S-warfarin, several herbal supplements also have significant effects on metabolism of warfarin. As mentioned earlier St. John's wort has clinically significant interaction with warfarin. Herbal supplements that may potentiate the effect of warfarin thus increase the risk of bleeding include angelica root, arnica flower, anise, borage seed oil, bromelain, chamomile, fenugreek, feverfew, garlic, ginger, horse chestnut, licorice root, lovage root, meadowsweet, passionflower herb, poplar and willow bark [105]. Anticoagulant effect of warfarin is also increased if combined with antiplatelet herbs such as danshen, and ginkgo biloba. Conversely, vitamin K containing

supplement such as green tea extract may antagonize the anticoagulant effect of warfarin.

The INR was increased in a patient treated with warfarin for atrial fibrillation when he started taking coumarin containing herbal products boldo and fenugreek. After discontinuation of herbal supplements, his INR returned to normal after one week [106,107]. Increased anticoagulation due to interaction between warfarin and danshen has been reported [108]. There is a case report of a 87-year-old African American man receiving warfarin therapy who was admitted to the hospital with an INR of 6.88. The INR increased to 7.29 during hospital stay although his previous INR values ranged from 1.9 to 2.4 (therapeutic range: 2−3). The patient admitted taking an herbal supplement called royal jelly a week prior to his hospital admission and the elevated INR in this patient was probably related to the use of royal jelly [109]. Consumption of coenzyme Q10 and ginger also appear to increase the risk of bleeding with warfarin therapy [110]. Chan et al. observed that patients with nonvalvular atrial fibrillation who received warfarin had little knowledge about the potential interaction of herbal supplements and foods with warfarin. As a result, patients who consumed herbal supplements at least four times per week had suboptimal anticoagulation control with warfarin [111].

Case Report: A 46-year-old woman receiving a total weekly dosage of 56 mg of warfarin showed INR between 1.6 and 2.2 in the past four months. While taking the same dosage, her INR was increased to 4.6 after she drank approximately 1.5 quarts (1420 mL) of cranberry juice for two days. Her INR was reduced to 2.3 two weeks after she discontinued drinking cranberry juice and her INR varied between 1.4 and 2.5 in following three months while taking 56 mg of warfarin per week. At a subsequent visit after drinking 2 quarts of cranberry juice (1893 mL) for 3 days her INT was again elevated to 6.5. At that time warfarin was discontinued and her INR was reduced to 1.86 after 3 days. Her INR was again 3.2 seven days later with 56 mg of warfarin per week. Her two episodes of highly elevated INR was most likely due to interaction of cranberry juice with warfarin [112]. Clinically significant warfarin-herbal interactions are listed in Table 18.5.

TABLE 18.5 Clinically significant warfarin-herb interactions.

Herbs that increase INR indicating increased efficacy of warfarin	Herbs that reduces INR indicating reduced efficacy of warfarin
Angelica Root, Arnica Flower, Anise, Bogbean, Borage Seed Oil, Bromelain, Boldo, Borage, Chamomile, Coenzyme Q10, Danshen, Dong Quai, Devil's Claw, Fenugreek, Feverfew, Garlic, Ginger, Grape seed, Ginkgo biloba, Horse Chestnut, Licorice, Meadowsweet, Passionflower Herb, Fish oil supplements, Evening primrose oil, Horse chestnut, Royal jelly, Saw Palmetto, Willow Bark	St. John's wort, Green tea extract, Milk Thistle, Goldenseal

Kava-drug interactions

Although liver damage is the most widely documented toxicity of kava, several cases involving severe CNS depression have been reported when the herbal is combined with other sedatives and hypnotics. One such case report described a 54-year-old male who became comatose after three days of kava ingestion while taking alprazolam, cimetidine and terazosin. It was thought that the adverse reaction was related to pharmacodynamic interaction between kava and alprazolam [23]. Kava lactones also inhibit CYP1A2, (56%), CYP2C9 (92%), CYP2D6 (73%) and CYP3A4 (78%) but have no effect on CYP2A6, and CYP2E1. Because of such inhibition, toxicities of drugs that are metabolized through these enzymes are increased if taken with kava [98]. Kava is a very toxic herb that should be avoided.

Drug interactions with ginkgo biloba

Ginkgo biloba is a popular herbal supplement promoted for sharpening memory and improving circulation. Ginkgo biloba is an inhibitor of platelet activating factors thus making this supplement dangerous when taken with warfarin or other anticoagulant drugs. However, ginkgo biloba can also induce liver enzymes thus causing treatment failure with drugs that are metabolized by the cytochrome P-450 liver enzymes. A 55-year-old man suffered a fatal seizure with no evidence of non-compliance (phenytoin and valproic acid) as evidenced by therapeutic range of both drugs for last one year. Autopsy report showed sub-therapeutic serum levels of phenytoin (2.5 μg/mL) and valproic acid (26 μg/mL). He self-medicated with ginkgo biloba. Ginkgo induces CYP2C9 and CYP2C19 activity that metabolizes phenytoin and valproic acid thus causing treatment failure [113]. Ginkgo biloba also has other clinically significant interactions with other drugs [114].

Other drug-herb interactions

Ginger and garlic supplements are popular but both supplement increases bleeding risk in patients taking warfarin. Ginger increases antiplatelet activity of

nifedipine, a calcium channel blocker. Feverfew reduces immunosuppression of cyclosporine and tacrolimus. Interestingly milk thistle which has protective effects on the liver and is often used in patients with liver disease or hepatitis C infection, protects the liver from toxicity of acetaminophen, cisplatin and cyclosporine [115]. Interaction of drugs with kava, ginkgo biloba, garlic and other herbal supplements are summarized in Table 18.6. In the opinion of this author anyone taking medication for treating a chronic disease should not take any herbal supplement unless approved by a physician.

HERBS ADULTERATED WITH WESTERN DRUGS

Although uncommon with herbal supplements prepared in the United States, herbal supplements coming from Asia and Indian Ayurvedic medicine may be contaminated with Western drugs. In this case drug overdose may occur in an unsuspected person taking such herbal supplements because presence of such Western drugs is not disclosed in package inserts. Some Chinese herbal supplements intended for diabetic patients may be contaminated with hypoglycemic agents and taking such product may cause severe hypoglycemia. This topic has been discussed earlier in this chapter. However, other Asian herbal supplements intended not for diabetes may also be contaminated with Western drugs. In one report, the authors analyzed 2069 samples of traditional Chinese medicines collected from eight hospitals in Taiwan, and in 618 samples (23.7%) encountered undeclared Western pharmaceuticals most commonly caffeine, acetaminophen, indomethacin, hydrochlorothiazide, and prednisolone [116]. Most of these herbal supplements were used to alleviate pain, inflammation or symptoms of arthritis. Pharmaceuticals such as acetaminophen, indomethacin and prednisolone can achieve those therapeutic effects. In addition, use of steroid such as prednisolone is dangerous without medical advice. Sibutramine, an oral anorexiant is often found as an adulterant in herbal slimming products and dietary supplements [117].

Case Report: A 33-year-old female with an 8 year history of epilepsy was managed with valproate, carbamazepine and phenobarbital but was never prescribed phenytoin. One month before admission she started consuming three proprietary Chinese medicines in addition to her prescription medicines. She followed instructions for taking Chinese medicines for almost a month and then became comatose and was admitted to the hospital. Serum drug level assays on the second day of admission surprisingly showed a toxic phenytoin level of 48.5 μg/mL (therapeutic range 10–20 μg/mL).

TABLE 18.6 Interaction of drugs with kava, ginkgo biloba, garlic and other herbal supplements are summarized in this table.

Herbal supplement	Interacting drug	Clinical effect
Garlic, Ginger	Warfarin	Potentiation of effect of warfarin (increased INR) with increased risk of bleeding due to pharmacodynamic interaction.
Garlic	Saquinavir, Ritonavir	Reduced plasma level due to induction of CYP3A4 (pharmacokinetic interaction)
Garlic	Chlorpropamide	Hypoglycemia because components of garlic have hypoglycemic properties.
Ginger	Nifedipine	Ginger increases antiplatelet activity of nifedipine.
Ginkgo biloba	Aspirin, Ibuprofen, warfarin	Increased risk of bleeding due to pharmacodynamic interaction
Ginkgo biloba	Phenytoin, valproic acid, omeprazole, ritonavir	Reduced plasma level and reduced efficacy. Most likely, a pharmacokinetic interaction.
Ginkgo biloba	Trazodone	Trazodone is an SSRI which may cause coma if taken with ginkgo. This is possibly a pharmacodynamic interaction because gingko binds with benzodiazepine and GABA receptors.
Kava	Levodopa	Reduced effect
Kava	Alprazolam, Paroxetine	Lethargic state possibly due to pharmacodynamic interaction.
Feverfew	Iron tablets	Reduced absorption
Feverfew	Cyclosporine, Tacrolimus	Reduced immunosuppression
Echinacea	Ketoconazole, Methotrexate	Increased liver toxicity
Echinacea	Simvastatin, Lansoprazole	Increased effect
Flaxseed oil, Saw Palmetto	Aspirin (325 mg)	Increased risk of bleeding
Bromelain	Naproxen	Increased risk of bleeding
Valerian	Alprazolam, Midazolam, Zolpidem, Zaleplon, Eszopiclone, Barbiturates, Alcohol	Increased sedation related to pharmacodynamic interaction.
Milk thistle	Acetaminophen, Cyclosporine, Cisplatin	Reduced liver toxicity associated with these drugs. This is a beneficial drug-herb interaction.

She was treated conservatively and after 10 days her clinical signs of phenytoin toxicity disappeared. Fortunately, she did not suffer any neurological damage. Analyses of three Chinese proprietary medicines showed the presence of 41 mg of phenytoin in Jue Dian Shen Ying Wan (orange capsule) while other two Chinese medicines were adulterated with carbamazepine and valproate. The patient consumed 6 orange capsules for almost a month causing her severe phenytoin toxicity. Unfortunately, the manufacturer's information stated that these preparations only contained Chinese medicines for controlling epilepsy [118].

Savaliya et al. reported that Indian Ayurvedic medicines may be contaminated with both steroidal and non-steroidal anti-inflammatory drugs. Dexamethasone and diclofenac were detected in 10 Ayurvedic products out of 58 preparations analyzed. In addition, piroxicam was detected in one product and dexamethasone in one product [119]. Many Indian Ayurvedic medicines are also contaminated with heavy metals such as lead, arsenic or mercury either due to manufacturing process or because a heavy metal (known as bhasma in Sanskrit) is a component of the Ayurvedic medicine.

Case Report: A 58-year-old woman from India residing in the United States presented to the emergency department with a 10-day history of progressively worsening post-prandial lower abdominal pain and nausea accompanied by vomiting. She was healthy but suffered from well-controlled non-insulin dependent diabetes mellitus and hypertension. On admission, her physical exam was unremarkable except for abdominal tenderness in the lower quadrants. Laboratory tests indicated a normochromic normocytic anemia with hemoglobin of 7.7 g/dL, hematocrit of 22.6%, and mean corpuscular volume (MCV) 87 fL with normal iron status. A CT scan of the abdomen and pelvis showed no specific abnormalities and the patient was discharged but she returned to the hospital five days later with worsening abdominal pain, nausea and bilious vomiting. Physical exam was remarkable for diffuse abdominal tenderness and pale conjunctivae. The laboratory evaluation was notable for anemia with hemoglobin of 8.8 g/dL, hematocrit of 23.5%, MCV of 87 fL and corrected reticulocyte count of 7%. The patient was admitted and review of her peripheral blood smear demonstrated normochromic, normocytic anemia with extensive coarse basophilic stippling of the erythrocytes. Her heavy metal screening tests showed an elevated blood lead level of 102 μg/dL (normal < 10 μg/dL). Zinc protoporphyrin (ZPP) was subsequently found to be elevated at 912 μg/dL (normal < 35 μg/dL). Her diagnosis was severe lead poisoning. At that point the patient disclosed that she had been taking an Indian Ayurvedic medicine called Jambrulin obtained from Unjha pharmacy through a family member in India. She had been taking 2 pills daily over a period of 5–6 weeks in an effort to enhance control of her diabetes. She stopped taking the medication approximately 2 weeks prior to admission because of the abdominal pain. The patient was instructed not to take Jambrulin and received dimercaptosuccinic acid (DMSA), an oral lead chelator at a dose of 10 mg/kg three times a day for five days followed by 10 mg/kg twice a day for two weeks. At the end of chelation therapy, her blood lead level was significantly decreased to 46 μg/dL and her symptoms were resolved. When Ayurvedic medicine pills were tested, they showed approximately 21.5 mg of lead per pill. The pills were also sent to the Connecticut Department of Public Health Adult Blood Lead Epidemiology and Surveillance Program and Public Health Laboratory and were found to contain approximately 3.5% lead by weight [120].

Heavy metals and pesticides are also common present as contaminants in commonly prescribed raw Chinese herbal medicines. Harris et al. reported that out of 334 samples representing 126 different Chinese herbal medicines analyzed all 334 samples contained at least one heavy metal (lead, arsenic, chromium, mercury or cadmium) while 115 samples (34%) had detectable levels of all five heavy metals tested. In addition, 42 different pesticides were detected in 108 samples (36.7%) [121]. It is also possible that poisoning after consuming raw herbal supplement may occur due to mistaken identity of the plant. Lin et al. reported an outbreak of foxglove leaf poisoning when nine people mistakenly drank tea prepared from foxglove leaves assuming the tea was prepared from comfrey leaves because comfrey leaves resemble foxglove leaves. Significant cardiac toxicity developed in three individuals and digoxin concentrations varied from 4.4 to 135.9 ng/mL in these nine individuals. Patients were also treated with Digibind and fortunately all patients recovered [122].

GRAPEFRUIT JUICE-DRUG INTERACTIONS

Furanocoumarins present in grapefruit juice inhibit the intestinal CYP3A4 enzyme but not the liver CYP3A4 enzyme. Therefore, the bioavailability of certain drugs is increased if taken after drinking grapefruit juice. Components of grapefruit juice also inhibit P-glycoprotein, organic anion-transporting peptides and sulfotransferases. Effects of drinking grapefruit juice may last up to 12 h [123,124]. Holmberg et al. reported

increased bioavailability of antiplatelet agent ticagrelor due to interaction with grapefruit juice [125]. However, grapefruit juice reduces the efficacy of clopidogrel because this drug must be metabolized by CYP3A4 and CYP2C19 into an active metabolite but grapefruit juice inhibits intestinal CYP3A4 and CYP2C19 [126]. Interestingly, grape juice has no effect if a drug which has a known interaction with grapefruit juice if taken orally is administered intramuscularly or intravenously. Clinically significant grapefruit-drug interactions are summarized in Table 18.7.

HERBS INTERFERING WITH DIGOXIN IMMUNOASSAYS

Chinese medicine Chan Su and Lu-Shen-Wan contain bufalin, which has structural similarity with digoxin. Significant interferences of Chan Su as well as Lu-Shen-wan with various digoxin immunoassays have been reported. Oleander containing herbs also significantly interfere with digoxin immunoassays due to the presence of oleandrin, which also has structural similarity with digoxin. Interestingly oleander poisoning can be rapidly detected by using digoxin immunoassays if a person is not taking digoxin [127,128]. More recently, significantly interference of herbal supplements containing lily of the valley extract with a digoxin immunoassay has been reported due to structural similarity of convallatoxin, the active ingredient of the extract with digoxin [129].

CONCLUSIONS

The popularity of use of herbal remedies among the general population is on the rise and such practice also increases risks of herbal supplement induced liver damage, other organ damage, and drug-herb interactions. Because of the perception that herbal supplements are safe, a majority of people taking herbal supplements do not disclose their use of supplements to their health care professionals. Mehta et al. reported that overall only 33% of herbal and dietary supplement users reported disclosing their use of herbal supplements to their conventional health care providers [130]. Therefore, clinical laboratory may play an important role in helping clinicians to identify a potential drug-herb interaction. For example, abnormal liver function tests in a healthy individual during a routine physical examination may indicate use of kava or other herbal supplements known to cause liver damage. If during routine drug monitoring the drug observed level is significantly lower than the previous measurements and if non-compliance can be ruled out, it may be an indication of a potential drug-herb interaction.

TABLE 18.7 Clinically significant grapefruit juice-drug interactions.

Drug class	Examples	Clinical effect
Calcium channel blockers	Felodipine, Manidipine, Nicardipine, Nifedipine, Nimodipine, Nitrendipine, Pranidipine, Verapamil	Significantly increased bioavailability
Benzodiazepines	Alprazolam, Diazepam, Midazolam, Triazolam, Quazepam	Significantly increased bioavailability
Statins	Atorvastatin, Lovastatin, Pitavastatin, Simvastatin	Significantly increased bioavailability
Cardioactive	Amiodarone	Significantly increased bioavailability
Immunosuppressant	Cyclosporine, Tacrolimus	Significantly increased bioavailability
Anticonvulsant	Carbamazepine	Significantly increased bioavailability
Antibiotic	Erythromycin, Clarithromycin	Significantly increased bioavailability
Protease inhibitors	Amprenavir, Saquinavir	Significantly increased bioavailability
Proton pump inhibitors	Lansoprazole, Omeprazole	Significantly increased bioavailability
Narcotic analgesic	Methadone, oxycodone	Significantly increased bioavailability
Analgesic	Acetaminophen	Significantly increased bioavailability
Antiplatelet agent	Ticagrelor	Significantly increased bioavailability
Antiplatelet agent	Clopidogrel	Clopidogrel is a prodrug which is converted into active metabolite. Reduced efficacy of clopidogrel due to inhibition of intestinal CYP3A4 and CYP2C19 responsible for bioactivation of clopidogrel.

The most probable cause is use of St. John's wort and on discontinuation of St. John's wort, the drug level usually returns to pre-herbal supplement use levels within two weeks. Similarly, observing an unusual INR during routine monitoring of a patient taking warfarin is also indicative of a potential interaction between warfarin and an herbal supplement. However, all drugs are not monitored and some drug-herb interactions can be identified only through the clinical effects of such interactions, such as treatment failure or drug toxicity. Because of the serious consequence from treatment failure from drug-herb interactions, transplant recipients, patients receiving HAART (highly active antiretroviral therapy) for AIDS treatment as well as patients receiving warfarin or any related anticoagulants must refrain from using any herbal supplements. Many Americans drink fruit juices including grapefruit juice but certain grapefruit juice-drug interactions have clinical significance [131].

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CHAPTER

19

Critical issues in hemoglobinopathy detection and serology testing for HIV and hepatitis infections

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INTRODUCTION

Clinicians depend on clinical laboratories for obtaining accurate results in immunology and serology testing and inaccurate results may have tremendous impact not only on the diagnosis but also on a patient. For example, a false positive human immunodeficiency virus (HIV) test result may have a devastating psychological impact on the patient. Various methods are used in immunology and serology testing including electrophoresis, immunoassays and various other analytical techniques. Even for a single analyte, multiple technologies may be available. As expected no method is free from analytical errors. In this chapter various sources of errors in immunology and serology testing are addressed. The emphasis is how to minimize errors as well as eliminating errors when possible.

CHALLENGES IN HEMOGLOBINOPATHY DETECTION

Multiple methodologies exist for testing for hemoglobinopathies in the clinical laboratory (Table 19.1). The common ones employed are conventional electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC) and isoelectric focusing (IEF). In hemoglobin electrophoresis red cell lysates are run in electric fields under alkaline (alkaline gel) and acidic (acid gel) pH. This can be carried out on filter paper, a cellulose acetate membrane, a starch gel, a citrate agar gel, or an agarose gel. Separation of different hemoglobins is largely but not solely dependent on the charge of the hemoglobin molecule. Change in the amino acid composition of the globin chains results in alteration of the charge of the hemoglobin molecule resulting in change of the speed of migration.

HPLC systems utilize weak cation exchange column chromatography. A sample of a red blood cell (RBC) lysate in buffer is injected into the system. Hemoglobin molecules are adsorbed onto the column as they are charged molecules in the buffer system. An eluting buffer is then injected into the system. The hemoglobin fractions then elute off the column. The time required for different hemoglobin molecules to elute is referred to as retention time. The eluted hemoglobin molecules are detected by light absorbance. HPLC permits the provisional identification of many more variant types of hemoglobin that can be distinguished by conventional gel electrophoresis.

In capillary electrophoresis a thin capillary tube made of fused silica is used. When an electric filed is applied, the buffer solution within the capillary generates an electro-endosmotic flow that moves toward the cathode. Separation of individual hemoglobins takes place due to differences in overall charges.

Isoelectric focusing (IEF) separates proteins in a gel medium that has a pH gradient consisting of ampholytes (zwitterions). With application of high voltage, narrow buffered zones are created with stable but different pHs. Slower moving proteins move through these different zones but stop migrating at their individual isoelectric points (pI). For hemoglobins, these will migrate to the zone where the pH of the gel is the

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TABLE 19.1 Common methodologies for detection of variant hemoglobins.

	Advantages	Disadvantages
Agarose gel electrophoresis	Cheaper to perform	Labor intensive and time consuming; common variant hemoglobins are distributed in the four major lanes
HPLC	Faster turnaround time; greater resolution of common variant hemoglobins	Expensive
Capillary electrophoresis	Faster turnaround time; common variant hemoglobins are distributed in fifteen zones	
Isoelectric focusing	Greater resolution	Harder to interpret; particularly suitable for small samples including dried blood spots and is thus often used for neonatal screening

same to that of that particular hemoglobin. Staining may or may not be necessary. This depends on the manufacturer's recommendations. Other less commonly used methodologies include DNA analysis and mass spectrometry.

Hemoglobinopathy screening is now done for all newborns in the US. Dried blood spots (DBS) collected from a heel prick is transferred onto filter paper. In others, the sample for hemoglobinopathy detection is usually whole blood collected in EDTA tubes.

Any one of the above methods can be used for screening purposes. Detection of abnormal hemoglobins requires validation by a second method. This allows for verification of the abnormality detected by the first method. In addition, relevant clinical history and review of the complete blood count (CBC) and of the peripheral smear provide important correlation in the pursuit of an accurate diagnosis.

In addition to the above mentioned tests, screening tests for sickle cell are available to identify individuals with either sickle cell disease or sickle cell trait. These tests are either the hemoglobin S solubility test or the sodium metabisulfite test. The principle of the hemoglobin solubility test is that deoxygenated hemoglobin S is insoluble in the presence of a concentrated phosphate buffer solution and will form a turbid suspension that is visualized. Red cells are first lysed and sodium hydrosulfite added to reduce the released hemoglobin. Hemoglobin S if present will result in a turbid solution. In the metabisulfite test, a drop of blood is placed on a microscopic slide and sodium metabisulfite added. The slide is then observed under the microscope for sickle cells.

Hemoglobinopathy diagnosis errors

The age of the patient is an important factor for hemoglobinopathy diagnosis. Common clinically significant hemoglobinopathies are due to defects in the beta globin chain. These include hemoglobin S and C and beta-thalassemias. At birth the newborn has active alpha and gamma globin chain production, which combine to produce fetal hemoglobin F. The gamma chain production diminishes from birth and beta chain production increases. Combination of the beta and alpha chains produce hemoglobin A, which predominates in healthy individuals. Significant amount of beta chain production takes place at around 6 months, therefore beta chain defects may not be picked up before 6 months of age.

Blood specimens for hemoglobinopathy diagnosis may be sent to outside clinical laboratories and thus specimens need to be transported by couriers. Sometimes the specimens are mailed. Unduly long transit times without refrigeration may result in artifactual bands on gel electrophoresis causing confusion in interpretation and delay in final diagnosis [1]. High heat and humidity can change the levels of hemoglobin A and S in DBS samples. Thus high heat and humidity needs to be minimized in transport and storage of such samples. The method of preparing the red cell lysate can sometimes be important. One study has demonstrated that for accurate quantification of hemoglobin H (composed of 4 beta chains) from electrophoresis on agarose gel at alkaline pH, the lysing agent required is carbon tetrachloride. If other lysates are used much less hemoglobin H was detected, which could result in missing its (HbH) detection [2]. When HPLC is used, a recognized problem is carryover from one sample to the next [3]. For example, if the first sample belongs to a patient with sickle cell disease (HbSS) then a small peak may be seen at the "S" window in the next sample. This can lead to diagnostic confusion as well as the sample to be re-run. Relevant to the final diagnosis is the transfusion history. Blood transfusion acquired hemoglobinopathies is an established phenomenon [3]. This potential for transfusion acquired hemoglobinopathy exists because heterozygous individuals show no significant abnormalities in routine testing required during the blood donor screening process [4]. The abnormal hemoglobin in recipients can account for between 0.8% and 14% of the total hemoglobin [3]. Transfusion histories thus remain vital in explaining such findings.

False positive sickle cell solubility test may be seen individuals with high RBC counts, very high WBC counts or very high platelet counts. Increased levels of globulins or lipids may also result in false positivity. There are certain hemoglobins other than hemoglobin S which may result in a false positive test. These include HbC-Harlem, and HbC-Georgetown.

Common hemoglobin disorders are HbS, HbE, HbC, HbD, HbG and HbO. HbS is the most common hemoglobinopathy in the US. HbE is the most common hemoglobinopathy in South East Asia. These two hemoglobinopathies (HbS and HbE) account for the majority of all hemoglobinopathies detected worldwide. Although we deal with a handful of hemoglobinopathies in the clinical laboratory, over a thousand hemoglobinopathies have been described. Thankfully, the vast majority of them are rare or clinically insignificant. Examples of common hemoglobin disorders are highlighted in Table 19.2.

TABLE 19.2 Common hemoglobin disorders.

Hemoglobin variant	Affected globin chain	Populations affected
HbS	Beta chain defect	Commonest variant hemoglobin seen in the US; mostly seen in African Americans
HbC	Beta chain defect	Implies ancestry from Western Africa. HBSC is a sickling disorder.
HbE	Beta chain defect	Commonest variant hemoglobin seen in Asia
HbD	Beta chain defect	Seen in African, Indian, Pakistani as well as English individuals. HbSD is a sickling disorder
HbG	Alpha chain defect	Commonest alpha chain variant. Found in African Americans and African Caribbean's. It is of no clinical significance
HbA2′	Delta chain defect	Commonest delta chain defect. Seen in 1−2% of African American population.
HbO	Beta chain defect	Wide geographic distribution: from Africa to Middle East to Eastern Europe. HBSO is a sickling disorder.

Hemoglobin A2: In the diagnosis of beta thalassemia trait, the proportion of HbA2 relative to the other hemoglobins is clinically important [5]. In certain cases HbA2 variants may also be present. In such cases the total HbA2 (HbA2 and HbA2 variant) needs to be considered for the diagnosis of beta thalassemia [5]. HbA2′ is the most common of the known HbA2 variants and has been reported in 1%−2% of African Americans [6]. HbA2′ has been detected in heterozygous and homozygous states and in combination with other Hbvariants and thalassemia [7−10]. The major clinical significance of HbA2′ is that for the diagnosis or exclusion of beta thalassemia minor the sum of HbA2 and HbA2′ must be considered. HbA2′ when present accounts for a small percentage (1−2%) in heterozygotes and is difficult to detect by gel electrophoresis [11]. It is however, easily picked up by capillary electrophoresis and HPLC. By HPLC HbA2′ elutes in the "S" window. In HbAS trait and HBSS disease, HbA2′ will be masked by the presence of HbS. In HbAC trait and HbCC disease, glycosylated HbC will also elute in the "S" window. In these conditions HbA2′ will remain undetected. Conversely, sickle cell patients on chronic transfusion protocol or recent, efficient RBC exchange may result in a very small percentage of HbS, which the pathologist may interpret as HbA2′.

It has been documented that the HbA2 concentration may be raised in HIV infected patients during treatment [12]. Other causes of a raised level of HbA2 level are thought to be very unusual [13]. Severe iron deficiency anemia can reduce HbA2 levels [14,15] and in some reports this has been shown to interfere with the diagnosis of beta thalassemia trait.

Hemoglobin F: Increases in hemoglobin F are associated with multiple pathologic states. These include beta thalassemia, delta beta thalassemia, and hereditary persistence of fetal hemoglobin (HPFH). The former is associated with high HbA2 and the latter two states are associated with normal HbA2 values. Hematologic malignancies are associated with increased hemoglobin F and include acute erythroid leukemia (AML, M6) and juvenile myelomonocytic leukemia (JMML). Aplastic anemia is also associated with an increase in HbF. In elucidating the actual cause of high HbF, it is important to consider the actual percentage of HbF and HbA2 values, as well as correlation with CBC and peripheral smear findings.

It is also important to note that drugs (hydroxyurea, sodium valproate, erythropoietin) and stress erythropoiesis may also result in high HbF. Hydroxyurea is used in sickle cell disease patients to purposely increase the amount of HbF, the presence of which may help reduce the clinical effects of the disease. Measuring the level of HbF may be useful in determining the appropriate dose of hydroxyurea. In 15−20% of pregnant women HbF may be raised to values as high as 5%.

HbF quantification may be an issue when HPLC is used. Fast variants (e.g., HbH or Hb Bart's) may not be quantified as they may elute off the column before the instrument begins to integrate in many systems designed for adult samples. This will affect the quantity of HbF. If there is an alpha globin variant that separates from HbA, then there will be a HbF variant that will often separate from normal HbF; however, it may not

separate from other hemoglobin adducts present, therefore the total HbF will not be adequately quantified. HbF variants may also be due to mutations of the gamma globin chain, and again this may result in a separate peak and incorrect quantification. Some beta chain variants and/or their adducts may not separate from HbF and this will lead to incorrect quantification [16]. Capillary zone electrophoresis has an advantage over HPLC in that hemoglobin adducts (glycated hemoglobins and the ageing adduct HbX1d) do not separate from the main hemoglobin peak, making interpretation easier than HPLC. If HbF appears to be greater than 10% on HPLC, its nature should be confirmed by an alternative method to exclude misidentification of HbN or HbJ as HbF [17].

Hemoglobinopathy S: Hemoglobin S hemoglobinopathy is the most common hemoglobinopathy detected in the U.S. Various possible diagnoses of patients with HbS hemoglobinopathy include sickle cell trait (HbAS), sickle cell disease (HbSS), and sickle cell disease status post RBC transfusion/exchange. Patients with sickle cell trait may also have concomitant alpha thalassemia and the diagnosis of HbS/beta thalassemia (0/+/++) is also occasionally made. Double heterozygous states of HbSC, HbSD, HbSO are important sickling states that should not be missed.

Patients with HbSS disease may have increased HbF. The distribution of HbF amongst the haplotypes of HbSS are HbF 5−7% in Bantu, Benin or Cameroon, HbF 7−10% in Senegal and HbF 10−25% in Arab-Indian [18]. Hydroxyurea also causes increase in HbF. This is usually accompanied by macrocytosis. HbF can also be increased in HbS/HPFH.

HbA2 values are typically increased in sickle cell disease and more so when measured by HPLC. This is because the post-translational modification form of HbS, HbS1d, produces a peak in the A2 window. This elevated value of HbA2 may produce diagnostic confusion with HbSS disease and HbS/beta thalassemia. It is important to remember that microcytosis is not a feature of HbSS disease and patients with HbS/beta thalassemia typically exhibit microcytosis.

HbSS patients and HbS/beta 0 thalassemia patients do not have any HbA, unless the patient has been transfused or has undergone red cell exchange. Glycated HbS has the same retention time (approximately 2.5 min) as HbA in HPLC [18]. This will produce a small peak in the A window and raise the possibility of HbS/beta + thalassemia.

HbS/alpha thalassemia is considered when the percentage of HbS is lower than expected. Classical cases of sickle cell trait have 60% of HbA and approximately 35−40% of HbS. Cases of HbS/alpha thalassemia will have lower values of HbS, typically below 30% with microcytosis. A similar picture will also be present in patients with sickle cell trait and iron deficiency. Common challenges in hemoglobinopathy detection are summarized in Table 19.3.

TABLE 19.3 Common challenges in hemoglobinopathy detection.

1	HPLC	Carryover of sample from one to next
2	Transfusion	Transfusion from donors who are for example HbAS (S trait) or HbAC (C trait)
3	Medication	Hydroxyurea raises HbF levels
4	Iron deficiency	Lowers HbA2 levels thus masking diagnosis of beta thalassemia trait
5	HIV	Falsely increases HbA2 levels
6	Agarose gel	Small percentages of abnormal hemoglobins (e.g., HbA2′) may be undetected
7	HPLC	HbA2′ elutes in the S window. Thus in sickle cell disease or trait, HbA2′ will be undetected.
8	HPLC	Fast hemoglobin variants (e.g., HbH, Hb Bart's) may not be quantified effectively. HbF quantification will also be affected.

CHALLENGES IN HIV TESTING

Testing for HIV can be broadly divided into screening tests and confirmatory tests.

Screening tests include standard testing, rapid HIV testing and combination HIV antibody and antigen testing. Confirmatory testing may be performed by a HIV-1/HIV-2 differentiation assay, Western blot, or detection of viral nucleic acid. The test of choice for confirmation is the HIV-1/HIV-2 differentiation assay.

Standard screening for HIV is performed by an enzyme immunoassay (EIA). The test is based on the detection of HIV-1 and HIV-2 antibodies in the serum. These antibodies are directed against HIV antigens that include p24, gp 120 and gp 41. Antibodies to gp 41 and p24 are the first detectable serologic markers following HIV infection [19]. EIA tests that detect IgM and IgG antibodies are referred to as third generation tests. These tests are able to detect the presence of HIV-1 and HIV-2 antibodies as early as three weeks after exposure. A positive standard screen needs to be confirmed, with a HIV-1/HIV-2 differentiation assay. If this test is unavailable an HIV Western blot may be performed.

HIV viruses are categorized into several groups (Table 19.4) including M, N, O and P. Group M is considered to be the pandemic strain and accounts for the vast majority of strains of HIV. Group O strains are from certain parts of Africa (Cameroon, Gabon and Equatorial Guinea). Groups N and P are from Cameroon.

TABLE 19.4 HIV types and groups.

HIV type	Group	Comment
I		Related to viruses found in chimpanzees and gorillas
I	M	M denotes "major". Commonest type of HIV−; responsible for the AIDS pandemic
I	N	N denotes for "non-M, non-O". Seen in Cameroon.
I	O	O denotes for "outlier". Most common in Cameroon; not usually seen outside West-central Africa.
I	P	P denotes for "pending the identification of further human cases": The virus was isolated from a Cameroonian woman residing in France.
II		Related to viruses found in sooty mangabeys

Group M viruses are divided into ten subtypes A-J. Subtype B is the most common one found in USA and Europe.

Two important issues regarding HIV screen tests are the ability of the test to detect non-M strains and the timing of the test post exposure. If the patient has not yet seroconverted, as expected the antibody is absent and the individual may have a false negative test. There are also rare patients with HIV infection who become seronegative even though these patients had shown earlier seropositive results after exposure to the HIV virus [20,21]. Some other causes of false negative results include:

- Fulminant HIV infection
- Immunosuppression or immune dysfunction
- Delay in seroconversion following early initiation of antiretroviral therapy.

A false positive serologic test for HIV is rare. However, false positive test results for HIV infection have been documented in individuals who have received HIV vaccines in vaccine trials [22]. Some of these individuals who became HIV positive on screen tests also had a positive Western blot. HIV RNA testing is an approach that should be used to resolve such issues. HIV testing methods are listed in Table 19.5.

TABLE 19.5 HIV testing methods.

Screening	Confirmatory
Standard testing (EIA)	HIV-1/HIV-2 differentiation immunoassay
Rapid HIV testing	Western Blot
Combination HIV antigen and antibody test	HIV RNA PCR

Rapid HIV antibody tests

Rapid HIV antibody testing is available in the United States. Results are available within minutes as the test can be done on site and can be read by the provider. Rapid tests may not be as sensitive as second-generation enzyme immunoassays (EIA). In one study with more than 14,000 specimens, rapid testing failed to detect 16 samples, which were positive by EIA and subsequently confirmed by Western blot [23]. A positive rapid test should be considered as "preliminary" and requires testing by confirmatory method. Reports of false positive do exist in the literature. A negative test may be reported as such and does not require further work up. However, early testing prior to seroconversion will naturally result in false negative results. Rapid tests performed on oral fluids appear to be less sensitive than testing done on finger stick blood samples. It has been reported that 1 in 12 infected people can have a false negative result when oral home based tests are performed.

Combined antibody antigen tests

Fourth generation tests have the ability to detect both HIV antibody as well as the p24 antigen. The sensitivity and specificity of such tests are generally excellent. The primary advantage of these assays is the ability to detect HIV infection prior to seroconversion. Combined HIV antigen/antibody tests can detect HIV-1, and HIV-2 as well as group M and group O infections. The combined antibody/antigen tests are now becoming the standard of practice for initial screening of patients for HIV infection.

Confirmation tests

The HIV-1/HIV-2 differentiation immunoassay is a rapid laboratory based test that is typically performed to confirm a positive fourth generation combined antibody antigen test and to distinguish between HIV-1 and HIV-2 infection.

The Western blot test has a much slower turnaround time. The HIV-1 Western blot cannot detect the O subtype virus. For detection of HIV-2 a separate HIV-2 Western blot needs to be performed. Thus, the preferred method of confirmation is the HIV-1/HIV-2 differentiation assay.

HEPATITIS TESTING

Hepatitis infection is a worldwide problem. Hepatitis A is a problem with many developing countries because the hepatitis A virus (HAV) may spread from contaminated water (fecal-oral route). However, hepatitis A

testing is straightforward; IgM anti-HAV denotes recent infection and IgG anti-HAV appears in the convalescent phase of acute hepatitis. Hepatitis E virus (HEV) is also an enterically transmitted virus. HEV can also be transmitted by blood transfusion, particularly in endemic areas. Chronic hepatitis does not develop after acute HEV infection, except in the transplant setting and possibly in other settings of immunosuppression. Fulminant hepatitis can occur, resulting in an overall case fatality rate of 0.5–3%. For reasons as yet unclear the mortality rate in pregnant women can be as high as 15–25%, especially in the third trimester. The diagnosis of HEV is based upon the detection HEV in serum or stool by polymerase chain reaction (PCR) or by the detection of IgM antibodies to HEV. Tests for antibodies against HEV alone are less than ideal since they have been associated with frequent false positive and negative results. The hepatitis D virus (HDV, also called the delta virus) is a defective pathogen that requires the presence of the hepatitis B virus (HBV) for infection. HDV can elicit a specific immune response in the infected host, consisting of antibodies of the IgM and IgG class (anti-HDV). Serology tests for Hepatitis B and C infection, which are the most important components of hepatitis testing, are discussed in detail in the following section.

Serology for hepatitis B

Serologic markers available for hepatitis B infection (Table 19.6) are HBsAg (hepatitis B surface antigen), HBeAg (hepatitis B e antigen), anti-HBc (antibody against hepatitis B core antigen; both IgG and IgM), anti-HBs (antibody against hepatitis B surface antigen), anti-HBe (antibody against hepatitis B e antigen) and HBV DNA. HBsAg is the first marker to be positive after exposure to hepatitis B virus. It can be detected even before the onset of symptoms. Most patients may clear the virus and HBsAg typically becomes undetectable within four to six months. Persistence of HBsAg for more than six months implies chronic infection. The disappearance of HBsAg is followed by the presence of anti-HBs. During the window period (after the disappearance of HBsAg and before the appearance of anti-HBs) evidence of infection can be documented by the presence of anti-HBc (IgM). Co-existence of HBsAg and anti-HBs has been documented in approximately 24% of HBsAg positive individuals. It is thought that the antibodies fail to neutralize the virus particles. These individuals should be considered as carriers of hepatitis B virus. There exists a subset of patients who have undetectable HBsAg who are positive for HBV DNA. Most of these patients have very low viral load with undetectable levels of HBsAg. Uncommon situations are infection with HBV variants that decrease HBsAg production or mutant strains that have altered epitopes normally used for detection of HBsAg.

TABLE 19.6 Hepatitis B serology.

Antigen	Utility
• HBsAg	First detectable agent in acute infection
• HBcAg	Not tested as it is not detectable in blood
• HBeAg	Indicates virus is replicating and patient is highly infectious.
ANTIBODY	
• Anti-HBc	First antibody to appear. Should be positive when other tests for hepatitis B are negative during the window period (HBsAg is negative and anti-HBs not yet detectable)
• Anti-HBe	Virus is not replicating.
• Anti-HBs	Patient is immune.

Individuals with recent infection will develop anti-HBc, IgM antibodies. Individuals with chronic infection and individuals who have recovered from an infective episode will develop anti-HBc, IgG antibodies. However, anti-HBc, IgM antibodies may remain positive for up to two years after an acute infection. Levels may also increase and be detected during exacerbations of chronic hepatitis B. This may lead to diagnostic confusion.

There exist some individuals who have isolated positive anti-HBc antibodies. The clinical significance of this finding is unclear. Some of these individuals have been found to have HBV DNA by PCR in samples from serum or liver. Transmission of hepatitis B has been reported from blood and organ donors who have had isolated positive anti-HBc antibodies. On the other hand there are reports that a certain percentage of these individuals who have anti-HBc are false positive. Presence of HBeAg usually indicates that the HBV is replicating and the patient is infectious. Seroconversion to anti-HBe typically means the virus is no longer replicating. This is associated with a decrease in serum HBV DNA and clinical remission. In some patients seroconversion is still associated with active liver disease. This may be due to low levels of wild type HBV or HBV variants that prevent or decrease the production of HBeAg.

Serology for hepatitis C

The tests for hepatitis C virus (HCV) include serologic assays and molecular assays for HCV RNA. The most commonly utilized screening assay to detect anti-HCV antibody is an enzyme immunoassay (EIA). The latest version is EIA-3, which has better sensitivity and specificity compared to EIA-1 or EIA-2. In addition, the mean time to detection of seroconversion is shortened by two

to three weeks. Rapid tests for detection of anti-HCV also exist. If an individual is positive for anti-HCV then the logical next step is to test for HCV RNA. If there is true infection both anti-HCV antibody as well as tests for HCV RNA should be positive. If the HCV RNA is negative then the possibilities include:

1. A false positive anti-HCV antibody test
2. If the individual is a newborn, the anti-HCV may be that of the mother with transfer of antibodies across the placenta
3. Intermittent viremia
4. Past infection

For establishing the diagnosis of past infection another test, the recombinant immunoblot assay (RIBA) may be undertaken. If the anti-HCV is a false positive test then RIBA will be negative. If it is a case of past infection the RIBA will be positive.

There may be individuals who have HCV RNA but anti-HCV antibody testing is negative. This can be seen in the immunocompromised individuals or in the setting of early acute infection. HCV RNA tests are positive earlier than anti-HCV antibody tests.

CONCLUSIONS

Clinicians rely on accurate results from clinical laboratories for diagnosis of hemoglobinopathy, HIV and hepatitis testing. A false positive of false negative test result has serious consequence to patient management. In this chapter, pitfalls in immunology and serology testing have been addressed with emphasis on both false positive and false negative test results and how to eliminate some of these errors in the clinical laboratory.

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CHAPTER

20

Sources of errors in immunology and serology testing

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INTRODUCTION

Clinicians depend on clinical laboratories for obtaining accurate results in immunology and serology testing and inaccurate results may have tremendous impact not only on the diagnosis but also on a patient. In this chapter various sources of errors in monoclonal gammopathy detection, CSF (cerebrospinal fluid) electrophoresis, and autoimmune serology are addressed. The emphasis is how to minimize errors as well as eliminating errors when possible.

DETECTION OF MONOCLONAL PROTEINS

A monoclonal protein (paraprotein, M protein) is a monoclonal immunoglobulin that is secreted by an abnormal clone of plasma cells [1]. The M protein can be an intact immunoglobulin, only light chains (light chain myeloma, light chain deposition disease or AL amyloidosis), or rarely only heavy chains (heavy chain disease).

Serum protein electrophoresis (SPEP) is an inexpensive, easy to perform screening procedure for detection of monoclonal proteins [2]. It is usually done by the agarose gel method or by the capillary zone electrophoresis method. Monoclonal bands are usually seen in the gamma zone but may be seen in proximity of the beta band or rarely in the alpha 2 area. Urine protein electrophoresis is analogous to the serum protein electrophoresis and is used to detect monoclonal proteins in the urine. Ideally it should be performed on a 24-h urine sample. Common problems encountered in SPEP are listed in Table 20.1.

When a monoclonal band is identified on SPEP, serum immunofixation and 24-h urine immunofixation is typically recommended. There are certain situations where a band may be apparent that in reality is not a monoclonal band. Examples include:

A. Fibrinogen is seen as a discrete band when electrophoresis is performed on plasma. This fibrinogen band is seen between the beta and gamma regions. If the electrophoresis is repeated after the addition of thrombin, this band will disappear. A standard serum immunofixation study will be negative. Immunofixation reagents for fibrinogen are also available to confirm the source of suspicious bands.

TABLE 20.1 Common problems encountered in serum protein electrophoresis (SPE).

1.	SPEP performed on plasma will result in a band due to fibrinogen. Subsequent immunofixation will be negative.
2.	A band may be seen at the point of application. Typically this band is present in all samples performed at the same time.
3.	If concentration of transferrin is high (e.g., due to iron deficiency) this may result in a band in the beta region.
4.	In nephrotic syndrome prominent bands may be seen in alpha-2 and beta regions that are not due to monoclonal proteins
5.	Hemoglobin-haptoglobin complexes (seen in intravascular hemolysis) may produce a band in the alpha-2 region.
6.	M proteins may form dimers, pentamers, polymers or aggregates with each other resulting in a broad smear rather than a distinct band.
7.	In light chain myeloma, the light chains are rapidly excreted in the urine and thus SPEP may fail to show a band.

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B. With intravascular hemolysis the released free hemoglobin binds to haptoglobin. The hemoglobin-haptoglobin complex may appear as a large band in the alpha 2 area. Serum immunofixation studies should be negative in such cases.
C. In patients with iron deficiency anemia, concentrations of transferrin may be high. This may result in a band in the beta region.
D. If electrophoresis is performed on a nephrotic syndrome patient, total protein and serum albumin is typically low. The condition also produces increases in alpha 2 and beta fractions. Bands in either of these regions may mimic a monoclonal band.
E. When performing gel electrophoresis a band may be visible at the point of application. Typically this band is present in all samples performed at the same time.

If the quantity of the M protein is low this may not be detected by serum electrophoresis. There are also certain situations where a false negative interpretation may be made on serum electrophoresis:

A. A clear band is not seen in cases of alpha heavy chain disease (HCD). This is presumably due to the tendency of these chains to polymerize or due to their high carbohydrate content [3–5].
B. In Mu HCD, a localized band is found in only 40% of cases [6]. Panhypogammaglobulinemia is a prominent feature of such patients.
C. In occasional cases of gamma HCD, again a localized band may not be seen [5,7,8,9].
D. When an M protein forms dimmers, pentamers, polymers or aggregates with each other or when they form complexes with other plasma components, this may result in a broad smear rather than a discrete band.
E. Some patients produce only light chains. These light chains are rapidly excreted in the urine [10]. Serum electrophoresis fails to show any band. Urine studies are typically more fruitful. When the light chains cause nephropathy and result in renal insufficiency, excretion of the light chains is hampered. It is then that a band may be seen in serum electrophoresis.
F. In some patients with IgD myeloma, the M protein spike may be small enough to be disregarded.

Hypogammaglobulinemia

Hypogammaglobulinemia may be congenital or acquired. Amongst the acquired causes are multiple myeloma and primary amyloidosis. Panhypogammaglobulinemia can occur in about 10% of cases of multiple myeloma. Most of these patients have a Bence Jones protein in the urine, but lack intact immunoglobulins in the serum [11,12]. Bence Jones proteins are monoclonal free kappa or lambda light chains in the urine. Panhypogammaglobulinemia can also be seen in 20% of cases of primary amyloidosis. It is important to recommend urine immunofixation studies when panhypogammaglobulinemia is present in serum protein electrophoresis.

Immunofixation studies

An apparent monoclonal protein on serum protein electrophoresis may or may not be a true monoclonal protein. Also, M proteins may not be apparent on electrophoresis. To confirm the former and because of the latter, if there is a high index of clinical suspicion, immunofixation (IFE) studies are required. Immunofixation can be performed on both serum and urine specimens. It is preferable to perform urine immunofixation on a 24-h urine sample. Immunofixation studies are more sensitive than regular electrophoresis and also determine the particular isotype of the monoclonal protein. However, IFE cannot estimate the quantity of the M protein (which the standard electrophoresis can). One source of possible error in urine immunofixation studies is the "step ladder" pattern. Here multiple bands are seen in the kappa (more often) or lambda lanes and are indicative of polyclonal spillage rather than monoclonal spillage into the urine.

Capillary zone electrophoresis

This is an alternative method of performing serum protein electrophoresis. Protein stains are not required. A point of application is not seen. It is considered to be faster and more sensitive compared to agarose gel electrophoresis. Classical cases of monoclonal gammopathy produce a peak, typically in the gamma zone. Other less obvious situations that may imply the presence of an underlying monoclonal gammopathy are:

- The upstroke off the gamma distribution curve and the downstroke forms an angle where they meet
- The gamma distribution curve is not smooth
- The beta two peak is >50% more than the beta one peak

These changes may be very subtle. Interpretation can be subjective. Interpretation by pathologists with a high index of suspicion will result in a high percentage of cases being referred for ancillary studies such as immunofixation. Others disregarding the subtle changes may potentially miss positive cases.

Free light chain (FLC) immunoassay

Patients with monoclonal gammopathy may have negative serum protein electrophoresis as well as serum

immunofixation studies. Reasons include very low levels of M proteins and light chain gammopathy where the light chains are very rapidly cleared from the serum by the kidneys. Because of the latter, urine electrophoresis and urine immunofixation is part of the work up for cases where monoclonal gammopathy is a clinical consideration. Urine electrophoresis and urine immunofixation studies are also performed to document the amount (if any) of potentially nephrotoxic light chains being excreted in the urine in a case of monoclonal gammopathy.

Quantitative serum assays for kappa and lambda FLC have increased the sensitivity of serum testing strategies for identifying monoclonal gammopathies, especially the light chain diseases [13–16]. Cases that appear as non-secretory myeloma can actually be cases of light chain myeloma. FLC assays allow disease monitoring as well as providing prognostic information for monoclonal gammopathy of undetermined significance (MGUS) and smoldering myeloma.

The rapid clearance of light chains by the kidney is reduced in renal failure. Levels may be twenty to thirty times higher than normal in end stage renal disease. In addition, the kappa/lambda ratio may be as high as 3:1 in renal failure (normal 0.26–1.65). Therefore patients with renal failure may be misdiagnosed as having a kappa light chain monoclonal gammopathy. If a patient has a lambda light chain monoclonal gammopathy, with the relative increase in kappa light chain in renal failure, the ratio may become normal. Thus, a case of lambda light chain monoclonal gammopathy may be missed. It is also important to be aware that the presence of circulating M proteins may interfere with other laboratory tests. The most common errors that occur are falsely low high density lipoprotein cholesterol, falsely high bilirubin, and altered values of inorganic phosphate. Other tests where altered results may occur include low density lipoprotein cholesterol, C-reactive protein, creatinine, glucose, urea nitrogen, and inorganic calcium. There is a potential for inappropriate clinical decisions based on altered lab values caused by monoclonal gammopathies.

CEREBROSPINAL FLUID (CSF) ELECTROPHORESIS

Qualitative assessment of cerebrospinal fluid (CSF) for oligoclonal bands is the most important diagnostic CSF study when determining a diagnosis of multiple sclerosis (MS). In the CSF of MS patients immunoglobulin levels are elevated relative to other protein components, which suggests intrathecal synthesis. The immunoglobulin increase is predominantly IgG, although the intrathecal synthesis of IgM and IgA may also be increased.

Oligoclonal bands are defined as at least two bands seen in the CSF with no corresponding band present in the serum. Thus, it is crucial to perform CSF and serum electrophoresis simultaneously, preferably in adjacent lanes. Oligoclonal bands may be found in 95% or more of patients with clinically definite MS [17]. However, they may also be seen in CNS infections (e.g., Lyme disease), autoimmune diseases, brain tumors and lymphoproliferative disorders. Thus it is important to realize that the presence of an oligoclonal band is not equivalent to MS.

The first step when interpreting CSF electrophoresis is to establish that the sample is indeed a CSF sample. The presence of the prealbumin band and the band in the beta 2 region due to desialated transferrin establishes that the sample is indeed a CSF sample. Both these bands are not present in the serum lane. The presence of oligoclonal bands may then be confirmed by performing immunofixation studies. An abnormality of CSF IgG production can be demonstrated in 90% of clinically definite MS patients [18]. There are various ways to document this. The CSF IgG level may be expressed as a percentage of the total protein, as a percentage of albumin, or by the use of the IgG index, calculated as (CSF IgG x serum albumin)/(CSF albumin x serum IgG). It is naturally important to correlate the findings of the electrophoresis with the IgG index, CSF studies and any pertinent imaging and clinical findings.

In MS the CSF is grossly normal and the CSF pressure is normal. The total leukocyte count is normal in the majority of patients. If the CSF white blood cell (WBC) count is elevated it rarely exceeds 50 cells/μL [19]. Lymphocytes are the predominant cell type. CSF protein levels are also usually normal. If there is a systemic immune reaction or a monoclonal gammopathy then bands will be seen in both the serum and CSF lanes. These bands will correspond with each other. These are not diagnostic oligoclonal bands.

ANTINUCLEAR ANTIBODIES

Antinuclear antibodies (ANA) are serologic hallmarks of systemic autoimmune diseases [20]. A positive ANA may be seen in individuals with systemic or organ specific autoimmune diseases and a variety of infections. They can also be found in otherwise normal individuals. Examples of organs implicated in organ specific autoimmune diseases are thyroid, liver and lung. Examples of infections where ANA may be found to be positive include infectious mononucleosis, hepatitis C infection, subacute bacterial endocarditis, tuberculosis and HIV infections [21–23]. If they are positive in

otherwise normal individuals these individuals are most likely women and elderly.

The ANA assay is performed by incubating human epithelial tumor cell line (Hep2) cells fixed with methanol and/or acetone with the patient's serum. Fluorochrome-labeled antihuman globulin is added which binds to the antigen antibody complex. The slide is then viewed through a fluorescent microscope. Antibodies present in the patient's serum will bind to the nuclear antigen and should also produce a pattern, which is noted. The dilution of the serum at which the reaction pattern disappears is also noted. The accurate interpretation of patterns requires considerable experience. Also, the pattern type has been recognized to have a relatively low sensitivity and specificity for different autoimmune disorders. As mentioned earlier false positives may be seen in normal individuals. The majority of these are present in low titer.

False negatives are also an established phenomenon due to technical and physical issues, which include method of substrate fixation, solubility of the antigen in question, and localization of the antigen outside the nucleus. In standard ANA assays using Hep2 cells some antigens such as SSA/Ro antigen are underrepresented and may cause false negatives in cases of Sjogren's syndrome or autoimmune myositis. Once the ANA is positive further testing is required. These tests include antibodies to double stranded DNA, antihistone antibodies, antibodies to chromatin and antibodies to other nuclear proteins and RNA-protein complexes.

Recently, screening for ANA with automated assays, such as EIA or bead-linked multiplexed immunoassays, whereby multiple antigens are affixed to a solid phase, has become common practice. Solid-phase assays are cheaper, quicker, quantitative, offer similar sensitivity and specificity as compared to the labor-intensive, time-consuming, semi-quantitative fluorescent immunoassays, and can identify the specific antigen targets for auto-antibodies using the same automated instrumentation. However, not all antigens are represented and therefore these automated ANA assays can be less sensitive than fluorescent immunoassays for some auto-immune diseases such as systemic sclerosis. Specificity is usually superior with solid-phase assays. Alternatively, fluorescent ANA testing systems with automated microscopy, image analysis, titer calculation, and pattern interpretation have been developed that offer similar accuracy and more reproducibility as compared to manual testing, although complex or mixed pattern interpretation remains difficult [24]. Ideally, a strategy involving combinations of fluorescent and solid phase ANA assays offers optimal sensitivity and specificity.

CONCLUSIONS

Clinicians rely on accurate results from clinical laboratories for diagnosis of hemoglobinopathy, HIV and hepatitis testing. A false positive of false negative test result has serious consequence to patient management. Moreover, a monoclonal band if missed can be also has serious patient safety concern. In this chapter, pitfalls in immunology and serology testing have been addresses with emphasis on both false positive and false negative test results and how to eliminate some of these errors in the clinical laboratory.

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CHAPTER

21

Sources of error in molecular diagnostic analyses

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INTRODUCTION

Molecular diagnostics has undergone a period of rapid development and growth in the last decade. The implementation of new high complexity tests and integration of new technologies into the clinical molecular diagnostics laboratory has been critical towards advancement to the goal of achieving precision medicine. Molecular diagnostics encompasses diverse fields such as infectious disease, genetics, pharmacogenomics, and oncology, however, the underlying principles and potential sources of error are common among these different applications. Common errors that may occur with diagnostic molecular assays are discussed here. As with other sections of the clinical laboratory, a strong program of quality control and quality assurance is necessary for the detection of problems, to monitor errors, and to implement methods to assure quality.

PRE-ANALYTICAL ISSUES

In molecular diagnostic testing, as in all laboratory testing, pre-analytical factors relating to specimen collection, transport, and storage, can have important effects on testing results. The analyte typically being assessed in most molecular testing is nucleic acids (e.g., DNA or RNA), and thus guidelines relating to specimen collection, transport, and storage focus on ensuring that the target nucleic acid is stabilized without the introduction of any interfering substances into the specimen that inhibit downstream applications. A guideline from the Clinical and Laboratory Standards Institute (CLSI) for specimen handling is available that outlines standardized methods for pre-analytical processes [1]. A wide array of specimen types with varying collection tubes or containers, and varying storage requirements are routinely handled in diagnostic molecular laboratories. These specimen types include blood, stool, urine, sputum, CSF, buccal swabs, amniotic fluid, endocervical swabs, bone marrow aspirates, formalin fixed paraffin embedded tissue, and fresh or frozen tissues.

Specimen collection

Specimen collection guidelines should be optimized to ensure that the correct specimen is collected based on the testing to be performed and the target nucleic acid. For genetics and pharmacogenetics testing, whole blood is generally utilized and is collected in EDTA or citrate tubes. For infectious disease assays, many specimen types can be submitted depending on the clinical syndrome and agent being tested for. Routine monitoring assays (e.g., viral load testing for HIV or HCV) are performed on plasma or serum. Laboratories should ideally adopt a single type of specimen because there are minor differences in viral loads between the two types of specimens. Viral load monitoring is done at defined intervals, and variability due to specimen type may lead to misinterpretation of results. For this reason, labs should adopt a single method (e.g., plasma or serum) and not introduce another specimen collection method. The timing of specimen collection is critical for many assays, especially viruses, where the analyte may be present transiently. Collecting a specimen during the acute phase of illness is highly recommended for respiratory viruses, arthropod-borne viruses, and many others. For respiratory viral assays, the optimal specimen type is usually a nasopharyngeal swab or aspirate, and throat swabs are usually discouraged by the laboratory.

Some specimens may be collected directly into fixatives or preservatives (e.g., cytology specimens for HPV testing, urine for BK virus testing). Sterile body

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fluids such as CSF, pleural fluid, or synovial fluids may be submitted to the lab in clean containers or tubes, without preservatives.

For oncology specimens, the primary specimen is tissue that has undergone formalin fixation and paraffin embedding (FFPE). A number of factors have been reported from tissue collection to fixation and embedding that may affect the quality and quantity of nucleic acids extracted from tissues that have undergone FFPE processing. Warm/cold ischemia time refers to the time from interruption of a tissue's blood supply to the time of preservation of the tissue. Warm ischemia time is the time the tissue remains at body temperature after the blood supply to that tissue has been interrupted, and cold ischemia time is the time the tissue has cooled prior to preservation. For polymerase chain reaction (PCR) assays using DNA, no significant difference has been reported based on ischemia time [2,3]. RNA however, is unstable and subject to degradation. Prolonged ischemia times may negatively impact both RNA quantity and quality [4]. This can be an important factor in RNA-based assays, particularly in those investigating gene expression. In addition to ischemia times, the fixative used for sample preservation is important. Neutral buffered formalin is the standard fixative used in most anatomic pathology laboratories. Specimens originating from cytopathology, such as touch preps and smears, and fixed in alcohol may be used as they also yield nucleic acids of suitable quantity and quality for molecular assays [5]. Other fixatives, such as Zenker's, Bouin's, or B5 cause metal-protein complexes or DNA depurination and are contraindicated when molecular testing is desired. The amount of time a sample is fixed in neutral buffered formalin is also important. Fixing samples in neutral buffered formalin results in DNA and protein crosslinking, and over-fixation can occur with more than 72 h of fixation, lowering DNA integrity and yield [6,7]. In addition, other treatments, such as decalcification, can have a profound effect on nucleic acid quality/quantity. Many decalcification solutions are acid-based, which results in damaged DNA of unsuitable quality for molecular testing. Decalcification using EDTA takes more time than decalcification using acid solutions, but does preserve the quality of DNA and thus may be an alternative to consider when molecular testing is required.

Specimen storage and transport

Specimen transport and storage considerations should be based on the type of target (DNA or RNA). RNA targets are usually very unstable and subject to degradation. These specimens should be transported immediately to the lab, held at 4 °C and processed as soon as possible to avoid target degradation. As another example for target specific transport requirements, an emerging assay in molecular oncology is testing for circulating tumor DNA to detect disease, identify targetable variants, and/or monitor treatment response. Samples for circulating tumor DNA have special requirements for transport to avoid lysis of white blood cells, and so samples should be processed within 6 h from collection [8]. Failure to process specimens in a timely manner is a common reason for false negative results in molecular assays.

Storage of specimens in preservative can be problematic, especially long-term storage. Specimens submitted for molecular testing must be stored in controlled conditions so that nucleic acids do not degrade during storage. To prevent degradation of target nucleic acids, fresh tissue or fluids may be transferred into a stabilizing reagent (for example, RNAlater or Trizol) that can then be used for nucleic acid extraction at a later time. These reagents are designed to lyse cells, inactivate proteins such as nucleases, and stabilize the nucleic acid during storage. Although successful molecular testing can be performed on stored FFPE tissue, some studies have found storage to negatively affect DNA quantity/quality [9–13]. Storage of FFPE tissue, and particular long-term storage, can result in DNA fragmentation, as well as accumulation of alterations to DNA sequences, most commonly a cytosine to thymine transition resulting from deamination of cytosine bases [14].

Specimen assessment

Another consideration, primarily for oncology specimens, is assessment of the lesional cell content as sufficient tumor cell percentage is necessary to ensure that a variant, if present, would be detected with the limit of detection established for the molecular assay requested. Assessment of tumor cell content in most laboratories is typically performed by a pathologist, although it may also be performed with digital imaging and software. Because assessments may be subjective and variable [15], many laboratories will often require a tumor cell content higher than the minimum required by the sensitivity of the assay. Frequently, the percentage of tumor in the tissue submitted for testing is insufficient, and enrichment of tumor is required prior to extraction of nucleic acids. Commonly this is performed by macrodissection, where a technologist will scrape areas of tumor using a scalpel or razor, or enrichment is performed by microdissection, in which tissue sections are stained and a dissecting microscope is used to assist the technologist in targeting small areas of tumor. Another option for tumor enrichment is laser capture microdissection; however, the cost, time, and training required can be a barrier to adoption for some laboratories.

Nucleic acid extraction

Most molecular diagnostic testing methodologies require isolated nucleic acids for optimal performance. While some methods can be performed directly on cells or tissues without an extraction step, most testing procedures will include a nucleic acid extraction and purification procedure prior to testing. During nucleic acid extraction, nucleic acids are released from cells (eukaryotic cells, bacterial cells) and virus particles prior to molecular detection. Purification of nucleic acids after cell lysis removes cellular debris, amplification inhibitors, inactivates or destroys nucleases (DNases and RNases) that can degrade nucleic acids, and leaves the nucleic acid in solution that is optimal for downstream applications. Purification can also be used to concentrate the nucleic acids so that rare targets such as bacteria or viruses can be detected. Thus, nucleic acid preparation methods are a critical component of molecular testing. The quality of purified DNA and RNA, as well as the concentration and purity affect hybridization, amplification and probe detection methodologies. Nucleic acid extraction and purification procedures can be a source of error in diagnostic testing. If intact nucleic acids are not isolated, false negative results can occur. In addition, the extraction and purification procedures can themselves be a source of interfering substances if reagents such as phenol or alcohols are carried over into the final eluted material containing the target nucleic acid.

The two main methods by which nucleic acids are extracted and purified are organic (e.g., phenol-chloroform) extractions or inorganic (e.g., silica binding) extractions. For phenol-chloroform extractions, cells are initially lysed with a chaotropic salt (guanidinium thiocyanate) and detergent, then phenol-chloroform is added to extract proteins, followed by precipitation of nucleic acids from the aqueous phase with ethanol [16,17]. Phenol and proteins must be carefully avoided when removing the aqueous phase to avoid contamination of the nucleic acids. Phenol-chloroform extractions are somewhat difficult for clinical laboratories, as they are time-consuming and labor-intensive, and have not been adapted to automated systems.

The silica binding method developed by Boom et al. relies on the binding of nucleic acids to silica in the presence of salt [18]. The silica-bound nucleic acid is separated from cellular debris, then washed and eluted into a low-salt solution. Silica binding methods result in highly pure nucleic acid that is largely free from contaminating proteins including nucleases, and inhibitory substances. This method is versatile in that silica can be bound to many different substrates such as membranes, filters beads, or magnetic particles, and many different products have been developed based on this technology. The Boom technology has also been successfully adapted to automated robotic systems, allowing for laboratories to process very large numbers of specimens. Automated systems are available from several manufacturers and have the advantage of workflow efficiency as well as reducing variability that can occur in manual extraction methods.

Additional considerations include special care when extracting RNA to avoid RNase contamination, and for FFPE samples, additional steps are required prior to nucleic acid extraction. Deparaffinization is typically performed using xylene, followed by rehydration in a series of ethanol solutions, but may also be performed using other methods, for example using mineral oil. Next, proteolytic digestion using heat and proteinase K is performed. This is a critical step to remove the protein crosslinking introduced by formalin fixation. Longer digestion times have been reported to result in increased yields of amplifiable DNA and RNA [19].

Finally, for most procedures, assessment of the amount and quality of isolated nucleic acids should be performed prior to testing. This can be performed by a spectrophotometer such as Nanodrop, or by a fluorometer such as Qubit, which provides more accurate results. For assays in which RNA quantity/quality are critical, measurement of RNA integrity number (RIN) can be assessed on an Agilent Bioanalyzer.

MOLECULAR METHODS

Hybridization methods

Hybridization methods rely on the use of labeled DNA or RNA as probes to detect nucleic acids in specimens. Nucleic acid hybridization is based on complementary base pairing between a labeled nucleic acid probe and the target nucleic acid. Direct probe hybridization is a relatively simple method, but requires the presence of the target in relatively high levels, so its use as a diagnostic tool is somewhat limited. Hybridization is used when the target nucleic acid is present in high enough concentrations to be detected reliably. When sensitivity is not a limiting factor, nucleic acid probe hybridization methods are excellent methods to use because they are relatively easy to perform, results can be obtained rapidly, and they are not susceptible to contamination like amplification methods. Hybridization techniques offer flexible formats (for example, hybridization can be performed on solid substrates, in liquid solutions or even *in situ* in tissues or cells).

Applications of direct probe hybridization in diagnostic microbiology include identification of bacteria in blood culture bottles using fluorescence *in situ* hybridization (FISH), or identification of cultured microorganisms such as fungi and mycobacteria

after transcription-mediated amplification (TMA). In diagnostic molecular pathology *in situ* hybridization is used to detect gene duplications and gene mutations (e.g., *in situ* hybridization for HER-2 amplification). Southern blotting, another hybridization-based method, is used in genetics assays to assess for large alleles, such as those that may be seen in triplet repeat disorders such as Fragile X syndrome or Huntington disease.

Sources of error in hybridization based assays include low signal if the hybridization conditions are not optimal (i.e., proper temperature is not maintained), or high background if the post-hybridization washes are not of the appropriate stringency.

Microarrays

DNA microarrays are increasingly being used in molecular diagnostics. Microarrays are microscopic groups of DNA spots attached to a solid substrate. The most common materials used as substrates are glass, plastic or silicon [20]. Microarrays are commonly referred to as gene chips or DNA chips. Microarrays are designed to contain a very large number of oligonucleotides on a single chip, allowing for collection of a large data set in one experiment. The principle of microarray chip testing is to apply target DNA or RNA to the chip, allow hybridization of complementary sequences, and then detect hybrids. Liquid detection formats (microbeads) analogous to flow cytometry are also available. Many different formats of labeled detection are available, including fluorescence, mass spectrometry, and electrochemical [21].

Microarrays have been successfully adapted to most of the disciplines within laboratory medicine when molecular testing requires analysis of a large data set with applications including microbiology [22–24], oncology [25], and genetic disease [26,27]. In the clinical microbiology laboratory, several microarray tests are commercially available for genotyping of human papillomavirus and hepatitis C virus, identification of bacteria with 16S rRNA probes, strain typing, and detection of antimicrobial resistance genes. In molecular oncology testing, gene expression microarrays are used for tissue of origin testing, copy number changes, and hematological malignancies [28–30]. Microarrays are particularly useful in genetic testing and can identify aneuploidies, unbalanced rearrangements, deletions, and duplications.

When using microarrays for diagnostic testing, common sources of error should be considered. Problems can occur with probe sequences, probe labels and detection methods. For fluorescently labeled probe assays, autofluorescence can interfere with correct data collection from fluorescently labeled probes on microarray substrates. Background signal may come from non-specifically bound probes or from autofluorescence (intrinsic signal from the substrate). Nonspecific hybridization may occur if stringency of hybridization is not optimized for all of the probes used on the microarray.

Amplification methods

Polymerase chain reaction (PCR)

PCR is based on the use of thermally stable DNA polymerase enzymes to copy a sequence of DNA by extending primers bound to a single-stranded DNA target. Repetitive cycles using heat to melt the double stranded DNA, followed by primer binding and extension of the primer, result in the synthesis of many copies of the target DNA [31,32], which can then be detected with probes. In endpoint PCR, the newly synthesized DNA is detected by a method such as probe hybridization or gel electrophoresis after the end of the reaction. In real-time PCR, the newly synthesized DNA is detected via DNA binding dyes or labeled probes as it is being synthesized. Real time PCR is highly advantageous to diagnostic medicine for several reasons: data is collected during the run, so results are obtained rapidly, and potential for contamination is reduced or eliminated, as reaction tubes are not opened after the PCR is complete.

Strand-displacement amplification (SDA)

SDA is a DNA amplification method that is performed without temperature cycling (isothermal). It relies on the use of a restriction enzyme to nick the DNA target, which is then copied by DNA polymerase. As the polymerase copies the target, the template strand is displaced [33]. SDA is used in the BD ProbeTec ET assay for the detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis*.

Transcription-mediated amplification methods

Nucleic acid sequence based amplification (NASBA) and transcription mediated amplification (TMA) are two methods that rely on the use of RNA transcription for amplification [34,35]. Primers that contain sequences recognized by RNA polymerases are used to hybridize to the target sequence, then extended by the RNA polymerase. These methods exhibit good sensitivity, similar to PCR. Transcription based methods are commercially available for infectious disease assays.

Amplification inhibitors

Inhibition of amplification (complete or partial failure to generate amplified DNA or RNA because of inhibition of enzymes used in amplification reactions) can be

a cause of negative results or inaccurate quantitation [36]. Presence of inhibitors can cause false negative results in qualitative assays or can reduce amplification efficiency, and can cause inaccurate results in quantitative assays. Inhibition of amplification reactions is a well-recognized phenomenon in the diagnostic laboratory, yet the reasons for inhibition are not always well understood [37]. Many substances from a variety of sources can inhibit PCR reactions; inhibitors can be present in the clinical specimen itself (for example, hemoglobin and lactoferrin, from blood specimens) or be carried over from the transport device (for example, heparin from a blood collection tube) or from the post-collection procedures such as nucleic acid extraction [36] (Table 21.1).

Some inhibitors are well-characterized and the mechanisms by which they function are understood. However, for some specimens and assays, inhibition may occur, but the inhibitory substances are not defined and/or the mechanism of inhibition is not understood. Inhibitory substances may bind directly to the target nucleic acids, blocking enzyme binding, or they may bind directly to amplification enzymes, preventing enzymes from binding the target. Inhibition can affect different amplification reactions differently, and to different levels [36,37]. Inhibitors are especially problematic in quantitative assays because they may cause aberrant results (inaccurate quantitation) or they may inhibit amplification altogether. Commercially available quantitative PCR assays use software algorithms that normalize the signal from the internal calibrators present in each reaction vessel. Patient test results are based on normalized values; if partial or complete inhibition has occurred it is detected by the system and interpreted correctly. Laboratories that develop in-house quantitative PCR should implement procedures using normalization to ensure accurate results in their quantitative PCR.

TABLE 21.1 Common PCR inhibitors.

General source	Inhibitory substance
Specimens	Blood – Hemoglobin, IgG, lactoferrin Sputum[a] Stool[a] – bile salts, complex polysaccharides Tissues – melanin, myoglobin, collagen Urine[a] – urea
Specimen collection tubes	EDTA Heparin
Nucleic acid extraction	Detergents (Sarkosyl, SDS) Proteinases Protein disrupting agents Guanidinium salts Phenol Alcohols

[a]*For many complex clinical specimens, inhibitors have not been well-characterized.*

Mechanisms of inhibition

There are several mechanisms by which inhibitors function, usually by targeting the enzymes involved in amplification, or interfering with binding of the enzyme to the target nucleic acid.

1. Blocking active site of enzymes. Some substances block active sites of enzymes used in amplification reactions.
2. Inactivating enzymes. Inhibitors also can act by denaturing or degrading the enzymes used in amplification (for example, if proteinases are present in the sample).
3. Binding target nucleic acids, preventing the target from being accessible to primers or enzymes.
4. Binding divalent cations. Inhibition can occur when divalent cations (Mg++) necessary as cofactors for polymerase enzymes are bound by EDTA.

Commonly encountered inhibitors and their sources

Inhibitors are present in clinical specimens, collection and transport devices, and can be introduced during processing or nucleic acid extractions. Amplification tests that are performed directly on specimens without a separate nucleic acid purification step are especially prone to inhibition.

Many clinical specimens such as urine, feces, blood and sputum are complex and contain inhibitory substances. Not all inhibitors have been characterized but the mechanism of some inhibitors have been reported. Hemoglobin is a potent inhibitor of PCR amplification and a well-characterized inhibitor of the Taq polymerase [38]. Immunoglobulin G is another inhibitor, thought to act by binding to target DNA [39]. Melanin is present in pigmented cells from skin and melanoma tumor samples. Melanin is a potent inhibitor of PCR and usually co-purifies with standard DNA or RNA extraction procedures [40]. Heparin and EDTA from specimen collection devices such as blood collection tubes are also PCR inhibitors. EDTA can potentially inhibit PCR by binding divalent cations that are used as cofactors by amplification enzymes [36]. Heparin is easily co-purified with DNA and a strong inhibitor of PCR, at levels as low as 0.032 U/mL in the PCR reaction [41]. Other PCR inhibitor sources are nucleic acid extraction procedures. Detergents used to lyse cells (e.g., Sarkosyl, SDS), protein disrupting agents (guanidine), enzymes used to digest cellular proteins (proteinases), organic extraction compounds (phenol, chloroform) and alcohols all may be inadvertently carried over from the extraction procedure into the purified nucleic acid

samples. If present in high enough concentrations, these substances can degrade polymerases, or physically block polymerase from copying target nucleic acids [36].

Monitoring for amplification inhibition

An effective way to determine if inhibitors are present in a specimen is to include an internal control in every reaction. Monitoring for inhibition is now a requirement for laboratories that are performing diagnostic assays using molecular methods. Commercially available, FDA approved diagnostic assays include internal amplification controls to monitor for inhibitors. Several different types of amplification controls can be used, and both internal and external control materials should be included in amplification assays. Internal controls are used to monitor the entire process, from extraction to assay interpretation and guidelines for choosing internal control materials are available [42,43]. If the result of the internal control is negative, the report can be modified to indicate that the specimen was inhibitory, rather than negative. For real-time PCR systems, amplification curves can be examined to determine if inhibition is occurring.

Strategies to prevent inhibition

For assays performed on plasma, guidelines for specimen collection should be followed carefully. Drawing the correct amount of blood into the tube will ensure that the EDTA is diluted to a level that will not inhibit downstream PCR testing after extraction of nucleic acids. Nucleic acid extraction methods shown to effectively eliminate inhibitors such as silica binding methods should be implemented, when possible. Amplification assays should be developed with inclusion of substances that can facilitate amplification and relieve inhibition include bovine serum albumin [44] and DMSO [36]. If the laboratory is monitoring for inhibitors using internal controls and detects an increased rate of inhibition, the assay can be re-optimized. Commercially available PCR assays now contain internal controls; inhibition of the internal control can be monitored and if significant inhibition occurs, the test may be reported as being inhibitory, rather than negative. Dilution of the sample can also be considered, as diluting the sample also dilutes the inhibitor. However, this may affect assay sensitivity, and if performed, should be within the validated performance specifications of the assay.

Nucleotide sequencing

Sanger sequencing

Sanger sequencing relies on the use of di-deoxynucleotides during amplification reactions. When a dideoxynucleotide is incorporated into the growing strand of DNA, the chain is terminated. This results in a mixture of terminated fragments, representing the entire length of the segment of DNA that is being analyzed. The fragments are electrophoresed, and detected by fluorescent label. The most convenient format for diagnostic laboratories is to use di-deoxynucleotides that are each labeled with a different dye; the resulting fragments can be electrophoresed in a single capillary or lane on a gel.

Next-generation sequencing (NGS)

NGS is a technology that allows massively parallel sequencing, making the interrogation of multiple targets simultaneously achievable. NGS has multiple clinical applications from genetics to infectious disease to oncology and has been adopted in many clinical laboratories. Different sequencing platforms are available. Two widely used platforms are the Illumina and the IonTorrent platforms, both of which utilize sequencing by synthesis technology. In Illumina platforms, fluorescently labeled nucleotides are sequentially added and imaged [45]. In IonTorrent platforms, unmodified nucleotides are added, but the release of H^+ during synthesis is measured [46]. Library preparations to enrich for target sequences are typically either amplicon-based, where multiplexed PCR amplification is used to amplify regions for sequencing, or hybrid capture based, where DNA is fragmented and probes are used to capture regions of interest. Adapters for sequencing are also added to the library for the sequencing reaction, as are indices which allow for sample identification.

Following sequencing, bioinformatics processing is performed, which critical in the evaluation of NGS data. Guidelines for validating NGS bioinformatics pipelines have been recently published by the Association for Molecular Pathology and the College of American Pathologists [47]. Analysis of NGS data generally starts with platform-specific base calling. These base calls are demultiplexed, and the reads are stored as FASTQ files. Reads are aligned to a reference sequence. This typically is the human reference genome, but in the case of infectious disease testing, may be viral or bacterial or fungal sequences. In most software, the alignment data is stored in binary alignment/map (BAM) format. Following alignment, variant calls are made. Additional annotation and filtering of variants is typically performed prior to clinical review. Recently, the Association for Molecular Pathology, American Society of Clinical Oncology, and College of American Pathologists published recommended standards and guidelines for the interpretation and reporting of somatic variants [48]. These guidelines recommend a four tiered classification system. Tier 1 variants are those with strong clinical significance, tier 2 variants are variants with

potential clinical significance, tier 3 variants are those with unknown clinical significance, and tier 4 variants are those that are considered benign or likely benign. For the interpretation of germline variants, guidelines from the American College of Medical Genetics and Genomics and the Association for Molecular Pathology are available [49]. This guideline recommends classification of variants into one of the following categories: pathogenic, likely pathogenic, uncertain significance, likely benign, or benign. Evidence to support classification for germline or somatic variants is obtained from various sources including population databases such as ExAC, gnomAD, 1000 genomes, cancer mutation databases such as COSMIC or cbioportal, germline databases such as OMIM, internal laboratory databases, professional guidelines, clinical trials database such as clinicaltrials.gov, and in silico prediction algorithms.

Sources of error in NGS testing can arise both from the library preparation/sequencing, as well as from the bioinformatics analyses performed. Sample source can play an important role in the ability to generate high quality NGS data. If nucleic acids are fragmented or of poor quantity/quality, artifacts can be generated. This may be seen in FFPE samples, for example. Low amounts of input DNA reduces the library complexity and can result in PCR duplicates (especially in amplicon based libraries). Storage of FFPE samples can introduce deamination errors, as discussed previously. Other errors that may arise are false negative calls, due to difficulty in aligning large insertions or deletions, or false positive calls in regions of homopolymer repeats. Low complexity regions and pseudogenes may present additional challenges. Bioinformatics tools (e.g., deduplication, read strand bias) and visual inspection of sequences and alignments can help identify some of these potential errors. Unique molecular identifiers (UMIs) are molecular barcodes used to tag molecules prior to library amplification; identification of reads that align to the same location and have the same UMI allows for accurate identification of PCR duplicates, as well as for the generation of a consensus read to correct amplification errors [50–53].

Case report: incorrect zygosity call for a variant detected by whole exome sequencing

A 7 year old boy with sensorineural hearing loss presented to genetics clinic for evaluation. Whole exome sequencing was performed and a heterozygous frameshift variant was identified in *STRC*, a gene that has been reported to be associated with deafness, autosomal recessive 16 (OMIM #603720). No other variants were identified in *STRC*. To further clarify the significance of a heterozygous pathogenic variant in an autosomal recessive disorder, the variant, alignment, and mappability were reviewed. *STRC* shares high homology with a STRC pseudogene (99.6% conserved). Long-range PCR followed by nested PCR and Sanger sequencing was performed which confirmed that the variant was present in the *STRC* gene and not in the pseudogene, and showed the frameshift variant to be present in a homozygous, not heterozygous, state.

COMMON CAUSES OF FALSE POSITIVE AND FALSE NEGATIVE RESULTS

Sequence mismatch between primer and target DNA: role of genetic variation

A mismatch between the primers or the probe sequence can result in false negative assay results, or can give inaccurate quantitative results in viral load assays. Mutations in the primer binding area can prevent amplification altogether or reduce efficiency of amplification. Microorganisms, especially viruses, are prone to this phenomenon because they can evolve rapidly. HIV and HCV viral load assays are subject to this phenomenon. Both of these viruses exist as viral populations with significant genetic variation. While quantitative assays for these viruses are usually targeted to conserved areas of the genome, there is enough genetic variation in the population such that occasional strains of these viruses may be missed altogether or underquantitated [54]. False negative molecular assays for bacterial pathogens can also occur because of sequence variation. PCR-based assays for *N. gonorrhoeae* may be negative when strains of the organism with genetic variation in the primer binding region are present in the specimen [55].

Sequence variation should always be a consideration when designing assays, especially "home brew" assays. For laboratories performing in-house developed tests, primer and probe sequences should be verified. This can be done by searching publicly available databases to ensure that known, common genetic variants are not present in the primer or probe sequence.

Case report: false negative result of PCR testing for Neisseria meningitidis

A 2-year old boy presented to the hospital with meningitis; empiric antimicrobial therapy had been started prior to obtaining CSF and blood specimens for culture. A Gram stain performed on the CSF revealed Gram-negative diplococci; culture was negative. Real-time PCR testing for *N. meningitidis* using primers for the ctrA gene was negative. Because the Gram stain was consistent with *N. meningitidis*, a second PCR assay using 16S rDNA primers was performed with positive results for *N. meningitidis*. During the time the testing was being performed, blood cultures grew *N. meningitidis*. The child recovered. Follow-up testing of the isolate from the blood culture showed sequence variation in

the ctrA gene, with polymorphisms present on one of the primer binding sequences, and a single nucleotide substitution in the probe binding area [56].

Mispriming/cross reactivity of primers and probes

Hybridization of primers to non-target sequences (mispriming) can occur under several conditions. Primers sequences should be checked by searching against a database such as GenBank to verify that they will not cross-react to other organisms or other target areas. Mispriming can also occur when assay conditions are not optimized (annealing and extension temperatures, composition of amplification mix, ion concentration, etc.). Nucleotide sequence homology in primer or probe binding regions can give false positive results. Hybridization probes used to detect amplified product may exhibit cross-reactivity to closely related species. For amplification assays that use labeled probes for detection of product, probe sequences should be checked for potential cross-reactivity to related species or genes, including pseudogenes if DNA is targeted.

Contamination

In general, molecular diagnostic methods, especially infectious disease assays, require very high analytical sensitivity. Because the amount of microbial DNA or RNA present in a clinical specimen may be very low, an amplification method is used to optimize sensitivity. Theoretically, nucleic acid amplification methods have the capability to detect a single molecule of analyte and many assays do approach this level of sensitivity, with some assays capable of detecting as few as 5 to 10 molecules of target analyte. But the extremely high analytical sensitivity of amplification methods also increases the risk of false positive test results due to presence of contaminating DNA in the reaction, where even a single molecule of DNA can serve as a template for amplification in a later reaction. Contamination of samples is a major consideration for laboratories performing amplification reactions, and procedures must be in place to prevent, monitor and control contamination. The molecular diagnostics laboratory must ensure that methods are in place to prevent, detect, monitor and correct for contamination events.

Sources of contaminating DNA include amplicon DNA, other specimens, and positive control materials. Contaminating amplicon DNA originates from DNA amplified during a previous amplification reaction. If reaction tubes are opened after amplification, (for example for gel analysis, microarray testing, sequencing etc.) amplified DNA can be aerosolized, contaminating the environment, and it can be carried directly into reagents and consumables. This contaminating DNA can then be carried directly into new reactions the next time assays are set up, causing false positive results. Nucleic acids from specimens themselves may also contaminate the environment during specimen handling, for example, when pipetting specimens into tubes for nucleic acid extraction procedures. Cross-contamination of samples containing the analyte prior to or during sample processing can lead to false positive results. When multiple samples are processed in batches, there is potential for aerosolization, splashing, etc. Finally, incorrect handling of positive controls (for example, plasmid DNA) may also result in contamination of patient samples, leading to false positive results.

Laboratory practices to prevent contamination in the molecular diagnostics laboratory include both physical design and layout of the laboratory, workflow practices, and policies and procedures designed to prevent contamination. Many good references are available for designing and implementing contamination control programs in the molecular diagnostic laboratory [57].

QUALITY MANAGEMENT

A comprehensive program for quality management specifically designed for the molecular diagnostics laboratory should be in place to ensure the accuracy of testing, accurate performance of test, tracking of trends, and rapid identification and correction of problems. Establishment of a quality management program is the principle method by which common interferences in the molecular diagnostic laboratory can be controlled, monitored, identified and corrected. The program should be comprehensive, including methods for validation of assays, ongoing quality control, and monitoring for problems. Assay validation is especially important, as the performance characteristics of the assay will be verified through this process [58] and any potential problems with the assay should be identified prior to clinical use. Ongoing quality management should include a strong program of quality control to identify problems in analytical phases of testing, and monitoring results, and should include participation in proficiency testing.

Quality control

Quality control (QC) is the portion of the quality management program devoted to detecting problems at the testing level. In molecular laboratories, quality control must include a method to ensure correct performance of nucleic acid isolation steps, amplification and probe controls, and detection steps. QC must also be included to monitor for inhibition if amplification assays are used,

and to check for contamination. Monitoring assay positivity rates can be a useful method for determining if a problem is occurring with an assay. An unusual increase in the number of positive tests may indicate contamination has occurred.

Quality control for qualitative assays

For qualitative assays, external control material should include both positive and negative controls. For qualitative assays with a single molecular target positive and negative controls should be included in each run of the assay. Positive and negative controls ensure that probes and primers are working correctly, and allow monitoring for contamination events. External controls should monitor the entire process (nucleic acid extraction and purification, amplification and detection). For more complex molecular testing (e.g., multiplexed PCR, microarrays, NGS) selection of quality control materials can be a challenge. External control materials may not be readily available for all of the markers being tested for and reported. Performing an external control for every analyte in the assay is also not practical. An option for quality control materials for these assays is to rotate different materials with each run, covering all the targets over a defined number of runs or kits. Statistical strategies and software for dealing with multidimensional data QC are being developed [59,60].

Quality control for quantitative assays

Quantitative assays should include positive controls at several levels in the reportable range of the assay, for example, a low positive, mid-range positive, and a high positive control. Results of the positive control samples should be monitored using statistical software to look for trends and to ensure that problems are detected.

CONCLUSIONS

The growth of the role of molecular diagnostics in clinical medicine and advances in technology have expanded opportunities for clinical laboratories to bring up new assays to support patient care and management. Each new assay brings potential sources of error, both new and old. Pre-analytical considerations in specimen collection, transport, storage and nucleic acid extraction are a constant and universal source of concern, while other sources of error, such as errors arising from processing data from NGS studies through bioinformatics pipelines, are more recent and specific to the technology involved. Awareness of these potential sources of error, combined with a robust quality control and quality assurance program, are essential to managing errors and reducing their occurrence.

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CHAPTER

22

Molecular testing for targeted therapies and pharmacogenomics

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INTRODUCTION

Molecular testing has applications in treatment of disease, as well as in diagnosis. Companion or complementary diagnostics link a deleterious variant to a specific therapy (targeted therapy). The term "Companion diagnostics" describes a laboratory test that is included on the drug's approved label and identifies individuals who will likely benefit from the specific drug. The term "complementary diagnostics" is used more broadly, for tests that can inform treatment decisions and may be directed to a class of drugs, and not implicitly stated on the drug label. Targeted therapies often refer to oncology settings and apply to drugs developed for cancer therapies targeting molecular mechanisms of tumor biology; however, variants within oncological pathways can render these therapies ineffective.

Pharmacogenetics testing for targeted therapies is evolving to include inherited conditions, to confirm that the molecular basis for disease is such that the therapy is likely to be effective. Pharmacogenetics often refers to germline settings, detecting variants in proteins such as enzymes or receptors that will predict efficacy or adverse events associated with different drugs. Pharmacogenetic targets are often enzymes involved in drug metabolism; inherited variants in genes coding for these proteins can alter their function. Inherited variants also play a role in treatments for infectious diseases. On the other hand, pharmacogenetics testing in oncology usually involves analysis of somatic variants of actionable genes involved in targeted therapies. We will use these terms as described above throughout this chapter. Targeted therapies and pharmacogenetics are keys for personalized medicine, the goal of which is to ensure that the right drug is given to the right patient at the right dose at the right time.

Molecular technologies are at the forefront of targeted therapies and pharmacogenetic testing. From detecting single known deleterious variants to multi-variant panels and sequencing, to quantitative PCR, molecular techniques can detect and quantify relevant targets. This chapter will provide brief descriptions of commonly used methods, including detection by next generation sequencing (NGS) strategies. Current applications of pharmacogenetic tests will be described, along with the challenges of detecting the genetic alterations and accurately interpreting the results. Inherited and somatic variations are addressed.

METHOD DESCRIPTION

There are various techniques applied in pharmacogenomics testing ranging from a single variant, multi-target assays and sequencing based technologies. The following is a brief description of each of these techniques.

Targeted single variant detection

Multiple methods are available for detection of single nucleotide base changes. A traditional method, still in use today, is the use of restriction enzymes that cut DNA at specific palindromic sequences. Variants may create or destroy a restriction site. In this method, PCR amplifies a specific region of interest, and products are treated with the restriction enzyme.

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The resulting differences in the PCR product sizes are resolved by electrophoresis.

Allele specific PCR is a variant detection technique based on the principle that DNA extension is efficient when the 3′ nucleotide of the primer matches the template, but extension is poor or non-existent when the terminal base is mismatched. Allele specific PCR is also known as amplification refractory mutation system (ARMS) or allele specific primer extension (ASPE). It is generally coupled to other technologies such as real-time (or quantitative) PCR (qPCR) or arrays. Incorporating real-time PCR into the allele specific analysis allows quick identification of contamination and decreases time to result. Importantly, real-time PCR reduces the number of false positives due to background or non-specific primer binding. A threshold difference is allowed between the quantity of housekeeping gene amplified and the quantity of the selected allele. Thus, by monitoring the amplification, a clear population of mutant alleles can be distinguished from wild-type or background. Allelic ratios of the fluorescence emitted by the wild-type and mutant reactions can determine homozygosity (of either the wild-type or the mutant) or heterozygosity.

Probe chemistries have been used with many variations for targeted variant detection. Two methods commonly employed in clinical laboratories are hybridization probes (LightCycler technology) and hydrolysis probes (TaqMan technology). In LightCycler technology, probes are designed to be a perfect match to either the variant or the wild-type sequence. They are typically 15–20 bases long with the variant located toward the center of the probe. Probes have been described with Förster (or fluorescence) resonance energy transfer (FRET) as the detection system [1,2]. In this approach, two probes, one over the variant of interest (the reporter probe) and one adjacent to the first (the anchor probe) are each fluorescently labeled, one with an acceptor fluorophore, and the other with a donor fluorophore. When the two probes are hybridized (at lower temperatures), the energy of the donor is transferred non-radiatively to the acceptor. The acceptor then emits at a longer wavelength, which is detected by the instrument. Single labeled probes as well as unlabeled probes have also been described [3].

To detect variants using LightCycler technology, PCR is performed, followed by a "melt" analysis. In a melt analysis, the temperature is slowly increased, with continual monitoring of fluorescence. As the temperature rises, the probes disassociate from their template, or melt, at a characteristic temperature, which is seen as a loss of fluorescence. A mismatch between the probe and the DNA amplicon will lower the temperature at which the probe melts from the DNA amplicon. A derivative of the melting curve easily visualizes melting "peaks", resolving the perfectly matched allele and the mismatched allele. Other variants within the region of the probes may be detected by a shift in the expected melt temperature (T_M). However, if T_Ms between the targeted variant and another variant are similar, the result may be a false positive for the targeted variant [4].

In hydrolysis (TaqMan) chemistry, probes are dually labeled with a fluorophore and quencher molecule. When the probe is hybridized to the product, the fluorescence is quenched. As PCR primers are extended, replicating the DNA template, the polymerase displaces and hydrolyzes the probe. As the probe is hydrolyzed, the fluorophore is released from the effect of the quencher, resulting in a fluorescent signal. In TaqMan systems, the wild-type and mutant allele are amplified separately by allele-specific PCR as described above. The ratio of the fluorescent signal of each amplification is used to determine zygosity. Other variants within the region of the probe may not be detected, but may interfere with amplification of one allele, resulting in a false negative or false positive.

Multi-variant panels

When testing for more than three or four variants simultaneously, methods capable of highly multiplexed reactions are used. Several commercial platforms are available for pharmacogenetic tests, namely, Thermo Fisher Science's OpenArray system, Autogenomics' INFINITY, and Luminex's xTag. Each of these platforms will be briefly discussed.

The TaqMan OpenArray Pharmacogenomics (PGx) panel (Thermo Fisher Scientific, Waltham, MA) is based on TaqMan chemistry, but uses a microfluidics system to assay multiple targets simultaneously. This real-time PCR combines amplification and detection. The predesigned panel assays 158 variants in known drug metabolizing enzymes and covers such genes as *CYP2D6*, *CYP2C19*, *NAT1*, *NAT 2*, and *TPMT*. Fluorescent ratios of normal (wild-type) and variant alleles are examined and cluster as Wild Type, Heterozygous variant or Homozygous variants. Available assays include variants in multiple genes involved with drug-metabolizing enzymes (http://tools.thermofisher.com/content/sfs/brochures/TaqMan%20OpenArray%20PGx%20Panel_Product%20Overview.PDF). Most targets are available as individual assays and can be combined as a custom array. Fig. 22.1 demonstrates results of a single sample tested for a multi-variant, multi-gene drug metabolizing panel.

xTag (Luminex Corporation, Austin, TX) is a bead array with oligonucleotides bound to microspheres. It begins with PCR, followed by ASPE. Biotin-dCTP is included in the subsequent amplification reaction and

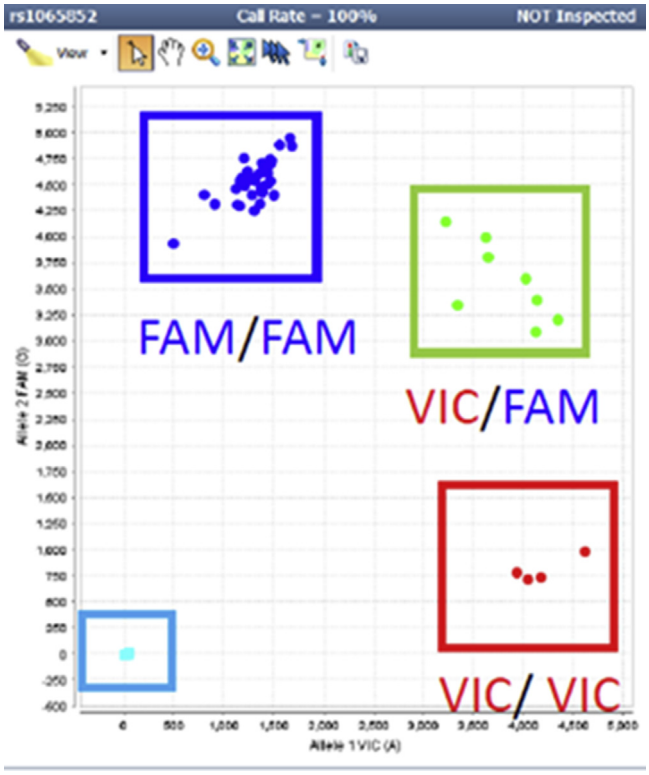


FIG. 22.1 Example results of TaqMan results for an ADME panel. Data from 120 variants (tested as separate microfluidic assays) in 39 genes are shown for one individual. For individual assays, reference and variant specific probes were labeled either with VIC or FAM fluorophores. In this example, this individual is heterozygous for eight variants in the tested genes. Homozygous variant calls are made from either VIC/VIC or FAM/FAM depending on the fluorophore labeling of the specific assay.

will only be incorporated into the product if the 3′ end of the primer perfectly matches the allele. The 5′ end of the primer has a tag sequence that hybridizes to the microspheres. The microspheres are labeled with fluorophores and tagged with a universal anti-tag sequence complementary to the allele-specific primer tags. The bead-ASPE hybridization products are incubated with an R-phycoerythrin-streptavidin conjugate, and fluorescence intensities are measured. Again, the allelic ratio between the wild-type and variant provides the genotype [5]. FDA approved products include assays for *CYP2D6* and *CYP2C19*.

The INFINITI (AutoGenomics, Inc., Carlsbad, CA) platform is a film microarray. Similar to the other platforms, DNA is first PCR-amplified for the specific regions of interest and the products are cleaned with exonuclease I and shrimp alkaline phosphatase (EXO/SAP). Allele specific primer extension follows, and the fluorescently-labeled products are hybridized to the film array. Labeled products bind specifically to their complementary microarray locations. Fluorescence levels are detected by a microscope and are converted into genotype data using allelic ratios. The automated instrument has FDA cleared products for Warfarin (*CYP2C9* and *VKORC1*) and *CYP2C19*, and RUO products for other genes/variants (http://www.autogenomics.com/?q=node/83).

Sequencing

Unlike variant-specific analyses, DNA sequencing allows for the detection of any variations within the amplified region of the gene. Classically, dideoxy (Sanger) sequencing has been the most popular sequencing method. Pyrosequencing has also been adopted for use in the clinical lab, particularly for oncology applications that require detection of low allelic fraction (low level somatic variation).

Sanger sequencing relies on the formation of the phosphodiester bond during DNA extension. Through DNA replication, the terminal 3′hydroxyl group of the DNA polymer reacts with the alpha phosphate group on the incoming nucleotide. Pyrophosphate is released, and a covalent phosphodiester bond is formed. If the 3′hydroxyl group is absent, the adjacent complementary nucleotide can bind to the polymerase, but a phosphodiester bond cannot be formed. Dideoxy nucleotide triphosphates (ddNTPs) do not contain a 2′ or 3′ hydroxyl group on the ribose ring of the nucleotide. Thus, these modified bases can be incorporated into the DNA polymer, but will terminate further polymerization.

Amplified DNA is used as a template for a unidirectional reaction; a single primer is added to each sequencing reaction. Fluorescently labeled ddNTPs (ddTTP, ddATP, ddGTP, and ddCTP) are each conjugated to a different fluorophore and therefore, each nucleotide provides a unique signal. At every base extended during the sequencing reaction, a minority of the polymers will incorporate the complementary ddNTP rather than the more abundant dNTP in the reaction. Incorporation of ddNTP will terminate further polymerization leading to an assembly of products that can be ordered from shortest to longest.

The products of the sequencing reaction are separated by capillary electrophoresis wherein smaller DNA molecules migrate faster than the larger molecules. A fluorescence detector is utilized to decipher the terminal nucleotide of the fragment. Single nucleotide resolution is achieved and a chromatogram is generated that assigns each nucleotide a specific color, facilitating interpretation of the sequencing result.

Sanger sequencing has traditionally been considered the current gold standard for variant detection. Sanger sequencing will recognize both rare and common variants, as well as small insertions, deletions, and duplications, although large deletions and duplications will not be detected. The technique is amenable to

high throughput production, and data analysis and interpretation are relatively straight forward. However, Sanger sequencing tends to be more time consuming when compared to variant specific detection. In addition, for applications where the population of target DNA is in the minority, such as during tumor characterization, the lack of analytical sensitivity can result in false negative typing.

Pyrosequencing, or real time DNA sequencing uses detection of pyrophosphate release [6]. Like Sanger sequencing, it exploits the natural mechanism of DNA polymerization to infer the DNA sequence. However, in contrast to Sanger sequencing, detection is accomplished as each nucleotide is incorporated, rather than by separation of a final set of DNA products. When the DNA phosphodiester bond is formed, pyrophosphate (PPi) is released. Pyrosequencing utilizes this PPi as a substrate for a series of enzymatic reactions that ultimately produce light.

As with Sanger sequencing, targeted pyrosequencing is preceded by PCR. The amplification reaction includes a biotinylated primer, which facilitates subsequent isolation of the amplicon strand to be sequenced. Pyrosequencing proceeds by sequential addition of each nucleotide to a sequencing primer. If the nucleotide added is complementary to the next base in the DNA sequence, it is incorporated into the polymer and PPi is released. Sulfurylase catalyzes the formation of ATP from PPi and adenosine phosphosulfate. Luciferase then catalyzes the reaction between ATP, O_2, and luciferin, ultimately releasing light. The light is collected by a CCD camera and recorded as peaks into a graph known as a pyrogram (Fig. 22.2). The amount of light generated is directly proportional to the number of the specific nucleotide incorporated. For example, if two GTPs are incorporated in tandem, they will produce twice as much light compared to the incorporation of a single GTP. Pyrosequencing can be applied to a range of pharmacogenomics applications, including detection of SNPs, copy number, triallelic polymorphisms, tandem repeats, and insertions/deletions [7]. Like dideoxy sequencing, it can detect all variants within a targeted region, but sequencing lengths are much shorter, typically less than 100 bases and often limited to only a few codons. Novel variants outside the targeted region will not be detected with this methodology. However, the analytical sensitivity of pyrosequencing, typically ~5%, outperforms that of dideoxy sequencing, typically ~25% [8]. This is particularly useful for oncology applications, where heterogenetic cell mixtures are common.

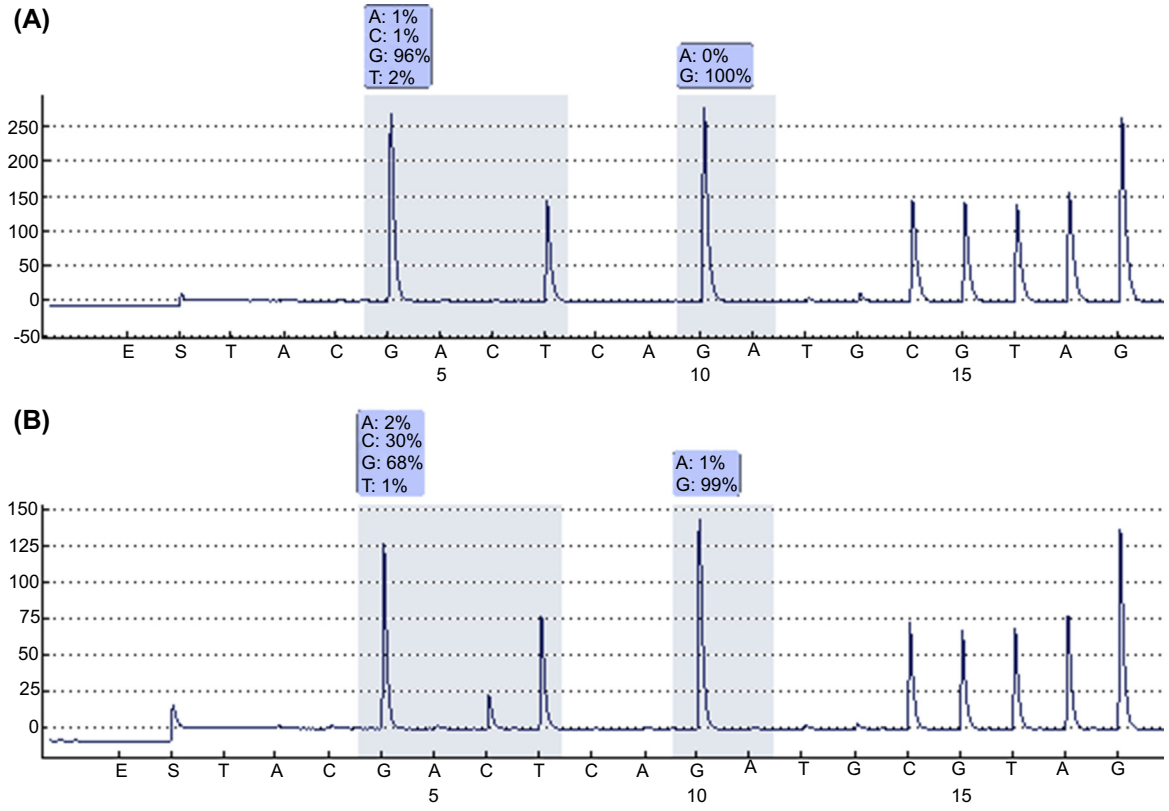


FIG. 22.2 Pyrosequencing results for *KRAS* codons 12 and 13 in (A) a tumor wild-type for codons 12 and 13 (sequence: GGT GGC), (B) a tumor harboring a c.35G>C, p.Gly12Ala variant, as evidenced by a reduced "G" peak at dispensation 4 and a novel "C" peak at dispensation 6.

Since all tumor tissue will have some portion of normal cells that likely do not harbor the somatic variant, increased analytical sensitivity can be essential. The added analytical sensitivity provided by pyrosequencing is the fundamental reason that it is utilized in the clinical lab. Additionally, targeted pyrosequencing is cost effective and less time intensive, as compared to other sequencing methodologies. Pyrosequencing is easily automated, accurate, and depletes the need for labeled nucleotides and a separation matrix.

Next generation sequencing (NGS) refers to large-scale DNA sequencing technology that allows for querying the entire genome (whole genome), the exons within all known genes (whole exome), or only exons of selected genes (target panel). Briefly, fragmented genomic DNA is read simultaneously by synthesizing a string of complementary fluorescently-tagged nucleotide bases. Base-calling algorithms use fluorescent images to infer the short DNA sequence reads that are then computationally assembled and aligned to the reference genome. Among other factors, the number of reads generated (depth of coverage) for a particular region will affect the ability to detect a genomic variant. NGS technology has been successfully utilized in the clinical setting to identify functional genetic variation associated with human disease. NGS applications for targeted somatic variants has been embraced by molecular oncology laboratories because multiple targets can be detected in one assay, preserving small samples and reducing time to result compared to sequential testing [9]. In addition, testing to a high depth of read allows detection of low percentages of somatic variants in the sample. Recently, NGS has been explored as a tool to discover common and rare variants in pharmacokinetic (absorption, distribution, metabolism and excretion [ADME]) and pharmacodynamic (drug target) genes. Several limitations exist specifically in regards to the use of NGS for ADME genotyping. These include the high homology between pharmacogenomics gene families like the cytochromes (CYP), SULT and UGT enzymes (www.pharmvar.org). Additionally, ADME genes are highly polymorphic with complex structural variations (*i.e.* the human leukocyte antigen genes; HLA) (www.pharmgkb.org). Haplotypes (a group of alleles inherited together), also known as star alleles (*e.g.* CYP2D6*1), are characterized by single nucleotide variants (SNVs), insertion-deletion variants (indels), structural variants (SVs) or a combination of these [10]. Although bioinformatics tools for accurate haplotype genotyping from NGS output data have been developed, none proved ready for broad clinical utilization [11–14]. Currently, from the NGS options available, panel-based NGS methodology for pharmacogenomics testing seems the best option and should be implemented with caution.

APPLICATIONS OF MOLECULAR TESTING

Applications may involve the detection of somatic variants acquired through mutagenesis or germline variants inherited parentally. Somatic variants are important in oncology whereas germline variants are often relevant for drug metabolism. Applications for each with the challenges in testing are described below. Table 22.1 summarizes the applications, the commonly tested variants and common platforms.

Targeted therapies for tumor/somatic variant detection

One of the hallmarks of cancer cells is the insubordinate activity of signaling pathways [15]. In almost all malignant tumors, either a tumor suppressor will be inactivated and/or an oncogene will be upregulated. The mechanism by which these somatic variants allow tumor cells to activate pro-growth signaling can affect the tumor's response to therapeutic agents. Examples of such a relationship are *KRAS* variants in EGFR-directed therapy for colorectal cancer, *BCR/ABL* translocations for the effectiveness of tyrosine kinase inhibitors against chronic myelogenous leukemia (CML), and *KIT* variants in gastrointestinal stromal tumors treated with imatinib.

The benefits for recognizing these mechanism-specific variants are: first, it benefits the patient by administering certain chemotherapeutic agents, which often have deleterious side effects, only when there is a greater possibility of the drug being effective, and second, it provides the pharmaceutical companies a foundation to select patients for clinical trials and also for targeting future drug discovery projects.

KRAS

Epidermal growth factor receptor (EGFR) is a transmembrane tyrosine kinase receptor that functions to activate signaling pathways involved in survival and proliferation. Activation of EGFR occurs when a ligand binds to the extracellular domain, triggering dimerization and consequential autophosphorylation of the cytoplasmic domain. EGFR activation stimulates both the mitogen activated protein kinase (MAPK) and the phosphoinositol-3 kinase (PI3K) pathways, ultimately triggering the expression of genes involved in growth and metastasis. Multiple metastatic cancers, namely colorectal, lung, and pancreatic cancers, are known to have aberrant expression of proteins involved in the EGFR pathway, namely due to gene duplications and point variants. The EGFR serves as the gatekeeper for

TABLE 22.1 Genes important in molecular testing for targeted therapies and pharmacogenomics.

Gene	Indication	Commonly used methods	Commonly tested variants	Comments
KRAS	Response to EGFR therapies	Targeted sequencing; pyrosequencing; allele-specific PCR	Exons 12, 13, 61	
bcr/abl	Response to imatinib	qRT-PCR; sequencing	Quantification of bcr/abl translocation, variants in the kinase domain	Quantitative PCR used for residual disease monitoring; sequencing used to predict resistance to therapy
KIT	Imatinib or tyrosine kinase inhibitors	Single variant detection; sequencing	D816V, Targeted exons 8, 9, 11, 13, 17,	Exons tested depend on cancer type
*HLA-B*57:01*	Abacavir sensitivity	Single variant detection	Presence or absence of HLA-B*5701 or HCP5 variant	
*HLA-B*15:02*	Carbamazemine sensitivity	Single variant detection	Presence or absence of HLA-B*15:02	
CYP2D6	Multiple drugs	Targeted variants	*2, *3, *4, *5, *6, *7, *8, *9, *10, *11, *12, 14, *15, *17, *29, *35, *41	Many rare variants will not be detected
CYP2C19	Clopidogrel sensitivity	Targeted variants	*2,*3,*17	
CYP2C9/VKCOR1	Warfarin sensitivity	Targeted variants	CYP2C9 *2, *3 VKCOR1 c-1639G>A	For sensitivity testing only; will not predict warfarin resistance
CFTR	Response to Ivacaftor; lumacaftor/ivacaftor	Targeted variants or sequencing	Growing list of variants with targeted therapies	
SMN1	Response to nusinersen	qPCR or other quantitative method	Deletion of *SMN1*	Testing for copy number is first tier test. Rarely SMA is caused by sequence variants

the MAPK and PI3K pathway activation, and therefore some cancers will harbor variants specific to the EGFR and not to downstream pathway components. Since the ligand binding site of the receptor is located on the extracellular membrane, the EGFR receptor has been an attractive therapeutic target using monoclonal antibodies. In particular, cetuximab and panitumumab, which function pharmaceutically to block the receptor, are frequently used in the treatment of colorectal cancer patients. However, only a subset (8–23%) of patients with metastatic colorectal cancer responds to these drugs. The primary factor governing the response or resistance to EGFR specific drugs is variants in proteins downstream of EGFR activation that results in constitutive activity, keeping the pathway turned on without initial signaling through the EGFR receptor [16]. Variants in *KRAS* that lead to such constitutive activity have shown the highest correlation to anti-EGFR drug resistance. *KRAS* genotype analysis of tumor tissue can therefore help predict if a patient will respond to EGFR inhibitors. More specifically, while a wild-type *KRAS* suggests the patient may respond, a variant in *KRAS* indicates drug resistance. It is important to note that the response rate to anti-EGFR therapy in patients with wild-type *KRAS* is <50%, suggesting that there are other factors involved.

KRAS variants that are known to convert the gene into an active oncogene are localized to hotspots in codons 12, 13, and 61. Multiple molecular assays have been developed to detect variants in these regions [8]. The most commonly utilized methods are Sanger sequencing, pyrosequencing, and allele specific PCR. Increasingly, *KRAS* is being included in NGS panels for somatic cell profiling [9,17]. Less commonly utilized are melt-curve analysis, StripAssay, and multiplex assays. These techniques differ in their diagnostic and analytical sensitivity.

For detection of somatic variants, interpretation of Sanger sequencing becomes more difficult, and often the analytical sensitivity is inadequate. This is a function of the starting population of cells, which is a mixture of tumor cells and non-tumor or normal cells. Often the signal to noise ratio is not high enough to distinguish if a variant is, in fact, present. The analytical sensitivity

of Sanger sequencing is ~20%, which can lead to false negative interpretations in heterogeneous cell populations. Thus, Sanger sequencing is less attractive for utilization in detection of somatic variants.

To increase analytical sensitivity in detecting somatic variants, other methods are often employed. For example, pyrosequencing has a sensitivity of ~5%. Monitoring the incorporation of each sequential nucleotide heightens the ability to detect small amounts of DNA polymerization. Consequently, the lower limit of analytical sensitivity is enhanced. In addition, this allows an estimate of the percent of cells in the population analyzed that harbor the detected variant. Multiple studies have shown that even in tissues with low tumor cell content, pyrosequencing provides sufficient analytical sensitivity and specificity to assess *KRAS* variant status in metastatic colorectal cancer tissue. In contrast to Sanger sequencing, most *KRAS* pyrosequencing assays will only analyze the codons of interest plus a few flanking nucleotides. However, while previously undescribed variants may not be detected, it is unclear if *KRAS* variants outside of codons 12, 13, and 61 bear any clinical significance. An example of a *KRAS* pyrogram is shown in Fig. 22.2.

Like pyrosequencing, allele specific PCR is highly sensitive and can detect as few as 10 copies of mutant alleles in proportions as low as 1% of the total DNA. Therefore, when compared with Sanger sequencing, the lower limit of detection is at least 10-fold more sensitive. In addition to an increased analytical sensitivity, allele specific PCR has the advantage of eliminating the post PCR processes required by sequencing, eliminating a major contamination source and reducing turn-around time. The primary disadvantage to allele specific PCR is that variants outside the targeted nucleotide will not be detected. The allele specific PCR methods to detect KRAS variants have focused solely on the seven most common variants in codons 12 and 13, which account for at least 90% of all KRAS variants. Approximately 10% of variants may be overlooked, particularly variants in codon 61, which can represent up to 8% of *KRAS* variants. Although the clinical implications of variants in codon 61 are not fully understood, at least one study has associated such variants with anti-EGFR therapy resistance. In general, variant detection between allele specific PCR and Sanger sequencing are highly concordant if the tumor cell percentage in the sample is sufficient. As such, the method of choice is typically a function of the proportion of tumor cells in the sample.

The typical allele specific PCR assay utilizes conventional probes such as TaqMan or molecular beacon to detect amplification. Scorpion primers are designer oligonucleotides that function as both the primer and the probe [18]. When utilized for *KRAS* detection, Scorpion probes provide an additional 10-fold decrease in the lower limit of detection (~0.1% or one target allele) when compared to conventional probes. While the sensitivity exhibited by Scorpion primers may initially seem attractive, the clinical significance of such a low limit of detection remains controversial. Further, the Scorpion assay is only available through a single commercial vendor and the cost is significantly higher compared to the reagents required for conventional allele specific RT PCR or DNA sequencing.

BCR/ABL

Chronic myelogenous leukemia (CML) is a myeloproliferative disorder that results in the proliferation of mature granulocytes and their precursors. The signature characteristic of CML is a unique chromosomal abnormality resulting from a reciprocal translocation between the long arms of chromosome 9 and 22, known as the Philadelphia (Ph1) chromosome [19]. The Ph1 chromosome is present in virtually 100% of CML cases, as it is the causative molecular abnormality of CML. The Ph1 chromosome fuses the 5′ end of the breakpoint cluster region (*BCR*) on chromosome 22 with the *ABL1* kinase protooncogene on chromosome 9, resulting in the loss of the native 5′ end of *ABL1*, which regulates kinase activity. The gene fusion results in an actively transcribed and translated protein called BCR-ABL1. BCR-ABL1 retains the kinase activity of ABL1, using phosphorylation events to trigger responses in multiple anti-apoptotic and pro-growth signaling pathways. However, the fusion protein loses its ability to be regulated, allowing for constitutive activity of ABL1, which ultimately leads to the pathogenicity.

Detection of the Ph1 chromosome is essential not only for diagnosis of CML, but because targeted therapy with Imatinib, a tyrosine kinase inhibitor with specificity toward BCR-ABL1, has proven to be a highly effective first line therapy. The methods utilized to detect the Ph1 chromosome are: conventional cytogenetics, fluorescence in situ hybridization (FISH), and reverse transcriptase (RT)-PCR. Cytogenetics uses a karyotype to visually scrutinize chromosomes and detect chromosomal abnormalities. Although cytogenetics is the gold standard for Ph1 detection, it requires a bone marrow biopsy and the manual evaluation of metaphase cells, making it a cumbersome and time-consuming technique with a sensitivity of ~1:20 cells. FISH, a probe-based detection method, has a slightly higher sensitivity, detecting from 1:100−1:500 Ph1 positive bone marrow cells, but is prone to false positive results. In contrast, quantitative (q)RT-PCR, which is based on extracting mRNA from whole blood and detecting the *BCR-ABL1* transcript, is relatively efficient, sensitive and specific.

However, qRT-PCR will not provide any additional information about bone marrow morphology or other chromosomal abnormalities.

The primary goals of CML treatment are to eliminate the Ph1 chromosome and *BCR-ABL1* gene expression. If treatment with Imatinib is successful, both will be achieved, but will still need to be monitored periodically. If treatment with Imatinib is not successful, or if relapse is detected, therapy must be altered. Conventional cytogenetics is not sensitive enough to be used in disease monitoring. qRT-PCR extends the limit of detection for cells expressing the Ph1 chromosome down to 1:100,000−1:1,000,000, and thus is 4−5 orders of magnitude more sensitive compared to cytogenetics [13]. In addition, qRT-PCR eliminates the need for the continued collection of bone marrow.

Using qRT-PCR rather than RT-PCR allows for a quantitative measurement to detect response and progression. Studies have shown that the concentration of *BCR-ABL1* transcript predicts the most important clinical outcomes: disease progression, drug resistance, and survival [20,21]. qRT-PCR is a powerful and efficient method for monitoring CML, but it is accompanied by few cautionary notes. Importantly, RNA is an inherently difficult macromolecule to extract and analyze as it is degraded by RNase, which is an exceptionally stable and abundant enzyme. In addition, RNA yields can vary from person to person. The latter is overcome by standardizing the amounts of BCR-ABL1 transcripts in a sample to the amount of an endogenous expressed housekeeping gene. This is necessary for accurate *BCR-ABL1* quantification, but can also be an additional source of bias in an assay. Depending on which housekeeping gene is measured, the relative expression, and hence quantitative assessment, of *BCR-ABL1* can vary.

A critical component to all tumor marker assays is precision. The assays must exhibit minimal fluctuations in order for consecutive results to translate into therapeutic meaning. The current limit of analytical precision for most *BCR-ABL1*qRT-PCR assays is ~0.5 logs. Therefore, if a patient has a rise in *BCR-ABL1* transcript that is less than or equal to 0.5 logs, it may simply be a result of analytical imprecision. Studies have shown that remaining within 0.5 log fluctuations of *BCR-ABL1* is a good prognostic indicator, and conversely a poor prognostic indicator if the transcript increases by more than 0.5 logs [22]. However, improving the precision of the assays may allow for a more fine-tuned assessment of patient status.

A universal problem with tumor markers is the lack of standardization and the large inter-laboratory variation observed. Currently, this also holds true for the *BCR-ABL1* transcript, where up to 8-fold differences in concentration are observed between laboratories evaluating identical specimens [23]. The variability is a result of procedural and/or vendor differences in RNA extraction, instrumentation, reagents, and housekeeping genes. Extensive efforts are being brought forth to create an international scale (IS) for *BCR-ABL1*, to harmonize quantitative BCR-ABL1 measurements across tests and laboratories. This will enhance patient care by facilitating inter-laboratory studies and allowing for patient portability [24].

A rise in *BCR-ABL1* transcript greater than 0.5 logs suggests that treatment is unsuccessful. Multiple variants in the kinase domain of BCR-ABL1 have been shown to confer drug resistance to Imatinib [20]. Identifying the specific variants present can guide therapy because certain variants are known to respond to alternative tyrosine kinase inhibitors, while other variants are known to be resistant to all currently available kinase inhibitors. Samples suspected of harboring such variants are subjected to nested PCR with the first round of PCR amplifying across the translocation, followed by a second round that amplifies the ABL1 region of interest from the fusion transcript. Amplicons are sequenced to identify variants.

KIT

KIT is a tyrosine kinase receptor involved in the development of melanocytes, interstitial cells of Cajal, hemopoietic progenitor cells, mast cells, and primordial germ cells [25]. Like all tyrosine kinase receptors, KIT is activated via ligand specific binding, with subsequent receptor dimerization, autophosphorylation, and downstream signaling leading to gene activation/repression and a physiological response. Although KIT follows the general convention of receptor signaling, KIT is relatively complex when implicated in cancer and scrutinized for its pharmacogenomics associations. This complexity is multifaceted and a result of KIT association with multiple tumor types, variant specific responses, and the appearance of secondary resistance. Further, there is an alternative tyrosine kinase receptor, PDGFRα, which is implicated in some of the same cancers.

As KIT has several roles in physiological homeostasis, it is not surprising that KIT variants are associated with multiple tumor types [25]. KIT variants are at the pathological root of many gastrointestinal stromal tumors (GISTs), melanomas, seminomas, systemic mastocytosis, and acute myeloid leukemia (AML) cases. The primary goal of detecting specific KIT alterations in these cancers is to provide prognostic and/or potential therapeutic information. In general, cancers with wild-type *KIT* alleles will not be responsive to Imatinib or other tyrosine kinase inhibitors. However, the presence of a KIT variant does not necessarily confer responsiveness. Multiple

KIT variants observed both within and between the cancer types further enhance the complexity. Pathogenic *KIT* alleles have been shown to encompass the whole spectrum of genetic variation; deletions, point variants, internal tandem duplications, insertions, and complex variants have all been reported. The type of cancer to be tested, and thus the expected variant(s), must be considered when designing the assay. In GISTs and melanomas, exon 11 is the most commonplace for variants, followed by exons 9, 13, and 17. The exon that harbors the variant influences drug dosing and prognosis. In GISTs with wild-type *KIT*, one may also want to evaluate the *PDGFRA* genotype [26]. D842V is the most common GIST-associated PDGFRα variant and is analogous to the D816V KIT variant described below. Cancers harboring this PDGFRα variant are completely resistant to all of the currently available tyrosine kinase inhibitors. In seminomas and systemic mastocytosis, identifying the KIT point variant D816V is critical [27]. D816V allows for constitutive kinase activity leading to cell proliferation and inhibition of apoptosis and infers resistance to tyrosine kinase inhibitors. For AML patients, variants in KIT confer poor prognosis [28]. These variants occur in exons 8 and 17. Most exon 8 variants are insertions/deletions, while the exon 17 variants are a mixture of point variants and insertions.

Secondary variants have been best defined in GISTs, where resistance to kinase inhibitors is observed in >85% of patients that were initially responsive to the drugs [26]. This resistance often develops in the original lesion, suggesting a clonal selection. More specifically, such variants were present in a subset of cells or a single clone within the tumor when treatment with the kinase inhibitor was initiated. By selectively killing the tumor cells that harbor only the primary variant, the minor clones are given the opportunity to expand. Screening for such minor clones before the initiation of therapy may allow the physician to predict the best front- and second-line therapies by understanding to what drugs the tumor will eventually become resistant.

Techniques utilized for detection of KIT variants will vary depending on the type of cancer, and if primary or secondary variants are implicated. One confounding issue in KIT testing will be for the laboratories to design testing panels that facilitate appropriate physician ordering and bolster subsequent result interpretation. Tiered testing might be an excellent cost and time efficient method to define the KIT status in a tumor population. In these cancer-specific cases, the most common variant would be evaluated first. If that variant is absent, continue to assess the alleles for the second most common variant. Alternatively, if that variant is present, the need for further testing is obsolete (unless secondary resistance is suspected). These algorithms should also direct testing to PDGFRα when implicated.

Host factors influencing response to infectious disease

Inherited factors can affect how humans respond to treatment of infectious diseases. Several loci in the Human Leukocyte Antigen (HLA) loci have been identified through genome wide association study, and have been replicated with additional studies. Because these are within human's immune response genes, a response associate with these loci is hypersensitivity.

HLA 5701

The HLA-B locus *5701 has been associated with abacavir sensitivity in treating HIV infections [29]. Symptoms related to abacavir sensitivity include fever, rash, fatigue, respiratory and gastrointestinal problems. Testing for the locus HLA-B*5701 will identify those who are HLA-B*5701 positive and likely to develop a severe reaction to abacavir, and in whom abacavir treatment should be avoided [30]. The HLA-B*5701 sequence is nearly identical to other HLA types, such as HLA-B*5702 or HLA-B*5706. High resolution HLA typing or HLA sequencing can be performed to definitively genotype this region. However, these tests are expensive and labor intensive. To find a solution that is simpler and less expensive, carefully designed primers to amplify only the HLA*5701 allele can be used to detect the presence or absence of the allele. This type of a test may not be able to distinguish between one copy (heterozygous) or two copies (homozygous). Care should be taken to not mistakenly call a closely related allele, since there also may be slight, although inefficient, amplification of related HLA alleles. The HLA-B*5701 allele is in linkage disequilibrium with the single-nucleotide variant rs2395029 in the *HCP5* gene [31]; therefore this can be used as a surrogate, and tested by a variety of methods suitable for single variant detection. The *HCP5* variant has close to 100% negative and positive predictive value when used to screen for abacavir sensitivity. The exception is a rare allele that is positive for the variant, but negative for HLA-B*5701. This result leads to a false positive, therefor avoiding treatment that may be safe. In additions, deletions that cover the HCP gene region have been reported [32]. If an undetected deletion is present, the result may be a false negative or a false positive, depending on which allele is deleted.

HLA-B*15:02

The presence of the HLA-B*15:02 allele is associated with severe hypersensitivity reactions to carbamazepine, an aromatic antiepileptic drug, resulting in Steven-Johnson syndrome (SJS) or toxic epidermal necrolysis (TEN). Hypersensitivity symptoms include skin

rash or blistering and erosion of mucous membranes and can be life-threatening. The allele has frequencies over 10% in Asian populations, increasing the risk for carbamazepine sensitivity in these populations. The HLA-BB*15:02 allele is strongly associated with SJS/TEN, and testing individuals of Asian descent is indicated, and if positive (heterozygous or homozygous) for HLA-B*15:02, carbamazepine should be avoided, and an alternative therapy selected. Other alleles (HLA-B*15:11 and HLA-A*31:0) may also play a role in carbamazepine sensitivity. The presence of the HLA-A*31:01 allele has been moderately associated with drug reaction with eosinophilia and systemic symptoms (DRESS) and has relatively high frequencies in other populations worldwide [33]. Testing is offered for HLA-B*15:02 alone or in a multigene targeted panel (https://www.ncbi.nlm.nih.gov/gtr/all/tests/?term=C3277286&filter=testtype:clinical).

PHARMACOGENETICS OF METABOLIC ENZYMES

Pharmacogenomics studies have demonstrated human genetic variation play a role in pharmacokinetics and pharmacodynamics. Polymorphic variants in absorption, distribution, metabolism and excretion (ADME) genes contribute to an individual's sensitivity, toxicity and/or resistance to a particular drug. These variants, often several alleles inherited together as haplotypes, are designated as star alleles. The Clinical Pharmacogenetics Implementation Consortium (CPIC) publishes consensus guidelines for clinical pharmacogenetic test results and dosing recommendations [34–42]. The personalized medicine goals of these guidelines are to provide clear pharmacogenetic reporting terms and therapeutic recommendations based on patient specific genotypes. Some examples of particular star alleles and their impact in drug response are included below.

CYP2D6

CYP2D6 is an enzyme from the cytochrome p450 superfamily involved in the metabolism of about 25% of commonly prescribed drugs. These include tamoxifen, beta-receptor blockers, analgesics, anticonvulsants, antidepressants and anti-psychotics, antihypertensives, and norepinephrine reuptake inhibitors. Variants in this enzyme can produce non-functional enzymes or enzymes with reduced-function. These variants are referred to as "*" alleles, representing a combination of nucleotide variants. The *1 allele is considered the normal, functional allele. Genotyping can identify variant alleles, although many are rare, and therefore may not be included in a clinically available assay. The combination of alleles results in four phenotypic categories; ultrafast metabolizer, extensive (normal) metabolizer, intermediate metabolizer and poor metabolizers. Depending on the drug, CYP2D6 may inactivate an active drug or metabolize a prodrug to its active form. If CYP2D6 activity is reduced, an active drug may build up toxic compounds; alternatively, a prodrug may result in ineffective treatment. Molecular assays detect common variants in the *CYP2D6* gene that affect protein function.

Several commercially available tests for *CYP2D6* are available and are discussed above. These include: Thermo Fisher Scientific TaqMan OpenArray, which can be customized for variants of interest and has separate assays for duplications and a known gene conversion, Luminex xTAG assay, which interrogates 17 allelic variants including the *5 deletion and duplication [5], and Autogenomics CYP2D6I assay [29] which detects 15 allelic variants including the *5 deletion and duplication alleles.

All the assays described are genotyping assays; they do not confirm if the variants detected are on the same chromosome (in *cis*) or on different chromosomes (in *trans*). Assumptions therefore are made to assign the variants based on reported combinations of variants on a single chromosome. For example, the non-functional *4 allele contains both the c.1846G>A variant, which affects splicing and results in a frameshift, and the c.100C>T variant in *cis*. In contrast, the reduced functional allele, *10 is defined by the c.100C>T variant alone. Although c.1856G>A can exist on a chromosome without c.100C>T (named *4M), this allele is rare. Thus, if the assay utilized detects both the c.1856G>A and the c.100C>T variants, it is presumed that these variants are present in *cis*. Heterozygosity for each of these variants predicts a *4 heterozygous allele (one copy of a non-functional allele). If allelic combinations aren't taken into consideration, this result could be misinterpreted as a *4/*10 compound heterozygous (one copy of a non-functional allele and one copy of a decreased-function allele). A true *4/*10 combination shows homozygosity for c.100C>T and heterozygosity for c.1846G>A.

Gene duplications add another level of complexity to CYP2D6 genotype interpretations. Although most molecular assays can detect the presence of a duplication, not all can assess which allele is duplicated (i.e. if the duplication arose from the WT or mutant allele). In addition, the number of copies is not determined. The AmpliChip assay was designed to reduce the diagnostic complexity associated with such genotypes by detecting seven duplications also known to contain non-functional or reduced-function variants (such as c.1846G>A and/or c.100C>T). Knowing which allele

is duplicated is important in predicting the phenotype, an example is a patient with the CYP2D6*1/*4 genotype and gene duplication. If the *1 is duplicated (*1XN/*4), the patient has at least two functional copies of the gene, predicting an extensive metabolizer phenotype. However, if *4 is the duplicated allele (*1/*4XN), the duplicated allele would still be non-functional and the patient would be predicted to have only one functional allele, consistent with an intermediate-to-extensive metabolizer.

Laboratories may report the * allele designation detected, or the nucleotide positions of base changes. If none of the targeted variants are detected, the wild-type or *1 allele is presumed. However, it should be noted that unless sequencing of CYP2D6 is performed to detect all variants, rare alleles may be missed by targeted variant analysis. Alternatively reports should state that "none of the targeted variants were detected."

Hemostasis (CYP2C19, CYP2C9, VKORC1)

Hemostasis is the ability of the body to properly control the spatial and temporal behavior of the clotting cascade. In certain pathologies, the risk of thrombotic events is high, and therefore pharmaceuticals are prescribed to reduce the risk of clotting. Dosing of such drugs is critical because there is a delicate balance between the risk of clotting and the risk of bleeding. Two of the most commonly prescribed oral anticoagulants are clopidogrel and warfarin, and the efficacy of both is known to be affected by genetic variations.

For more than 60 years warfarin has been front line therapy for the treatment or prevention of thromboembolisms in patients at risk for clotting. Despite its wide-spread use, adverse events resulting from inter-individual variability are common. Mechanistically, warfarin executes its function by inhibiting the activation of vitamin K, required for the gamma carboxylation of many of the factors involved in the coagulation cascade. In the absence of gamma carboxylation, these factors are unable to cleave their substrates, and as such, the coagulation cascade is partially arrested. Two distinct genes are implicated in the pharmacological response to warfarin: *CYP2C9* and *VKORC1* [43]. The FDA has mandated that the warfarin package insert contain a table that integrates *CYP2C9* and *VKORC*1 genotype with drug dose.

CYP2C9, a member of the cytochrome P450 family, is responsible for the rate limiting step to breakdown warfarin into its inactive metabolite. *CYP2C9* is a highly polymorphic gene, and some of these SNPs reduce the catalytic efficiency. *CYP2C9*2* and *3 are well defined alleles that cause a decreased warfarin clearance rate. Cumulatively, they are present in about 20% of Caucasians. Patients harboring either of these alleles are predicted to require a lower loading and maintenance warfarin dose, and are also at greater risk for bleeding complications as a result of warfarin administration.

Vitamin K is a cofactor for the enzymatic addition of gamma carboxylate functional groups onto various proteins, including many of the principal components of the coagulation cascade. The result of this reaction is the oxidation of vitamin K. To function as a cofactor for subsequent gamma carboxylation reactions, vitamin K must be converted back into its reduced form. VKORC1 is the enzyme responsible for such vitamin K recycling, and is the mechanistic target of warfarin; warfarin binds to VKORC1 and prevents VKORC1 from reducing vitamin K. Polymorphisms within the *VKORC1* warfarin binding site can alter the binding affinity of the enzyme for the drug. The most common of these, −1639G>A, is associated with warfarin sensitivity.

Combined, *CYP2C9* and *VKORC1* have been shown to be predictive of warfarin dosage requirements for ~50% of patients. Multiple FDA cleared platforms for detection of the common *CYP2C9* and *VKORC1* variant alleles are available, and all function with extremely high (>99%) sensitivity and specificity [44,45]. The fundamental issues regarding *CYP2C9* and *VKORC1* testing are bureaucratic in nature [33]. Controversy exists regarding the clinical utility, and hence reimbursement poses a major issue. Despite the endorsement for genotype testing by the FDA, AACC, NACB, CAP and others, the Centers for Medicare and Medicaid Services are reluctant to provide any reimbursement for *CYP2C9* or *VKORC1* testing until a large scale, prospective, randomized study has shown clinical utility of testing.

Further studies are also needed to design the most effective scheme for incorporating *CYP2C9/VKOR1* genotype testing into the treatment plan [46]. Algorithms have been proposed, but it is unclear whether it is imperative for the genotyping to be completed with expedited turn-around-time, or if it is sufficient to begin a patient on a dose appropriate for their height/weight/age and adjust their dose as the genotype results become available. In either case, the international normalized ratio (INR) should be used in tandem to the genotype results to assure that the proper therapeutic target is achieved.

The use of platelet antagonists, such as aspirin and clopidogrel, has been shown to improve outcomes in patients with acute coronary syndrome and percutaneous coronary interventions [47]. Clopidogrel functions by irreversibly binding to the P2Y12 platelet receptor, preventing ADP activation of platelets. About 25% of patients do not respond to clopidogrel; a large part of the variability is attributed to the *CYP2C19* gene. Clopidogrel is a prodrug, requiring a two-step enzymatic reaction

to be converted into the active metabolite. CYP2C19 is a catalyst for both of these reactions, and like many members of the *CYP* family, is a highly polymorphic gene. Although multiple polymorphisms have been identified in *CYP2C19*, only 5 have been shown to alter the kinetic properties of the enzyme. *CYP2C19*2, *3, *4,* and **5* are associated with loss of function and a decreased metabolic rate of clopidogrel; *CYP2C19*17* is associated with a gain of function and an increased metabolic rate of clopidogrel. Although robust and accurate FDA-cleared and laboratory developed tests can identify these variants, the main challenge in testing for *CYP2C19* variants lies in the clinical relevance of these alleles, which is currently highly controversial [48,49].

In 2010 the FDA mandated a box label warning be placed in the clopidogrel package insert to caution patients that there is diminished effectiveness of the drug in patients who are poor metabolizers, and available genetic testing for the *CYP2C19* gene can identify such persons. The label suggests that if genetic testing reveals that the patient is a poor metabolizer, alternative treatment or treatment strategies should be considered. Immediately following the FDA endorsement, multiple national cardiovascular associations contested the decision, stressing that there was not sufficient evidence to support these claims. The controversy over the utility of *CYP2C19* testing remains [50]. Over 30 clinical trials and at least a dozen meta-analyses reveal conflicting data, bolstering the debate. In summary, clopidogrel is a widely prescribed drug that can reduce adverse thrombotic events in at risk patients. There is an association between *CYP2C19* genotype and clopidogrel response: poor metabolizers will have a decreased serum concentration of the active metabolite and will have platelets with higher *ex-vivo* reactivity to ADP than predicted of a patient on clopidogrel. However, the association of genotype with the risk of cardiovascular events remains incredibly controversial.

PRECISION MEDICINE AND PEDIATRICS

Building upon the knowledge and ability to detect genetic variation in single gene disorders, clinical genome sequencing may one day be performed *in lieu* of traditional newborn public health programs [51,52], but this approach is not without some controversy [53]. Clear examples of the ability to tailor therapies to patients based on their genetic makeup include personalized medicine for treatment of cystic fibrosis (CF) and spinal muscular atrophy (SMA). Ivacaftor (Kalydeco) and lumacaftor/ivacaftor (Orkambi) are two US. Food and Drug Administration (FDA)-approved drugs prescribed for people with CF who have specific pathogenic variants in the cystic fibrosis transmembrane conductance regulator (*CFTR)* gene. The CFTR protein is a chloride channel present at the surface of epithelial cells in multiple organs. The F508del (p.Phe508del) pathogenic variant is the most common variant detected in CF patients. This variant causes protein misfolding, defective cellular processing and trafficking and ultimately leads to protein degradation and reduced CFTR at the cell surface. Although some mutant-CFTR (F508del-CFTR) escapes degradation, once at the surface F508del-CFTR is unstable and has defective channel properties compared to wild-type CFTR protein. Many other variants (see FDA label for list of *CFTR* gene variants that produce a CFTR protein responsive to ivacaftor) in the *CFTR* gene also lead to reduced CFTR levels at the cell surface and/or defective channels on the cell surface. Ivacaftor is a potentiator of the CFTR protein; it facilitates increased chloride transport of the CFTR protein located at the cell surface. Lumacaftor improves the conformational stability of F508del-CFTR, resulting in increased processing and trafficking of mature protein to the cell surface. The overall level of ivacaftor-mediated CFTR chloride transport is dependent on the amount of CFTR protein at the cell surface and how responsive a particular mutant CFTR protein is to ivacaftor potentiation. Combination therapy in patients with p.F508del and another known ivacaftor-responsive variant has shown increased quantity, stability and function of mutant CFTR molecules and corresponding clinical benefit.

(Kalydeco - https://www.accessdata.fda.gov/drugsatfda_docs/label/2017/203188s019lbl.pdf, Orkambi - https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/206038s010lbl.pdf).

SMA is a progressive disease characterized by muscle weakness and atrophy due to the degeneration and loss of the anterior horn cells in the spinal cord [54]. The *SMN1* and *SMN2* genes both code for the survival motor neuron (SMN) protein. Normally, most functional SMN protein is produced from the *SMN1* gene, with a small amount produced from the *SMN2* gene. Patients with SMA have pathogenic variants in *SMN1* (most commonly deletion of exon 7) leading to impaired SMN production. The number of copies of the *SMN2* gene varies and is thought to be a disease modifier. SPINRAZA (nusinersen) is an FDA-approved antisense oligonucleotide (ASO) designed to treat SMA caused by pathogenic variants that lead to SMN protein deficiency. Nusinersen binds to a specific sequence in the intron downstream of exon 7 of the SMN2 transcript to increase exon 7 inclusion in SMN2 messenger ribonucleic acid (mRNA) transcripts and production of full-length SMN protein (https://www.accessdata.fda.gov/drugsatfda_docs/label/2016/209531lbl.pdf).

The above successes in personalized medicine in the pediatric field are exciting, however, it is critical for

practitioners to be aware that dosing guidelines for pediatric patients with autism spectrum disorder and/or attention deficit disorder with hyperactivity based on genotype are not well defined [55]. Efficacy and toxicity evidence does exist for a few genotypes and drugs in the pediatric setting and they include *CYP2D6* and *CYP2C19* and tricyclic antidepressants as well as SSRIs to avoid (https://www.pharmgkb.org/). In as much, the information needed to predict and measure the responses to therapeutic agents does not only include things such as age and weight, but will soon include genomic data.

CASE STUDIES

The following scenarios illustrate a few of the challenges in pharmacogenetic testing. They are typical types of cases received in the clinical laboratory.

Case 1 involving CYP2D6: A male in his 30's is nonresponsive to psychiatric medication, and a sample is sent for *CYP2D6* testing. His genotype showed a *17 decreased function allele and the *2a functional allele. The assay also detected duplication but could not determine which allele was duplicated. Without knowing the duplicated allele, a phenotype could not be accurately predicted. If the *2a allele was duplicated, he would have at least two copies of a functional allele, and one allele with decreased function. The predicted phenotype would be an extensive or rapid metabolizer. If the *17 allele was duplicated, he would have a duplicated decreased function allele, and one normal function allele. The predicted phenotype would be an extensive metabolizer. A family study was performed and demonstrated that the *2a allele and the duplication was inherited from his mother, while the *17 allele was inherited from his father. With this information, he was predicted to have at least an extensive metabolizer phenotype and is likely a rapid metabolizer. A rapid metabolizer phenotype could explain his nonresponsiveness because the medication could be quickly eliminating, before it is able to be effective.

Case 2 involving SMA: A 2-week old female tested positive for SMA by newborn screening. To confirm the disease and determine if therapy with SPINRAZA will likely be effective, a sample is sent for *SMN1* and *SMN2* quantitative testing. qPCR is performed, targeting single nucleotide variants in exon 7 and exon 8 that distinguish between these highly homologous genes. No copies of *SMN1* exon 7 were detected, yet three copies of *SMN2* exon 7 were present. Testing for exon 8 revealed one apparent copy of *SMN1*, and two apparent copies of *SMN2*. The most likely interpretation is that *SMN*1 is deleted on one chromosome, and a gene conversion event between *SMN2* and *SMN1* occurred on the other chromosome between exon 7 and exon 8. Although exon 8 appears to have one copy of *SMN1* exon 8, the gene conversion allele is functionally an *SMN2* gene. This result confirms no functional copies of *SMN1* consistent with a diagnosis of SMA. In addition, three copies of the *SMN2* gene are expected to modify the severity of disease to some extent. Given the presence of *SMN2*, SPINRAZA is likely to be effective in allowing expression of full length SMA protein, providing enough transcript and protein to allow for normal development.

CONCLUSIONS

In summary, molecular testing is available for a number of inherited and somatic applications. Still, challenges remain in the technologies to accurately detect the variations, as well as in their interpretation. The main challenge is to show clinical utility, that patient outcomes are improved by pharmacogenetic testing. Evidence is easier to obtain for the oncology applications, as is seen in the number of tests developed and used clinically. Showing improved outcomes is difficult for metabolic enzymes because of pathway redundancy with other enzymes involved, and rarity of variants to show differential effects between heterozygotes and homozygotes. As studies continue to show validity and utility, molecular testing for targeted therapies and pharmacogenetics will be at the forefront of personalized medicine.

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CHAPTER

23

Challenges and sources of inaccuracy in biochemical genetics testing

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INTRODUCTION

Inborn errors of metabolism (IEM) are biochemical genetic disorders of intermediary metabolism. The majority of these disorders are due to single gene mutations resulting in an enzyme deficiency, membrane transporter or other functional protein defect. Many inherited metabolic disorders can result in a rapid deterioration of a patient leading to high morbidity and mortality. Symptoms such as vomiting, seizures, ataxia, poor feeding, hypotonia and lethargy in a child with IEM are generally non-specific. Laboratory testing is needed to confirm the clinical suspicion and to make the final diagnosis. Laboratory tests can be broadly divided into routine tests, which are available in most hospital laboratories, and specialized tests, which are performed by a limited number of laboratories. Initial laboratory tests needed for patient management include blood gases, pH, glucose, electrolytes, ammonia, lactate, renal function tests, liver function tests, urinalysis and basic hematological tests. Specialized tests include plasma amino acid profile, urine organic acid profile, plasma acylcarnitine profile, free fatty acid profile, pyruvate, acetoacetate, 3-hydroxybutyrate, mucopolysaccharides, oligosaccharides and enzyme activity assays. Functional assays and mutational analyses may also be needed to make the definitive diagnosis. Proper specimen collection and processing for many of these tests can be challenging. Inappropriate specimen collection, handling and processing can lead to a delayed diagnosis or a misdiagnosis. In addition to preanalytical challenges, there are a number of analytical and post-analytical challenges in biochemical genetic testing [1–3]. A number of these challenges are listed in Table 23.1.

PREANALYTICAL CHALLENGES

There are a number of preanalytical challenges in clinical biochemical genetics testing. These challenges include proper communication among healthcare providers to order the right test(s), as well as proper collection, processing and handling of the patient's specimen(s). Many metabolic disorders are identified through newborn screening. To avoid delay in clinical care and follow-up testing, timely and prompt communication between the newborn screening laboratory and the primary care physician (or pediatrician) and a metabolic specialist is of the utmost importance. Depending on the urgency of the results, the family and patient needs to be contacted for follow-up testing and clinical assessment immediately [4,5]. The importance of correct patient preparation and sample collection and handling cannot be overemphasized. For many metabolic tests, patient preparation is important. For example, the patient should fast before sample collection for an amino acid profile. Amino acid results from a postprandial sample may not be properly interpreted. Samples for lactate and ammonia should be collected and transported to the laboratory on ice [3,6]. Specimen draws from newborns, particularly those who were born prematurely, is challenging and may require a well-trained phlebotomist. Drawing a large volume of sample from a newborn, which may be required for biochemical genetics testing, can be another challenge. Hemolysis which is known to interfere with many laboratory tests is higher in the samples drawn from newborns.

Commonly collected specimens for inherited metabolic testing include whole blood, serum or plasma, urine and cerebrospinal fluid. Whole blood is collected

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TABLE 23.1 Pre-analytical, analytical and post-analytical challenges in biochemical genetics testing.

Pre-analytical	• Coordination and communication among healthcare providers for selection of right tests • Collection of the right specimen (s) • Proper handling and transportation of samples • Obtaining reliable patient information such as medication and nutritional history, fasting status etc
Analytical	• Unavailability of FDA approved commercial tests • Complexity of methods and their validation • Lack of certified materials such as quality controls and standards • Lack of standardization of methods • Limited reference ranges • Significant variation among biochemical genetic laboratories • Lack of experienced and qualified personnel • Limited proficiency testing and quality assurance schemes
Post-analytical	• Preanalytical and analytical challenges affecting results interpretation • Lack of experienced and qualified personnel for interpretation of test results • Communication of results among healthcare providers

on filter paper for newborn screening or in tubes for harvesting serum, plasma, leukocytes and red blood cells. Amniotic fluid, skin fibroblasts, and liver and muscle tissue may also be used for the diagnosis of certain metabolic disorders. Serum/plasma is the most commonly used specimen for initial and follow-up testing of metabolic disorders. Urine is the specimen of choice for the diagnosis of organic acidurias and renal transport disorders. When urine is used, the results are generally normalized to creatinine concentration to account for sample dilution. CSF is the specimen of choice for the diagnosis of neurotransmitter disorders and certain other diseases such as non-ketotic hyperglycinemia. Table 23.2 provides sample collection and handling for the commonly used tests in the diagnosis and management of metabolic patients.

ANALYTICAL CHALLENGES

Laboratory analyses in patients with inherited metabolic disorders can be divided into two broad categories: routine and specialized testing. Routine testing is available in most hospital-based healthcare facilities and includes common chemistry and hematological analyses performed on automated analyzers. Routine testing is most often performed using well established FDA approved methods and seldom poses any problems. On the other hand, specialized tests such as amino acids, organic acids, acylcarnitines, mucopolysaccharides, oligosaccharides, glycoproteins neurotransmitters, enzyme assays and DNA analysis are not available in a routine hospital laboratory and need to be referred to specialized centers. These analyses require specialized techniques such as gas-chromatography mass spectrometry (GC−MS), liquid-chromatography mass spectrometry (LC-MS), tandem mass spectrometry (LC-MS/MS) and electrophoresis. Unlike routine tests, most biochemical genetics tests require extensive sample preparation and tests are performed most often manually. Some laboratories may perform quick spot screening tests for a presumptive diagnosis in a sick patient. When used in conjunction with clinical context and correlated to the patient's symptoms, screening tests can provide quick and useful information. However, it is important to keep in mind that these tests lack specificity and sensitivity, and may provide a high rate of false positive and negative results.

Method evaluation, quality control and quality assurance

Unlike routine chemistry and hematology tests which are FDA approved and well established, most biochemical genetics tests are not FDA approved and are developed in the laboratory under the category of "Laboratory Developed Tests (LDTs)". LDTs in biochemical genetics laboratories pose unique challenges because quality control and standard materials are not available for many important diagnostic analytes. For example, many organic acids and acylcarnitines of clinical interest are not available as pure compounds. Their estimation is based on other structurally similar "surrogate compounds" and are, at best, semi-quantitative. Regardless of these limitations and challenges, like FDA approved tests, the performance of LDTs should be evaluated for accuracy, precision/imprecision, reportable range, analytical measurement range/linearity, clinical reportable range, analytical sensitivity/limit of quantitation analytical selectivity/specificity and reference ranges [7,8]. This leads to wide variations in quantitative and interpretive results among different laboratories [1,3,9].

Quality control (QC) and quality assurance (QA) in biochemical genetics laboratories pose unique challenges. As discussed above, quality control and standard materials for many biochemical genetics tests are not available. In routine assays, quality control is generally maintained by analyzing 2−3 quality control samples with each batch of patient samples. Patient results are deemed acceptable if the results of quality control samples are within an acceptable range, generally calculated from an average +2 or 3 standard deviations of repeated QC results. This method of quality control works well when measuring a single analyte, but may

TABLE 23.2 Specimen collection and handling.

Analyte	Specimen type	Specimen handling
Acylcarnitines	Plasma (EDTA or Heparin)	Freeze
Acylglycines	Urine	Freeze
Alkaline phosphatase	Serum or Plasma (Heparin)	Refrigerate if specimen cannot be analyzed in 4 h.
Amino acids	Plasma (Heparin) or Urine (Random), Cerebrospinal fluid (CSF)	Fasting blood specimen is preferred. Refrigerate if testing done in-house. Freeze for shipping.
Ammonia	Plasma (Heparin)	Send immediately on ice. Postprandial specimen is preferred. Recollect if specimen is hemolyzed. Freeze plasma if analysis cannot be done in 4 h.
Complete Blood Count (CBC)	Blood (EDTA)	Refrigerate if specimen cannot be analyzed the same day.
Creatine kinase	Serum or Plasma (Heparin)	Hemolysis will falsely increase creatine kinase. Refrigerate if specimen cannot be analyzed in 4 h.
Creatinine/Urea	Serum or Plasma (Heparin)	Stable at room temperature for 3 days.
Electrolytes	Serum or Plasma (Heparin)	Hemolysis will cause increase in potassium.
Galactose-1-Phosphate Uridyltransferase (GALT)	Blood (EDTA or Heparin)	Stable for 4 weeks at 4 °C
Glucose	Plasma (Fluoride, Heparin)	Send as soon as possible. Heparin plasma is acceptable if specimen can be processed and analyzed within 30 min. Fasting specimen when possible.
Homocysteine	Plasma (Heparin or EDTA)	Separate plasma within 4 h. Fasting preferred
Lactate	Plasma (Heparin or Fluoride)	Send immediately on ice. Separate plasma immediately. Freeze if specimen cannot be analyzed within 4 h.
Lactate Dehydrogenase	Serum or Plasma (Heparin)	Hemolysis will cause false increase in lactate dehydrogenase
Lipids	Serum or Plasma (Heparin)	Refrigerate
Liver enzymes	Serum or Plasma (Heparin)	Hemolysis will increase AST
Mucopolysaccharides	Urine (random)	Refrigerate
Organic acids	Urine (random)	Refrigerate if done in-house. Freeze for shipping. Scope of testing varies between laboratories.
pH and Blood gases	Whole Blood (Heparin)	Send immediately on ice.
Pyridoxal-5-Phosphate	CSF	Freeze
Pyruvate	Whole Blood (Heparin)	Add specimen to pre-chilled tube containing perchloric acid.
Sialic Acid	Urine	Freeze
Succinyladenosine	CSF	Freeze
Sulfocysteine	Urine	Freeze
Uric Acid	Serum or Plasma (Heparin) Urine (random or timed collection)	A large number of drugs interfere in uric acid methods. Consult laboratory.
Urinalysis	Urine (random or timed)	Tests generally include pH, specific gravity, protein, glucose, bilirubin, urobilinogen, leukocyte esterase and cell count
Very-long chain Fatty Acids	Plasma (Heparin or EDTA)	Fast overnight

The table provides general information that can vary among laboratories.
Modified and reprinted with permission from Garg U, Smith LD. Introduction to laboratory diagnosis and biomarkers in inborn error of metabolism. In: Garg U, Smith LD, editors. Biomarkers in inborn errors of metabolism: Elsevier; 2017. pp. 1–24.

not work well when a multi-component analysis is done. Many biochemical genetics tests such as amino acid, acylcarnitine and organic acid profiles involve multi-component analysis; more than 30–100 analytes in a single analysis. For example, if an average +2 standard deviation is used to establish the acceptable range, the chances of the result of a quality control sample within the quality control range is 95%, and the probability of

the two controls within the quality control ranges is $0.95 \times 0.95 = 0.9025$ or 90.25%. If it is a 20 component analysis with two controls for each component, the probability of all controls being within the quality control ranges is only $(0.95)^{20\times2} = 0.128$ or 12.8%. This simplified calculation may overestimate the failure rate because the quality control measurements in a multicomponent analysis are not completely independent, but makes the point of a high failure rate of quality controls in a multicomponent analysis. Using wider quality control ranges such as an average + 3 standard deviations will result in higher acceptance of analytical runs but may miss subtle changes in patient values. Sometimes the average plus/minus is a fixed percentage, such as mean + 20%, that may be used to establish quality control ranges. In addition to the acceptability of quality control sample results, it is important to interpret the data in context with specific analytes. For example, detection of succinylacetone on a urine organic acid profile may be clinically significant despite the lack of a succinylacetone control. Therefore, an analyst should be able to judge the performance of a method for clinical use despite minor deviation from quality control rules. These limitations make a laboratory diagnosis involving inherited metabolic disorders challenging and complicated. It is important that the laboratory results are interpreted in context with the patient's clinical information. Results interpretation may be more challenging for reference laboratories which generally don't have adequate patient information.

To aid quality assurance in a biochemical genetics laboratory, many external proficiency testing programs such as those from Centers for Disease Control and Prevention (CDC), College of American Pathologists (CAP) in the United States [10] and European Research Network for Evaluation and Improvement of Screening, Diagnosis and Treatment of Inherited Disorders of Metabolism (ERNDIM) in Europe [11] are available. The CDC provides dried blood spot samples for quality control and proficiency testing with emphasis on newborn screenings. CAP proficiency testing includes organic acids, amino acids, carnitine, acylcarnitines and mucopolysaccharides. ERNDIM provides the most extensive quality control materials and external proficiency testing schemes. It provides proficiency testing for organic acids, amino acids, carnitine, acylcarnitines, purines and pyrimidines, white cell cysteine, mucopolysaccharides and many other analytes. It also provides lyophilized material for a number of analytes.

POST-ANALYTICAL CHALLENGES

Once the analysis has been performed, biochemical genetics laboratories face a number of post-analytical challenges. The interpretation of test results is crucial and should be done in context to the patient's clinical information by trained and qualified personnel [1,12]. Comparison of patient results with "normal or reference values" can be challenging as there is scarcity of age- and sex-based reference ranges. For proper interpretation, pattern recognition of multiple changes is more important than the individual values in a profile. Communication with healthcare providers and expert guidance on reflex and follow-up testing may be needed to confirm initial findings or to rule-in or rule-out subtle changes.

CHALLENGES IN THE DIAGNOSIS OF SPECIFIC DISORDERS

Due to a large number of metabolic disorders, it is only possible to discuss the challenges in laboratory testing for a selected number of disorders. Disorders of amino acids, organic acids, fatty acid oxidation and lysosomal storage make up the majority of cases of inherited metabolic disorders [13–16], and are discussed below.

Amino acids disorders

Over 50 inherited metabolic disorders of amino acids have been described. Amino acid profiling is a commonly used test in the patients suspected with inherited inborn error of metabolism. Depending on the suspected disorder, plasma, urine and/or cerebrospinal fluid amino acids are analyzed. Plasma is a preferred sample for the diagnosis of most amino acids disorders, urine is the specimen of choice for renal transport disorders, and CSF is used for the quantification of amino acids that effect the central nervous system. Three commonly used methods for the quantification of amino acids are ion-exchange chromatography with post-column derivatization with ninhydrin, HPLC-tandem mass spectrometry (HPLC-MS/MS) and pre-column-derivatization reverse phase HPLC with UV or florescent detection.

Ion-exchange chromatography with post-column derivatization with ninhydrin method has been in use for many decades and is still the most commonly used method for the assay of amino acids [17]. In this method, various amino acids are separated using cation-exchange resin column and different lithium-citrate buffers of varying pH and concentrations. Column temperature may also vary to achieve better separation. Post-column eluent reacts with ninhydrin at 120–135 °C. Primary amino acids make a purple complex which is measured at 570 nm and imino acids (proline and hydroxyproline) yield a yellow complex which can be measured at 440 nm. Secondary amino acids such as sarcosine react with ninhydrin, but they have a lower sensitivity.

Measurement at two (570 nm and 440 nm) wavelengths increases the sensitivity and facilitates the identification process. Primary amino acids have a 570 nm/440 nm ratio of approximately 6, whereas the sulfur containing amino acids (cystine, sulfocysteine) have a ratio of approximately 1. The method is very stable and reproducible and needs minimal sample preparation. Most of the normal and pathological reference ranges have been established using this method. Major drawbacks of the ninhydrin method are a long analytical run time (over 2 h/sample) and interferences from exogenous compounds. The number of laboratories using this method is decreasing in favor of HPLC-tandem mass spectrometry or HPLC-UV or fluorescent detection.

HPLC-tandem mass spectrometry is becoming the method of choice for amino acids analysis. This method is highly specific and provides faster analysis time (15–30 min/sample). Commonly used HPLC-MS/MS involves reverse phase column and electrospray ionization. HPLC-MS/MS methods, with and without the derivatization of amino acids, have been described. Methods involving derivatization are preferred as they provide better sensitivity and specificity. Butylation is the most commonly used HPLC-MS/MS method for the analysis of amino acids [18]. In this method, amino acids are derivatized to butyl esters using butanol/HCl at 65 °C. Quantification involves the use of deuterated internal standards and multiple reaction monitoring (MRM) or neutral ion loss. Tandem mass spectrometry is the most commonly used method for the neonatal screening of amino acids disorders. To achieve high throughput in newborn screenings, samples are directly injected into the mass spectrometer without a column. Without a column, isobaric compounds such as leucine, isoleucine, alloisoleucine and hydroxyproline are indistinguishable and give a single peak. Commercial HPLC-MS/MS amino acids assays from various vendors, including Waters (Milford, MA) and AB Sciex (Framingham, MA), are available.

HPLC methods with UV or fluorescent detection have also been described. These methods commonly use pre-column derivatization and reverse phase column. Ortho-phthalaldehyde (OPA) has been used in the derivatization of amino acids; however, this reagent does not react with proline, hydroxyproline and sulfur-containing amino acids. Furthermore, OPA-derivatized products are not very stable. Due to these limitations, this method has not been used in routine analysis. Other derivatizing reagents, dansyl chloride and 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate have also been used. 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate reacts with primary and secondary amines to form highly stable fluorescent derivatives [19]. The derivatives can be detected by fluorescent detection or mass spectrometry.

TABLE 23.3 Various conditions which can affect different amino acid concentrations and, thus, results interpretation.

Condition	Specimen type	Amino acid (s) affected
Postprandial (animal protein)	Blood	↑ Arg, Lys, Ser, Thr, ILe, Leu, Val
Hemolysis	Blood	↓ Arg, Gln, Asn; ↑ Orn, Glu, Asp
Extended Storage	Blood	↓ Cystine, Gln
Valproate, Carbamazepine	Blood, CSF	↑ Gly
Starvation/Ketosis	Blood	↑ Leu, Ile, Val
Methotrexate, Nitrous oxide, Unspun sample at RT	Blood	↑ Homocysteine
Bacterial contamination	Urine	↑ Ala, Gly, Pro; ↓ Phe, Tyr, His, Trp, Ser
Liver Dysfunction	Blood	↑ Met, Phe, Tyr, Orn
Ampicillin/Amoxicillin Treatment	Urine	Interferes with Tyr. May also interfere with Met, Phe, ASA (Ninhydrin method)
Hyperornithinemia-Hyperammonemia-Homocitrullinuria (HHH)	Blood	Homocitrulline coelutes with methionine

Amino acid results should be interpreted in context with patient information as many conditions can influence the results and, thus, interpretation (Table 23.3). Certain treatments, such as with amoxicillin and betaine, can interfere with amino acids analysis (Figs. 23.1 and 23.2). In Hyperornithinemia-Hyperammonemia-Homocitrullinuria (HHH), homocitrulline coeluted with methionine (Fig. 23.3). In some conditions, the clinically significant peak may be very small and needs special attention. In homocystinuria, the homocystine peak is very small (Fig. 23.4). Looking for homocysteine-cysteine dimer and performing total homocysteine is helpful. Pattern recognition may provide better interpretation rather than individual amino acid values.

Organic acid disorders

Organic acidemias are a diverse group of inherited metabolic disorders that are characterized by the accumulation of organic acids in body fluids. There are over 50 well characterized organic acidurias. Since organic acids are excreted in urine, the urine is the specimen of choice for the diagnosis of organic acidurias. Gas chromatography mass spectrometry (GC–MS) is the method of choice for the screening of organic aciduria. Using GC–MS, more than 100 organic acids can be screened in a single analytical run. Other methods,

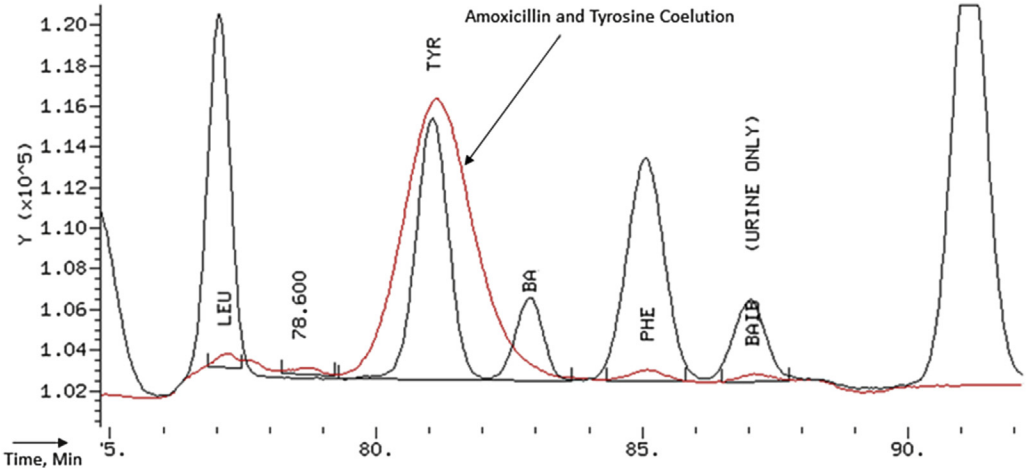


FIG. 23.1 Urine amino acid profile from a patient being treated with amoxicillin. Tyrosine and amoxicillin coelute (Red tracing). For peak identification, black tracing is an overlay from a calibrator.

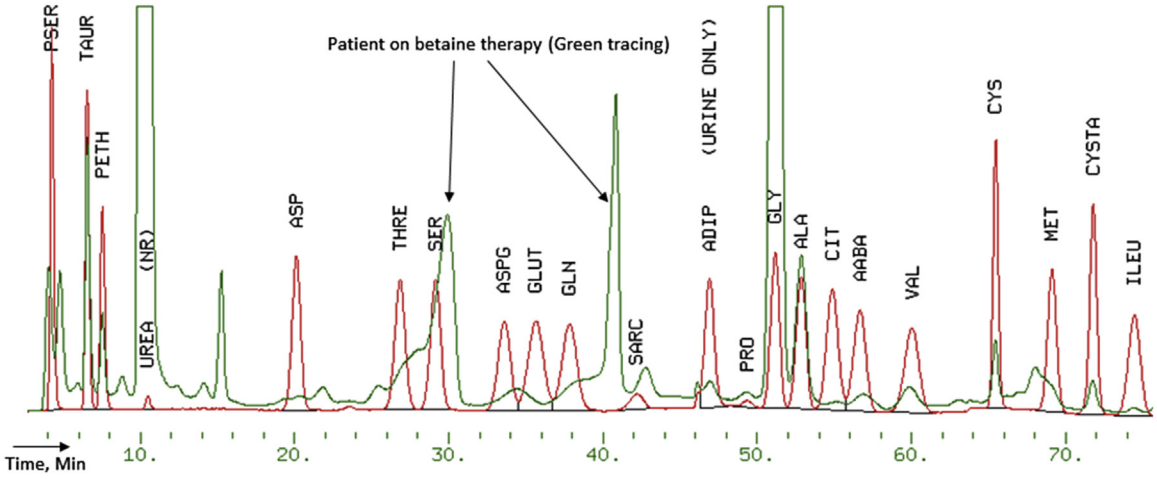


FIG. 23.2 Plasma amino acid profile from a homocystinuria patient being treated with betaine. Threonine, serine, aspartate, glutamate, glutamine retention times are affected. For peak identification, red tracing is an overlay from a calibrator.

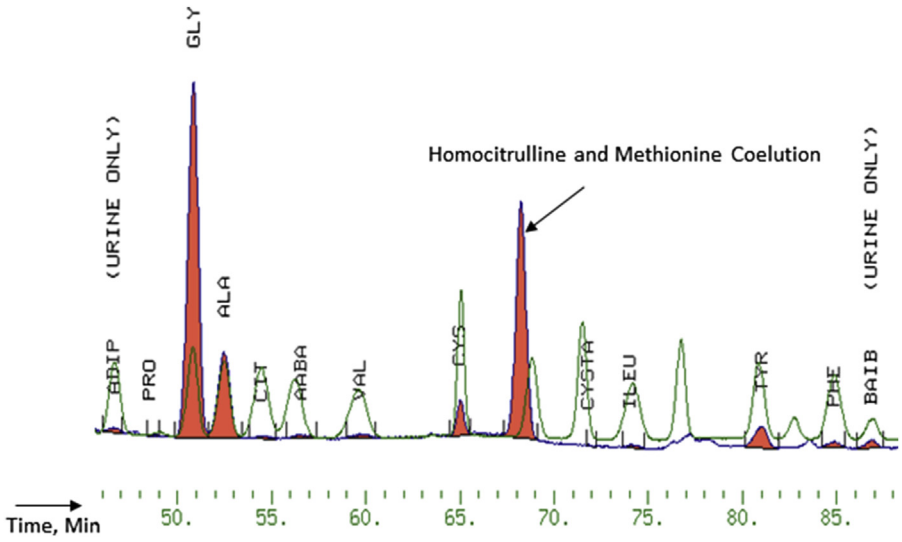


FIG. 23.3 Plasma amino acid profile from a HHH patient. Homocitrulline and methionine coelute. For peak identification, green tracing is an overlay from a calibrator.

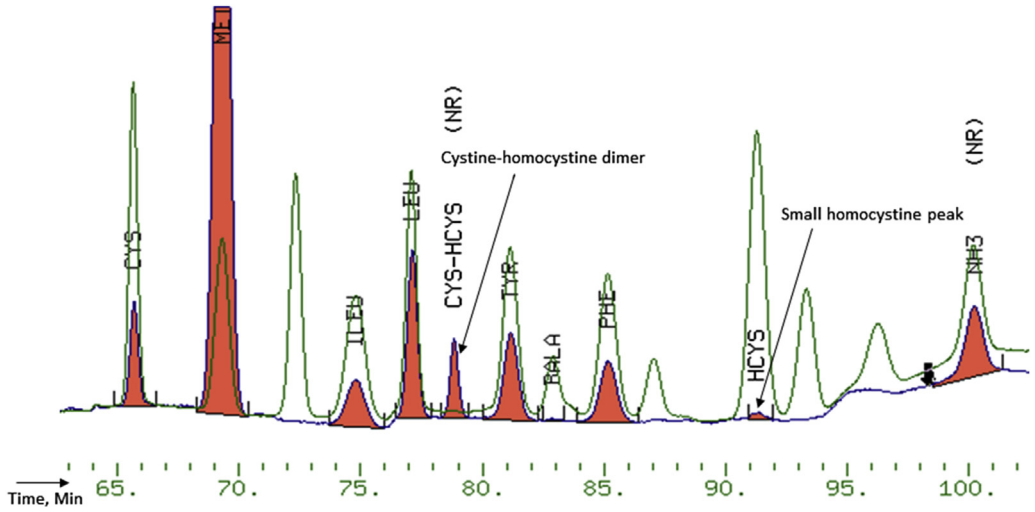


FIG. 23.4 Plasma amino acid profile from a patient with homocystinuria. Homocystine is a very small peak. This patient had a total homocysteine level of 160 μmol/L. Note cysteine-homocysteine dimer peak which can assist in identification of a patient with homocystinuria.

such as capillary electrophoresis and HPLC-MS/MS, are used for the analysis of a limited number of organic acids. In a typical organic acid GC−MS analysis, using liquid-liquid extraction (ethyl acetate and/or ether), organic acids are extracted under acidic conditions (pH < 2). Some laboratories prefer oximation of 2-ketoacids. Oximation stabilizes 2-ketoacids and facilitates their detection and quantification. Important 2-ketoacids include pyruvate, acetoacetate, succinylacetone and branched-chain ketoacids. Since organic acids are relatively polar and non-volatile, they are derivatized before their analysis by GC−MS. N, O,-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) is a commonly used derivatizing agent and forms trimethylsilyl (TMS) derivatives of organic acids. The organic acid-TMS ethers are injected into GC−MS and separated using a capillary column coated with nonpolar stationary phase. Mass spectrometry involves electron impact ionization and mass scanning in the range between m/z 50 and 500−600. Identification of organic acids is made through comparing unknown spectra with the known spectra. Quantitation is based on comparing signals of unknowns with that of calibrators and internal standards.

The analysis of organic acids and the interpretation of the results may be challenging. If a sample analysis cannot be performed within 24 h, samples should be frozen at −20 °C and shipped frozen to a reference laboratory. Certain drugs, such as valproate and carbamazepine, can interfere in the analysis. A GC/MS chromatogram showing various valproate peaks is shown in Fig. 23.5. Another challenge is the subtle but

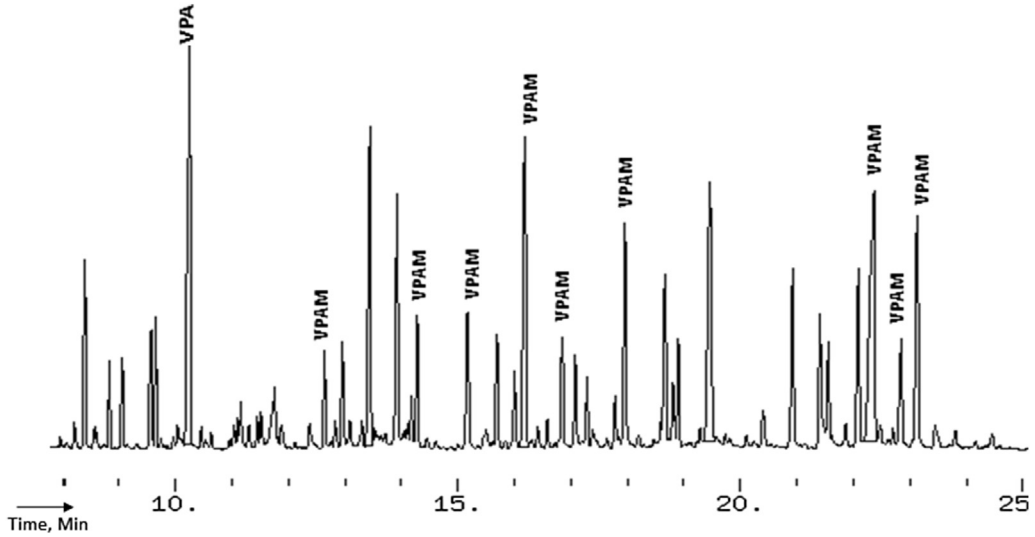


FIG. 23.5 Urine organic acid profile from a patient being treated with valproate. Multiple peaks are seen from valproate (VPA) and valproate metabolites (VPAM).

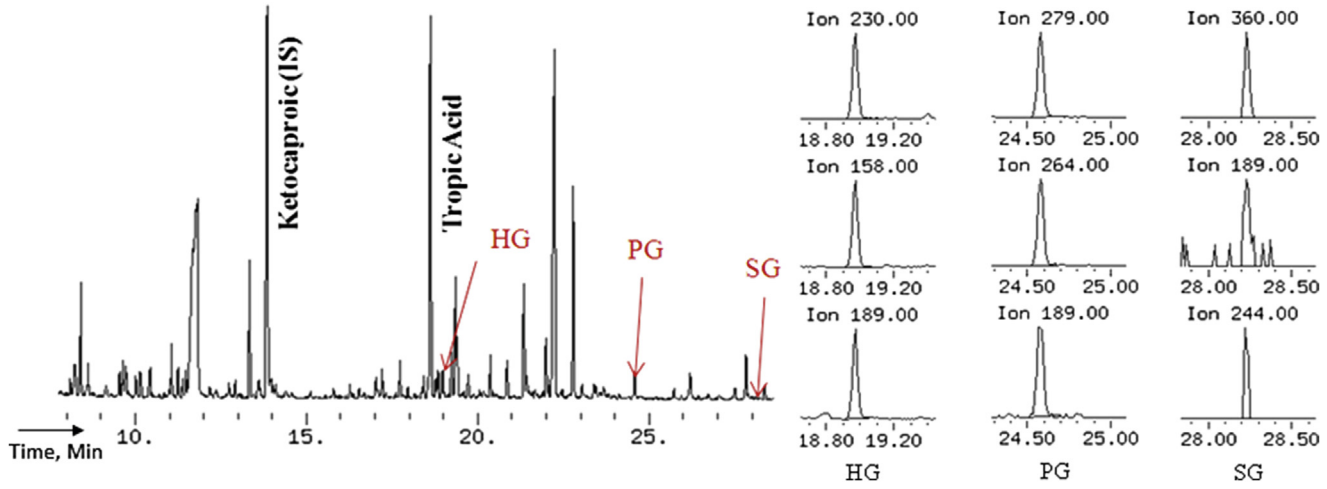


FIG. 23.6 Urine organic acid profile from a patient with medium chain acyl-CoA dehydrogenase (MCAD) deficiency. Total ion chromatogram shows very small peaks of hexanoylglycine (HG), propionylglycine (PG) and suberylglycine (SG). These peaks can be easily identified by selected ion extraction.

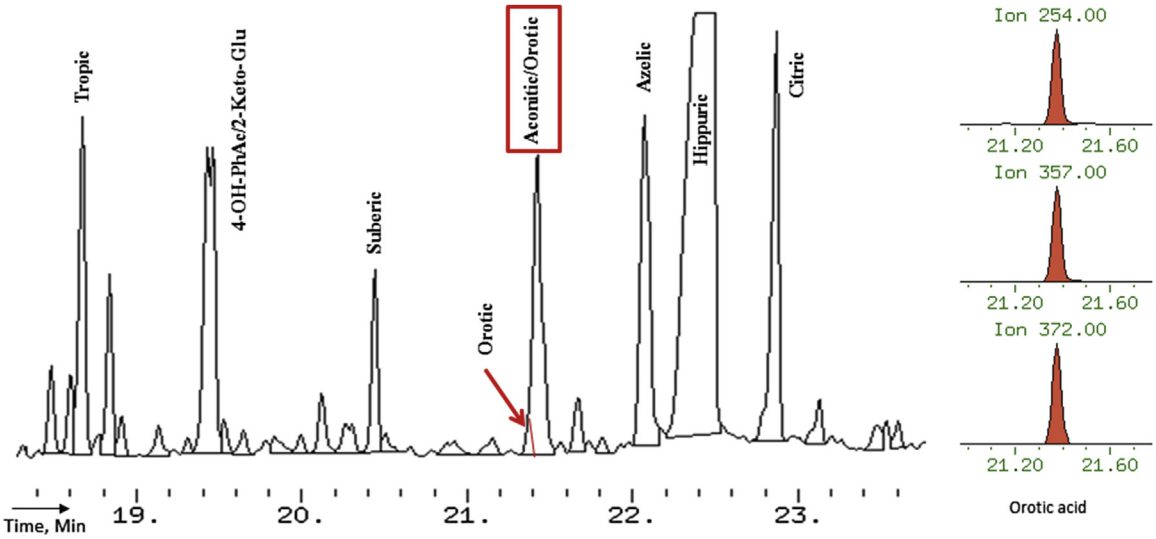


FIG. 23.7 Urine organic acid profile from a patient with ornithine transcarbamylase (OTC) deficiency. Ornithine peak is small and coelutes with acotinic acid. Selected ion extraction can easily identify orotic acid.

clinically important elevations seen in certain disorders. These small elevations are easy to miss when performing full ion scans, but can be detected using selected ion monitoring. Figs. 23.6 and 23.7 illustrate the clear detection of small elevations when using selected ion monitoring. Table 23.4 lists certain challenges in the analysis of organic acids [20,21].

Fatty acid oxidation defects

Mitochondrial fatty acid oxidation (FAO) is a principal mean of producing energy, particularly during a fasting state. It produces NADH and FADH which are used to produce ATP by oxidative phosphorylation. FAO also generates ketone bodies, acetoacetate and 3-hydroxybutyrate, which are utilized by the brain to produce energy. FAO defects lead to fasting intolerance and accumulation of fats in various tissues such as the liver, spleen and muscle. Plasma or a whole blood acylcarnitine profile is the best test for the diagnosis of FAO defects. An acylcarnitine profile can also diagnose a number of organic acidurias. In fact, in newborn screening, certain FAO defects and organic acidurias are screened through a whole blood acylcarnitine profile. Tandem mass spectrometry with electron spray ionization is the primary method for the analysis of acylcarnitines [22]. In a typical method, acylcarnitines are extracted from plasma or whole blood on filter paper

TABLE 23.4 Challenges in organic acid analysis.

Disorder/condition	Elevated compound(s)	Issue	Possible solution (S)
Glutaryl-CoA dehydrogenase deficiency	3-Hydroxyglutaric acid	Small peak, May coelute with 2-hydroxyglutaric acid	Selected ion extraction, Plasma acylcarnitine profile
Medium-chain acyl-CoA dehydrogenase deficiency	Hexanoylglycine, Suberylglycine, Phenylpropionylglycine	Small peaks particularly in non-fasting patient	Selected ion extraction, Plasma acylcarnitine profile
Orotic Aciduria, Ornithine transcarbamylase (OTC) deficiency	Orotic acid	Small peak, Coeluting peaks	Selected ion extraction
Tyrosinemia Type 1	Succinylacetone	Small peaks, Multiple peaks	Specific ion extraction, Oximation
Canavan disease	N-Acetylaspartate	Small peak	Specific ion extraction
4-Hydroxybutyric aciduria	4-Hydroxybutyric acid	Small peak, co-elutes with urea	Urease treatment, Specific ion extraction
Malonic aciduria	Malonic acid	Small peak, co-elutes with urea	Urease treatment, Specific ion extraction
Drug Treatment: Carbamazepine, Ethosuximide, Levetiracetam Phenobarbital, Salicylate, Valproate	Drugs and their metabolites	Interfering peaks	Specific ion extraction, Levetiracetam coelutes with Tiglylglycine
Bacterial contamination	3-Hydroxypropionic acid, 4-Hydroxyphenylacetic acid, D-Lactate	Spurious peaks	Proper sample collection and storage

by methanol and/or acetonitrile containing isotopically labeled acylcarnitines as internal standards. The extract is then dried and acylcarnitines are butylated using butanol/HCl. Commonly used MS/MS analysis involves flow injection of butylated extract, electrospray ionization and precursor ion scan. Quantitation is based on comparing signals of unknowns with that of calibrators and internal standards. Multiple reaction monitoring (MRM), particularly for low molecular weight acylcarnitines, may also be carried out. In flow injection analysis, isobaric compounds are not separated and may result in various types of interferences. Certain challenges in the analysis of acylcarnitine are listed in Table 23.5.

TABLE 23.5 Challenges in acylcarnitine analysis.

Interferences in acylcarnitine analysis (Butylation method):
- Glutamic acid – Falsely high C2
- Cefotaxime – Falsely high C16:1
- Formiminoglutamic acid (FIGLU, mass 287) - Falsely high C4 due to isotopic interference from 288
- Plasticizer - Falsely high C4

Isobaric compounds that are indistinguishable by flow injection:
- Isobutyrylcarnitine/Butyrylcarnitine
- Isovalerylcarnitine/2-Methylbutyrylcarnitine/Pivaloylcarnitine
- 3-hydroxyisovalerylcarnitine/2-Methyl-3-hydroxybutyrylcarnitine
- Tiglylcarnitine/3-Methylcrotonylcarnitine

Lysosomal storage disorders

Lysosomes are intracellular organelles that contain hydrolytic enzymes which are responsible for the degradation and recycling of macromolecules such as glycosaminoglycans or mucopolysaccharides, oligosaccharides, glycoproteins and lipids. Lysosomal storage disorders are due to defects in specific enzymes within the lysosome, or defects in the transport of enzymes into the lysosome. This leads to an accumulation of macromolecules within lysosomes, resulting in organ dysfunction. Over 50 inherited lysosomal storage disorders have been identified. Diagnosis of lysosomal disorders is made by measuring specific compounds and/or enzyme activities. Genotyping is also frequently performed to diagnose - or to confirm the diagnosis of - lysosomal disorders. Specific compounds are analyzed by thin-layer, paper or high-performance liquid chromatography. Enzyme assays are performed on leukocytes, skin fibroblasts or tissue biopsies. Genotyping is done on DNA isolated from blood or tissue cells. Since glycosaminoglycans or mucopolysaccharides, oligosaccharides, glycoproteins and lipids are heterogeneous group of compounds, their analysis can be challenging. The measurement of enzyme activities can also be challenging and is performed only by a limited number of laboratories. Many states screen for selected lysosomal disorders in dried blood spot.

In addition to the challenges discussed above, certain lysosomal disorders present with a unique issue of pseudodeficiency [23,24]. In pseudodeficiency, which is due to allelic variants, enzymatic activity is significantly reduced in a healthy person. Pseudodeficiency for at least nine lysosomal enzymes, including arylsulphatase A (ARSA gene), β-hexosaminidase (HEXA gene), α-iduronidase (IDUA gene), α-glucosidase (GAA gene), α-galactosidase (GLA gene), β-galactosidase (GLB1 gene), a-fucosidase (FUCA1 gene) and β-glucuronidase (GUSB gene) have been reported. Although this condition is rare for most lysosomal disorders, its frequency for arylsulphatase A is estimated to be 7.3–15%. Therefore, it is important to keep pseudo-deficiencies in mind to avoid diagnostic confusion.

CONCLUSIONS

Biochemical genetic laboratories face unique preanalytical, analytical and post-analytical challenges that can lead to inaccurate and erroneous results which may result in wrong diagnosis or missed diagnosis of inborn errors of metabolism. Attention must be paid to various processes and procedures for the correct diagnosis of inborn errors of metabolism.

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CHAPTER

24

Sources of pre-analytical, analytical and post-analytical errors in the microbiology laboratory

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INTRODUCTION

The Centers for Medicare & Medicaid Services (CMS) issued a section of the federal regulation entitled "Standards and Certification: Laboratory Requirements" which enacts the Clinical Laboratory Improvement Amendments (CLIA) law passed by Congress in 1988 (CLIA '88). These regulations establish the quality standards for laboratory testing performed on human specimens. The regulations have defined the "path of workflow" for clinical laboratory specimens as the totality of all pre-analytical, analytical, and post-analytical processes. This workflow begins with test specimen acquisition, ordering, testing and culminates with reporting of the results [1].

Submitting specimens to the clinical microbiology laboratory requires attention to many details during the pre-analytic, analytic and post-analytic stages of the specimen life span. Appropriate collection is required for applicable interpretation of results, which helps direct proper therapy for the patient, and keeps cost for collection materials down to a minimum.

Each laboratory is required to have a policy with criteria for acceptable submission, handling and if necessary, referral to a CLIA-certified laboratory. This policy should include patient preparation, specimen collection, labeling, storage, conditions for specimen transportation, processing, acceptability and rejection, and documentation of date and time the specimen was received [2]. This chapter describes the types of clinical laboratory errors that can occur and at which points they can occur. Quality improvement measures can be implemented in order to reduce and prevent these errors.

PRE-ANALYTICAL ERRORS

The pre-analytical stage is the most error-prone of the microbiology workflow process. The pre-analytical stage includes test selection, test ordering, specimen collection and labeling, transport, storage and processing. The following sections describe the appropriate steps that should be taken to ensure the optimal process of the pre-analytical stage.

Test selection

The laboratory medical director is responsible for deciding which tests are available for ordering at their facility. Laboratory tests are chosen based on the applicability for the patient population in terms of disease prevalence and treatment options for improved outcomes due to early detection, in addition to a satisfactory test sensitivity and specificity [3]. When considering the implementation of a new test, a study on cost-effectiveness can provide the necessary information to determine whether the test is beneficial to both the patient and laboratory. Assay efficiency and effectiveness should also be taken into consideration [4]. In his article, Hernandez describes the need to allow testing based on cost per specific outcome rather than a test solely driven by financial considerations [4].

Tests ordered by healthcare professionals should be clinically indicated and necessary to help the clinician optimize patient care. Communications with a clinical microbiologist during daily "round discussions" were shown to decrease antibiotic costs at a teaching hospital when compared to limited consultation at a satellite

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facility [5]. In addition, the clinician and/or infectious disease specialist may speak to a pharmacist or infection prevention specialist for recommending laboratory testing when appropriate [6].

The clinical microbiology laboratory is, at times, pressured to perform exhaustive, expensive and clinically irrelevant testing which can mislead the physician into an erroneous diagnosis and inadequate therapy [7]. The microbiology laboratory director can assist with antimicrobial stewardship to provide appropriate diagnostic testing in the microbiology laboratory. The goals of this type of stewardship are to select the right test for that particular patient, to generate an accurate and clinically relevant result within the appropriate time and to optimally influence clinical care while conserving patient and health care costs.

Test ordering

Requesting an order should be written or submitted via an electronic format by an authorized person. The requisitions must include the person ordering the test, the laboratory submitting the specimen, patient name, sex, age or date of birth, source of specimen, time and date of collection and for PAP smears, the patient's last menstrual period with an indication of whether the patient had a previous abnormal report [8]. Paper requisitions that arrive to the laboratory without the appropriate information are rejected. Under CLIA regulations, a verbal test order may be acceptable, and the laboratory must solicit written or electronic authorization within 30 days of the receipt of the order. Electronic test ordering has decreased errors in ordering [9].

The test that the clinician is requesting should be clear on the requisition or electronic system. A test order for "viral PCR" is not acceptable because molecular tests for viruses are tested individually, e.g., HSV, CMV and VZV. Each test requires an individual order. The specimen that is collected from the patient should be appropriate for each test. For example, the most common diagnostic instrument used for TB PCR is approved by the Federal Drug Administration (FDA) for the testing of sputum samples. Any other patient specimens, such as cerebral spinal fluid or pleural fluid, should not be tested on the same instrument because they are not approved and releasing results with these specimens would be considered a laboratory-developed test (LDT). The microbiology laboratory must conduct an extensive verification for the fluids which were not indicated for the intended use by the FDA [10].

Clinicians should be made aware of tests that are available in the clinical microbiology laboratory, especially when new tests are introduced. Laboratory professionals should provide education to the hospital staff so that they are aware of these tests as well as provide educational interventions to reduce overutilization of testing. In addition, clinicians must be aware of the timing of various infectious diseases as some tests should be ordered during certain seasons during the year. For example, testing for influenza is warranted during the peak of flu season, which is between October and February, but other respiratory viruses such as enterovirus should be considered during the summer. In addition, the use of a molecular influenza testing method is preferable to an antigen test for the inpatient hospital population. Molecular influenza tests have a higher sensitivity and have rapid turnaround times [11]. Appropriate types of tests will depend on the time in the disease course for the patient, especially acute versus chronic or pre-treatment with antibiotics.

Specimen collection

The collection container for specimens will vary depending on the type of specimen and test to be performed. All containers must be sterile and improper collection will cause the rejection of specimens. Some types of samples have an optimal time better suited for collection. For example, sputum samples collected for the purpose of isolating acid fast mycobacteria should be collected first thing in the morning for acid fast smears and culture. Many microbiology laboratories are better staffed during daytime hours with less personnel during nights and weekends. A schedule of test runs and other available delivery and receiving times for specimens should be disseminated by the laboratory so that the specimens collected are not sedentary [12].

The proper collection of specimens is important because a poorly collected specimen may lead to failure in isolating the organism or in isolating normal flora that will give erroneous results. Certain sites should not be cultured due to high frequency of colonizing normal flora, such as those found on Foley catheters or indwelling catheters [13]. A cotton swab is not an optimal choice for wound or body fluid cultures. While a routine culture may be ordered and set up, bacteria that have attached to the cotton may not release onto the media and will not grow. Special swabs, flocked swabs, have been developed that are made from nylon fibers and are designed to release all material into media which can then be cultured onto various specialty plates including those that are optimized for the growth of anaerobes, fungi, and acid fast mycobacteria. When a tissue is sent for biopsy, the largest amount of tissue will yield the highest amount of bacterial growth [12,14]. Specimens received in biohazard bags are not considered sterile. Information should be distributed to the clinicians on the volume required to perform a test.

Specimen labeling

All specimen containers must be labeled with two patient-specific identifiers. These may include patient name, date of birth, hospital number, social security number, requisition number, accession number, or unique random number [2]. Anytime an unlabeled specimen is received by the laboratory, it will be rejected. An unlabeled specimen received in a biohazard bag with a paper requisition inside the bag is not acceptable. There is a possibility for mix-up with the non-labeled specimen container and a label or requisition printed on the same printer. Some specimens are not easily recollected, such as a brain biopsy and in these cases, a physician can sign a release form acknowledging the patient sample was unlabeled or the specimen label does not match the requisition.

Specimen transport

Laboratories may require the transfer of specimens to either a central laboratory or an outside reference laboratory and care must be taken to ensure the appropriate specimen transport conditions. These include transport in the appropriate temperature conditions as well as protection so that the specimen containers are not damaged and do not leak. Couriers may use hard pack coolers with ice packs for specimen transport from one location to another. If a specimen requires overnight transport, labs must check with the individual reference laboratories to determine appropriate temperature conditions.

Special transport media is available to preserve microorganisms during the transport process. For example, Cary-Blair media is used to preserve stool specimens, viral transport media and universal transport media will preserve nasopharyngeal swabs and fluids, tissues, and lesion swabs for the detection of viruses, *Mycoplasma* spp. and *Ureaplasma* spp. Anaerobic transport media is usually a semi-solid media that contains sodium thioglycollate, a reducing agent that provides an oxygen free environment.

Specimen storage

Miller et al. published a comprehensive Infectious Diseases Society of America (IDSA) guideline for the ideal collection, transportation times, and types of containers for various specimens [13]. Room temperature is acceptable for most specimens before they reach the laboratory, except for viral transport media used for viral culture, which should be transported on ice. Samples must be processed onto appropriate media or prepared for serological or molecular methods within 2 h. Specimens that need to be set up for aerobic and anaerobic bacterial culture and virus culture should be processed immediately in order to preserve organism viability.

Specimens collected should not be left at room temperature overnight, especially if refrigeration is required to preserve the specimen. The viability of bacteria and/or viruses will decrease depending on various factors including temperature and storage conditions [13]. Additionally, normal flora may replicate and cause overgrowth in the specimen causing false positive results [15,16]. Serum and plasma collected in appropriate tubes should be separated from blood to preserve antibodies and prevent bacterial growth if testing will be performed at a reference laboratory and not performed the same day it was collected [12,17].

Specimen processing

After receipt of the specimens in the laboratory, standard precautions should be used to handle the specimens to reduce possible exposure to pathogens. Most cultures can be processed in biosafety laboratory two (BSL2) conditions, which is suitable for processing specimens that can pose a moderate hazard to lab personnel and the environment. Processing of each specimen should occur within a Class I or II biosafety cabinet that will provide personnel protection from harmful agents as well as protection to avoid contamination of the specimen that is being handled. Personnel who work in the clinical microbiology laboratory must have specific training in handling pathogenic agents [18].

Laboratory acquired infections can occur through exposure from a variety of different ways. These include fixing slides for Gram stains, streaking culture plates outside of a biosafety cabinet, morphology of colonies of bacteria not looking as expected and therefore opening plates outside a biosafety cabinet and looking too closely, or results not consistent with select agent, sniffing plates, or clinician aware of possible exposure but failed to notify laboratory [19].

Deviations from any of the above pre-analytical stages are cause for specimen rejection. Laboratorians must remain vigilant with the pre-analytical processes to reduce any adverse results, which can affect the analytical stage of processing and eventually patient care. Review of systems by the manager or laboratory director is useful to identify the root cause of any identifiable mistakes and errors that occur in the laboratory.

Case study

An internal medicine resident was interested in ordering a cryptococcal antigen test in the CSF for a patient with HIV. The resident searched for the test in the

ordering system and found two tests, one that was called "crypto ag" and one called "cryptosp ag." The resident clicked on the test that read "cryptosp ag" and went home. The following day at rounds with the infectious disease fellows, pharmacology residents, and clinical microbiologists, the resident was told that they actually ordered an antigen for *Cryptosporidium* in the stool. Ordering a test in which the source is in question and cannot be found easily is a clue that it is the wrong test. The test should not be ordered with the assumption that someone will notice a comment about testing on a specimen other than what is posted in the laboratory test directory.

While the organisms in this example are similar in spelling, the organism that is under consideration as well as the sample that should be submitted for testing is very different. This issue may be corrected by making the name of the test considerably different from each other and by making the source for specimen testing clear. The clinicians should work with the laboratory professionals to help make the test name easier to understand by the ordering clinicians.

ANALYTICAL ERRORS

The analytic phase consists of the analysis of the specimen. After the specimen is processed, the diagnostic procedure along with the all products used will provide the results of the testing. In the clinical microbiology laboratory, identification of bacterial isolates can be performed with biochemical methods; however, automated systems have replaced the identification of many common organisms. More recently, rapid automated systems such as matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) and molecular methods have become more commonplace. Prior to the installation of new instruments, a verification of the instrument performance must be completed with a certain number of controls. Throughout the lifespan of the instrument in the laboratory, performance validations will need to be completed to ensure the results reported are consistent and accurate.

Susceptibility testing for antibiotics can be accomplished with automated systems, Etests, microbroth dilution, and Kirby Bauer disks. Quality control must be performed weekly for those testing systems that are used frequently, and daily if used infrequently. Manual testing requires the use of a QC organism that falls within the correct range of minimal inhibitory concentration for Etests or micrometers in diameter for Kirby Bauer disks. If the QC organism is out of range, patient results may not be released until the cause of the result is identified. Some microorganisms develop resistance over time and the laboratory must go back to a frozen stock of the QC isolate to achieve in-range results. In addition, Etests and Kirby Bauer disks must be stored at an appropriate temperature so that they fall within range when tested. Some instrument software is customized for each individual laboratory to recognize unusual susceptibility results and will alert the technician to confirm or double check the result.

Case study

A broncho-alveolar lavage (BAL) specimen from a patient with cystic fibrosis grew *Pseudomonas aeruginosa.* Additional antimicrobial susceptibility testing with ceftolozane/tazobactam is requested. The susceptibility of the patient isolate of *P. aeruginosa*, was tested for minimal inhibitory concentration (MIC) using an Etest alongside a QC organism. The following day the MIC was interpreted as sensitive for the patient. However, the QC organism was out of range for the MIC required. The technician reported the result anyway and decided the QC could be repeated another time.

The technician should have repeated both the patient isolate along with the QC organism before reporting the result. Reporting the result with an incorrect QC may potentially harm the patient if the result of the susceptibility testing its actually resistant. The out of range results could be due to the improper storage of the Etest strips, the plate media, or the incubation parameters.

POST-ANALYTICAL ERRORS

The post-analytic phase describes the steps taken after the testing is performed including reporting of test results, report reviews, results archiving and specimen storage.

Reporting

Results from testing performed should be reported accurately and promptly with enough information for correct interpretation by the clinician or health professional who will follow-up with the patient. The microbiology laboratory information management system, or LIS, must have the flexibility to report the genus and species of an organism. In some cases, more than one organism is isolated from a specimen and should be reported in an organized manner. The computer software may be used to report a standardized description of organism names and text to aid in description of the result [20].

Some microbiology laboratories may choose to add text to describe the consistent sensitivity of streptococci to penicillin and therefore explain the absence of testing

for antibiotic sensitivities. Adding these comments can help the clinician understand the results and avoid unnecessary phone calls. Additional antibiotic susceptibility testing may be requested by the clinician in the case that the patient has a penicillin allergy.

Another case in which additional text may be added is when the genus or species name of an organism has recently changed based on taxonomic revision by genome sequencing. The ease of identifying these organisms has increased, especially with the incorporation of the MALDI-ToF and genetic sequencing instruments. Updating the bacterial taxonomy in the clinical microbiology laboratory can cause confusion if not addressed in a timely manner. CAP requires that laboratories assimilate "taxonomic changes that potentially affected the choice of appropriate antimicrobials to report and/or the interpretative breakpoints to use." [21] The text may include the previous name of the organism and provide the new name. For example, the guidelines for the organisms that were once known as the "H" in the HACEK organisms, *Haemophilus* spp., has been reclassified into the genus *Aggregatibacter* spp. The change affected the interpretations for susceptibility testing that are specific to this genus and which do not affect the rest of the organisms of the group: *Cardiobacterium* spp., *Eikenella corrodens*, and *Kingella* spp [22].

An accurate Gram stain can provide early empiric treatment options for an infection before the organism is identified. Some bacteria do not follow the rules of the Gram stain and may lead the laboratory technician to report an incorrect Gram stain report. For example, *Bacillus* spp. can appear Gram negative and *Acinetobacter* spp. may appear Gram positive. False negative Gram stains may occur due to inadequate specimen or smear preparation. These errors can have a significant adverse impact on patient care [23]. Gram stain competency is required by all technicians who read Gram stains. Gram stain consistency must be monitored especially for those technicians who work alone on a shift.

In addition, the identification of an organism may be reported before the antibiotic susceptibility results and may assist in providing empiric treatment options for the patient. After the bacterial isolate grows, antibiotic susceptibility results are provided for individual organisms and a minimal inhibitory concentration may be included along with an interpretation of whether the organisms are sensitive, intermediate, or resistant to the antibiotic. These interpretations must be released accurately in order to best treat the patient and not provide inaccurate results, which may lead to the incorrect treatment.

Laboratory management should have a procedure to review results frequently to catch any results that are not appropriate. Unusual or inconsistent antimicrobial susceptibility results should be confirmed and/or retested. In the CLSI M100 document, Appendix A is called "Suggestions for confirming resistant, intermediate, or non-susceptible antimicrobial susceptibility test results and organism identification" and is a guideline that can easily be followed in the laboratory [24].

TURNAROUND TIME

The laboratory must have a procedure in place that provides the amount of time for which a result will be reported. These times should be reasonable based on the amount of time it takes the laboratory to perform the test. An example of a rapid test is an antigen test, which do not take long to complete, usually between 15 and 20 min and can be reported as soon as they are read. Molecular testing also has a short turnaround time, provided the instruments do not require a large number of specimens for batching and/or a small number of organisms that are received by the laboratory. Results for antibiotic susceptibilities provided as soon as possible will promote appropriate antibiotic use and has the potential to decrease healthcare costs [25].

Corrected reports

In the event that a result is incorrectly reported, corrections must be made and the ordering physician must be notified. Incident reports should be reviewed with staff and documentation of corrective action should be recorded. Communicating this critical result to the clinician will have an impact for the patient. While some errors are considered low-risk, others may have severe ramifications [23]. Microorganisms that are resistant to an antibiotic and reported as sensitive is considered a critical error and should be mitigated by making a corrected report and contacting the clinician or nurse in charge of the patient. Corrected reports are monitored and reviewed by the lab manager who will track the errors and ensure the lab technicians improve their methods for reporting.

RESULTS ARCHIVING AND SPECIMEN STORAGE

All results should be retained for at least 2 years. Electronic reporting has allowed this process to become easier for storage and especially for retrieval. Depending on the regulatory agency that has certified the laboratory, specimens are to be stored for some amount of time. For example, CAP requires the retention of body fluids, CSF, and serum to be held for 24 h so that the specimens can be retained for repeat or additional

testing when needed. The Association of Public Health Laboratories (APHL) has recently published guidelines that suggest how long original specimens should be held along with a positive final product, or isolate, that may be used in the future for quality control purposes or new test validation [26]. Bacterial isolates can be held for a longer period in the case that the health care provider requests additional susceptibility testing.

Case study

A 16-year-old female was admitted through the Emergency Department for a suspected community acquired pneumonia. A Gram stain on sputum cultures was reported as "Gram positive cocci". The patient was empirically started on vancomycin. The following day, the cultures were growing *Staphylococcus aureus* and the isolate was set up on an automated system for antibiotic susceptibility results that were reported the following day. The susceptibility report indicated that the bacterial isolate was resistant to vancomycin.

While not all *S. aureus* will look like clusters in a Gram stain, when bacteria form clusters, reporting this information will help the clinicians guide therapy, as it will suggest the bacteria may be a *Staphylococcus* species. Additionally, it is helpful to know when the bacteria appear as chains (indicate a *Streptococcus* spp.) or as a diplococcus (which can indicate organisms such as *Neisseria* spp. or *Streptococcus pneumonia*). The other issue with this report is the fact that the susceptibility result indicates resistance to vancomycin. This should have alerted the laboratory staff to an unusual result, as vancomycin resistant *S. aureus* (VRSA) are rare. A secondary test should be performed to confirm vancomycin resistance and if the isolate is not resistant, a corrected report will need to be issued.

QUALITY IMPROVEMENT

Various quality improvement processes may aid in the prevention of laboratory errors. The review of all systems in the laboratory: pre-analytic, analytic, and post-analytic should be a continuous program for laboratories to identify and defer problems that arise.

In order to assure that the technologist has appropriate training to identify the organisms and to operate the laboratory instruments, federal regulations require competency assessment by laboratory personnel, as published in subpart M, CLIA '88. This assessment includes six procedures including, direct observation of patient test performance, monitoring test result reporting and recording, review of quality control, proficiency testing results and maintenance records, direct oversight of instrument maintenance performance, assessment of test performance, and assessment of problem solving skills. CLIA requires competency assessment in the first 6 months of the employees hire date and once per year thereafter. Some hospitals may require certification by the American Society for Clinical Pathology (ASCP), while others may accept appropriate education and experience for hiring.

In addition, laboratories accredited by a CMS approved agency have a specific requirement for proficiency testing (PT). PT is to be performed by the appropriately qualified technicians in the laboratory on non-waived and not on waived tests. However, it is recommended for laboratories to demonstrate accuracy of testing when only waived tests are being performed. Test are differentiated into waived when cleared for home use by the U.S. Food and Drug Administration while non-waived testing have arduous regulatory requirements with more difficult methodologies [27].

A CMS-approved accreditation program must accredit each clinical microbiology laboratory. One of the most widely used programs is the College of American Pathologists, or CAP. A CMS approved program must enroll in PT, which are sent to the microbiology laboratory three times per year for each test performed in the laboratory. The tests are performed in the same manner as patient specimens and results are sent to the program agency for grading. When a laboratory fails to attain the minimum satisfactory score for a testing event in two consecutive tests, training must take place for the incorrect analyte. In the case of continued unsuccessful scores, the laboratory must perform two consecutive PT events successfully or may be required to cease testing.

In addition to monitoring the work completed by the laboratorians, as mentioned above, validation and verification of all instruments should be performed. Validation and verification of each test performed in the laboratory must be recorded and kept for the life of the test and 2 years post-retirement. In 2015, CLIA instituted the individualized quality control program, or IQCP, which allows each clinical laboratory to develop a quality control (QC) program that works best for the specific testing performed. Each test requires at least two levels of external quality control each day of patient testing. No IQCP is needed if the manufacturers QC instructions require this level of testing. If the manufacturer QC instructions are less stringent, then an IQCP can be implemented. Each IQCP requires a risk assessment for the entire testing process: pre-analytical, analytical, and post-analytical, quality control plan, and a quality assessment program. The risk assessment identifies and evaluates potential problems or errors that may occur in the testing processing. The quality control plan includes the number, type and frequency of testing

and criteria for acceptable results of quality controls. The quality assessment may include PT scores, patient result reviews, specimen rejection logs, and turnaround time reports, records of preventative measure, corrective actions and personnel competency.

While there is limited information for outcome data in clinical microbiology, outcomes have historically been measured into clinical and financial areas [28,29]. Clinical outcomes include those that affect the patient under medical care. These can be measured by length of stay, mortality, morbidity, and infection rate among others [10]. Financial outcomes can be measured by costs to the patient, the healthcare institution, and the insurers [10]. Driving the testing with financial gain is not necessarily advantageous to the patient. Laboratories should strive to have a "patient first" approach to provide high quality services to patients [9]. Outcome studies in the future can be conducted to understand the utility of new technologies. One author suggests that studies should be objective, systematic, have controlled clinical outcomes examined, and the results should be published in peer-reviewed journals [29]. Understanding the usefulness of the tests chosen by the laboratories will provide the foresight to ensure that the quality of testing is positively affecting patient care.

Possibilities for errors in the microbiology laboratory are vast, but with the overview of processes and suitable checks in place, these errors can be greatly reduced. Laboratory professionals must seek to maintain appropriate education of health care professionals and laboratorians in order to process specimens correctly. Assessment of all processes will benefit the laboratory, health care institutions, and patients to achieve quality of laboratory testing.

CONCLUSIONS

Errors in microbiology laboratory may occur in the pre-analytical, analytical and post analytical stage. Pre-analytical errors may occur from specimen collection, improper labeling or during improper transportation. Although most errors occur at pre-analytical stage, caution must also be exercised to eliminate errors during analytical and post-analytical phase.

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CHAPTER

25

Sources of errors in hematology testing

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INTRODUCTION

To understand sources of errors in hematology it is important to understand the steps involved in providing blood count values as well as interpretation of peripheral blood smears [1].

Blood specimens for complete blood counts (CBC) are typically collected in vacuum tubes that contain the anticoagulant ethylenediaminetetraacetic acid (EDTA). The blood collected in the vacuum tube is analyzed on the automated hematology analyzer for CBC results. These automated instruments have various channels. Different channels are used to obtain different counts. One channel is used for red blood cell (RBC) count and platelet count. Another channel is used to obtain the total white blood cell (WBC) count and hemoglobin level. In this channel the red cells are lysed. Some instruments have a separate channel for hemoglobin. Other channels are for WBC differential count, reticulocyte count, nucleated red cell count. Different methodologies exist to obtain the actual counts. These include impedance (based on the measurement of changes of electrical resistance produced by a particle suspended in a conductive medium as it passes through an aperture of known dimension), conductivity measurement with high frequency electromagnetic current, light scatter and fluorescence-based (flow cytometric) methods. For the measurement of hemoglobin concentration, the red cells are lysed and hemoglobin (also methemoglobin and carboxyhemoglobin) are converted to cyanmethemoglobin. The absorbance of light at 540 nm is measured to provide a hemoglobin level [2]. Each time a cell passes through the aperture a pulse is produced. The pulse height is proportional to the cell volume. The distribution curves for the volume are separated from each other with a moving discriminator. Cells with a volume between 2 and 30 fL are counted as platelets. Cells with a volume of 40−250 fL are counted as red cells. Other than the actual counts RBC and platelet histograms are also provided. Each cell volume is measured directly and the mean corpuscular volume (MCV) is calculated by averaging the volume of all the cells or by drawing a perpendicular line from the peak of the RBC histogram to the base line. The RDW (red cell distribution width, which is a measure of anisocytosis) is calculated from the RBC histogram at 20% of peak height.

The lysing reagent causes WBC to lose cytoplasm and the cell membrane collapses around the nucleus. This allows differentiating the cells as the nuclear size differences are accentuated. WBCs are counted between the range of 30 fL to 300 fL. Typically three peaks are seen in the WBC histograms. The first peak represents the lymphocytes and the third peak represents the neutrophils. All other white cells are represented as the second peak.

As discussed earlier the automated instruments are actually measuring red cell counts, volumes, and hemoglobin levels. The RDW and MCV are calculated by the instrument from the red cell volume histogram. Values for hematocrit, MCH (mean corpuscular hemoglobin), and MCHC (mean corpuscular hemoglobin concentration) are actually calculated by the instrument as following:

$$\text{Hematocrit} = \text{MCV} \times \text{RBC count}$$

$$\text{MCH} = \text{Hemoglobin}/\text{RBC count}$$

$$\text{MCHC} = \text{Hemoglobin}/\text{Hematocrit}.$$

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ERRORS IN HEMOGLOBIN MEASUREMENT AND RBC COUNT

Hemoglobin measurement is based on absorption of light at 540 nm. If the sample is turbid this may produce higher hemoglobin levels. Examples of such state include hyperlipidemia [3], patients on parenteral nutrition [4], hypergammaglobulinemia and cryoglobulinemia. Turbidity from very high WBC counts can also falsely elevate hemoglobin levels. Smokers have high carboxyhemoglobin that may falsely elevate the measured hemoglobin level. Based on a study of 1221 men, Shin et al. concluded that subjects with low testosterone and free testosterone levels also showed low hemoglobin and hematocrit suggesting that testosterone may play a significant role in erythropoiesis. However, this is a source of error not interference [5].

Large platelets may be counted by some instruments as red cells. Also, red cell fragments greater than 40 fL will be counted as whole red cells. In both situations the RBC count will be falsely high. Cold agglutinins will cause red cell agglutination *in vitro* and result in low RBC counts. If cold agglutinins are suspected, the sample should be warmed to obtain an accurate RBC count. Artificial hemoglobin containing solutions will increase hemoglobin levels without an increase in the RBC count [6].

ERRORS IN MCV AND RELATED MEASUREMENTS

If there is red cell agglutination then red cell clumps will be counted as single red cells but the volume of the estimated cell will be much higher. This may result in falsely high MCV values. If large platelets are counted as red cells then these platelets typically have less volume than a normal red cell. This would also result in falsely low MCV values but possibly increased mean platelet volume. If the patient is in a state of high osmolarity, the cytoplasms of the red cells of such patients are also hyperosmolar. When diluents are added to the blood in the analyzer, water will move into the red cells causing them to swell in size. MCV values will be higher than that in the *in vivo* state. Examples of hyperosmolar states are uncontrolled diabetes mellitus, hypernatremia, and dehydration [7]. The converse would occur in hypo osmolar states.

A true decrease in the MCHC is seen in iron deficiency anemia. However, in states of high osmolarity as discussed above there may be a false increase in MCV with decrease in MCHC. Values for hematocrit, MCH and MCHC are obtained by calculation using hemoglobin levels, RBC counts and MCV values. If there is an error in any of these values, the calculated values will also be inaccurate.

ERRORS IN WBC COUNTS AND WBC DIFFERENTIAL COUNT

Falsely-high WBC counts are more common than falsely-low WBC counts. There are several situations where the WBC count may be falsely elevated. One of the most frequent situations is high WBC count in the presence of significant number of nucleated red cells (NRBC). If an accurate WBC count is required, then a corrected WBC count needs to be performed. This can be done by some hematology analyzers by running the sample again in the "NRBC mode" or performing a manual count. Platelet aggregates and non-lysis of red cells are other causes of spuriously-high WBC counts. If the high WBC count is due to non-lysis of red cells, this may be a tip-off for hemoglobinopathies. Target cells seen in hemoglobinopathies are typically resistant to lysis. Platelet aggregates may be due to EDTA, and redrawing blood in a citrate tube may be the solution in such cases. Erroneous WBC counts with spurious leukocytosis can be seen with the presence of cryoglobulins and microorganisms. Spurious leukopenia can be seen with cold agglutinins and EDTA-dependent leukoagglutination [8].

As discussed earlier, WBC histograms have three peaks. The first peak represents lymphocytes and this peak represents WBCs with the lowest cell volume. Then it is easy to understand that when there are giant platelets or nucleated red cells or red cells resistant to lysis these may be counted as lymphocytes in some instruments, giving rise to a falsely high lymphocyte count. Hemoglobinopathies and target cells are important causes of non-lysis of red cells.

Presence of malarial parasites in red cells has been also known to increase the lymphocyte count.

In myelodysplastic syndrome if the myeloid series is affected, then hypolobated and hypogranular neutrophils can be present. Automated analyzers may no longer count these dysplastic neutrophils as such; instead they may be counted as lymphocytes.

Basophilia is typically seen in chronic myelogenous leukemia. Basophils are cells with coarse granules that may even obscure the nucleus. If the analyzer falsely recognizes all the dense granules of basophils as one single nucleus, then these cells could be counted as lymphocytes.

It is thus apparent that there can be multiple situations where the lymphocyte count is inappropriately elevated. Although falsely low lymphocyte counts are rare, falsely low neutrophils can be encountered more frequently. If there is an error in the neutrophil count it is more likely to be a falsely-low count rather than a high count. Neutrophil aggregation is a documented phenomenon and can result in a low neutrophil count.

Neutrophils have fine granules whereas the granules of eosinophils are larger. Basophils have quite large granules. If neutrophils have hemosiderin granules they may be counted as eosinophils. If eosinophils are hypogranular then these eosinophils may be counted as neutrophils. Red cells infected by malarial parasites may contain malarial pigments. Malaria infected red cells are resistant to lysis. These red cells with malarial pigments may be counted as eosinophils.

A key difference between lymphocytes and monocytes is the difference in size, monocytes being significantly larger. Reactive (activated) lymphocytes typically have more abundant cytoplasm when compared to a non-reactive lymphocyte. Their size approaches that of a monocyte. These lymphocytes thus may be counted as monocytes.

Also, it has been reported that abnormal lymphocytes such as those seen in chronic lymphocytic leukemia, lymphoblasts and leukemic or lymphoma cells can be miscounted as monocytes. When there is left shift in the WBC series, we tend to see slightly more immature cells such as bands and metamyelocytes. Cells that are more immature are naturally larger and may also be counted as monocytes. Storage of blood at room temperature and delay in running the sample on the analyzer may also contribute to inaccurate WBC differential values.

When differential counts obtained by automated analyzers are compared to differential counts performed manually, a difference in results is relatively frequent. Most often, they are clinically inconsequential. However, it is always important to correlate significantly abnormal results with morphological review of the peripheral smear.

ERRORS IN PLATELET COUNT

In certain situations, hematology analyzers are known to provide a falsely-low platelet count when the true platelet count is adequate. This may give rise to suboptimal clinical management.

Partial clotting of specimen or platelet activation during venipuncture may cause platelet aggregation. Both mechanisms may lead to low platelet counts. Checking the specimen for any clot, analyzing the histograms as well as review of the smear are all important steps to avoid misleading low platelet counts. There are various other mechanisms to explain falsely low platelet counts, otherwise referred to as pseudothrombocytopenia: anticoagulant-induced pseudo thrombocytopenia, platelet satellitism, giant platelets, and cold agglutinin-induced platelet agglutination.

Falsely elevated platelet counts are much less common than falsely low counts. Fragmented red cells or white cell fragments may all be counted as platelets giving rise to high platelet counts. Fragmented red cells can be seen in states of microangiopathic hemolysis such as disseminated intravascular coagulation (DIC). White cell fragments can be seen in leukemic or lymphoma states. Patients with leukemia, especially acute leukemia, need supportive therapy in the form of blood component transfusions. If the platelet count is falsely elevated in a patient with acute leukemia the decision to transfuse platelets may be delayed, with undesirable clinical consequences. Falsely-high platelet counts may also be seen in presence of cryoglobulins and microorganisms present in blood [9]. Grabbe et al. described a case of a treated malaria patient with a false normal platelet count. The blood smear revealed small red cells, infected by trophozoites of Plasmodium falciparum that interfered with the platelet count [10]. Giant platelets if present may produce falsely low platelet count using automated analyzers [11].

ERRORS IN SPECIFIC HEMATOLOGY TESTING

In the following section, specific selected test errors that are often seen in hematology laboratory are discussed in more detail.

Cold agglutinins

Cold agglutinins are polyclonal or monoclonal autoantibodies directed against red blood cell **i** or **I** antigens and preferentially bind erythrocytes at cold temperatures [12]. These autoantibodies are typically immunoglobulin M subtype, which may be associated with malignant disorders (for example, B cell neoplasm) or benign disorders (such as post-infection, and collagen vascular disease) and can be manifested clinically as autoimmune hemolytic anemia [13]. In the hematology laboratory with automated analyzers, cold agglutinins typically present as a discrepancy between the red blood cell indexes [13,14]. The agglutinated erythrocytes may be recognized as single cells or may be too large to be counted as erythrocytes; subsequently measured mean corpuscular volume is falsely elevated and the red blood cell count is disproportionately low. The measured hemoglobin is correct due to its independence on cell count, whereas the calculated indexes are incorrect as following: the hematocrit (red cell count x MCV) is low, while the MCH (hemoglobin/red cell count) and the MCHC (hemoglobin/hematocrit) are elevated. Hemagglutination may be grossly visible to the unaided eye [12] and microscopic examination of the peripheral blood smear would show erythrocyte clumping [13].

By rewarming the blood sample to 37 °C, the erythrocyte agglutination is alleviated and correct values may be obtained [13]. More severe cases of cold agglutinin may require saline replacement of the plasma if rewarming the sample fails to resolve the red blood cell index discrepancy.

Spurious leukcopenia due to cold agglutinins is also encountered occasionally with automated hematology analyzers. The mechanism is postulated to be an IgM autoantibody directed against components of the granulocyte membranes [15]. Cold agglutinin-induced leukopenia should be recognized as a potential cause of pseudogranulocytopenia so that white blood cell counts can be accurately reported and unnecessary evaluation of patients for leukopenia can be avoided.

Cryoglobulins

Cryoglobulins are typically IgM immunoglobulins that precipitate at temperatures below 37 °C, producing aggregates of high molecular weight [12]. The first clue to a diagnosis of cryoglobulinemia is laboratory artifacts detected in the automated blood cell counts [16]. The precipitated cryoglobulins of various sizes may falsely be identified as leukocytes or platelets causing pseudoleukocytosis and pseudothrombocytosis. At the same time, the RBC indexes are generally unaffected. Correction of the artifacts for automated counts can be obtained by warming the blood to 37 °C or by keeping the blood at 37 °C from the time of collection to the time of testing. Peripheral blood smear typically shows slightly-basophilic extracellular material, and leukocyte cytoplasmic inclusions are occasionally found.

Pseudothrombocytopenia

Pseudothrombocytopenia is caused by various etiologies, including: giant platelets [17], anticoagulant-induced pseudothrombocytopenia [18], platelet satellitism [19,20], and cold agglutinin-induced platelet agglutination [21]. Pseudothrombocytopenia may occur with giant platelets. Due to their large size, the giant platelets are excluded from electronic platelet counting causing pseudothrombocytopenia. This scenario is of particular clinical importance in patients with rapid consumption of platelets in the peripheral circulation such as in: disseminated intravascular coagulation, acute immune thrombocytopenic purpura, or thrombotic thrombocytopenic purpura. Effective platelet production by bone marrow in these cases will present with many large platelets in peripheral blood, many of which may not be identified by automated analyzers. An accurate platelet count can be obtained with a manual count using phase contrast microscopy.

Anticoagulant-induced pseudothrombocytopenia is an *in vitro* platelet agglutination phenomenon generally seen in specimens collected into EDTA [18]. It has been reported both in healthy subjects and in patients with various diseases (including collagen vascular disease, and neoplasm), and has an overall incidence of approximately 0.1% [17,22]. Although the agglutination is most pronounced with EDTA, it may occasionally occur with other anticoagulants as well, such as heparin, citrate or oxalate [18]. Since the platelet aggregates are large, the automated hematology counters do not recognize them as platelets, leading to lower platelet counts. In some cases, the aggregates are large enough to be counted as leukocytes by automated instruments, causing a concomitant pseudo-leukocytosis. The platelet aggregation in pseudothrombocytopenia is usually temperature-sensitive [18], with maximal activity at room temperature. The EDTA-induced pseudothrombocytopenia is mediated by autoantibodies of IgG, IgM and IgA subclasses [23] directed at an epitope on glycoprotein IIb [24]. This epitope is normally hidden in the membrane GP IIb/IIIa due to ionized calcium maintaining the heterodimeric structure of the GP IIb/IIIa complex. EDTA, through its calcium chelating effect, dissociates the GP IIb/IIIa complex with GP IIb epitope exposure. It is noted that in Glanzmann's thrombasthenia, a disorder characterized by the quantitative and/or qualitative abnormality of glycoprotein IIb/IIIa, pseudothrombocytopenia does not occur [24]. Interestingly, in recent years, Abciximab (a GP IIb/IIIa antagonist) has been found to be associated with pseudothrombocytopenia [25]. If anticoagulant-induced pseudothrombocytopenia is suspected a peripheral blood smear should be examined for platelet clumping [18].

Platelet satellitism has features similar to anticoagulant-induced pseudothrombocytopenia. In the presence of EDTA, platelets bind to leukocytes and form rosettes. The binding is usually to neutrophils [22] but binding to other leukocytes has also been reported [23]. The automated analyzers do not identify platelets that bind to leukocytes, resulting in pseudothrombocytopenia. Platelet satellitism is mediated by autoantibodies of IgG type directed at GP IIb/IIIa on the platelet membrane and to an Fc gamma receptor III on the neutrophil membrane [26].

Platelet agglutination due to cold agglutinins causing pseudothrombocytopenia is a rare condition. The platelet agglutination is anticoagulant-independent, usually occurs at 4 °C, and is mediated by IgM autoantibodies directed against GP IIb/IIIa [24]. Since these autoantibodies have little activity at temperatures above 30 °C, they are not associated with any clinical significance.

Spurious leukocytosis

The presence of microorganisms in the peripheral blood can result in spuriously high WBC counts or differentials by automated analyzers. Organisms that have been shown to be associated with this artifact include: *Histoplasma capsulatum*, *Candida* sp., *Plasmodium* sp., and *Staphylococcus* sp. [27]. Spurious leukopenia due to EDTA is sometimes encountered [28,29]. Leukoagglutination has been reported as a transient phenomenon in neoplasia (especially lymphoma), infections (infectious mononucleosis, acute bacterial infection, etc.), alcoholic liver diseases, and auto-immune diseases (rheumatoid arthritis, etc.). It can also occur in the absence of any obvious underlying disease, even though an inflammatory condition is often found. Other EDTA-dependant counting errors are well known: platelet clumps, and platelet-to-neutrophil satellitism. Association of neutrophil clumping and platelet satellitism has also been observed [30].

False positive osmotic fragility test

The osmotic fragility test is useful for diagnosis of hereditary spherocytic hemolytic anemia [31,32]. Spherocytes are osmotically fragile cells that rupture more easily in a hypotonic solution than do normal RBCs. Since they have a low (surface area/volume) ratio, they lyse at a higher solution osmolarity than do normal RBCs with discoid morphology. After incubation in a hypotonic solution, a further increase in hemolysis is typically seen in hereditary spherocytosis. Cells that have a larger (surface area/volume) ratio, such as target cells or hypochromic cells are more resistant to lysing in a hypotonic solution.

Conditions associated with immunologically-mediated hemolytic anemias may present with many microspherocytes in peripheral blood. Consequently, the fragility test can be positive in immunologically-mediated hemolytic anemias besides hereditary spherocytosis, but the former would have a positive direct Coombs test and the latter would not.

ERRORS RELATED TO SAMPLE COLLECTION, TRANSPORT AND STORAGE

EDTA is the typical anticoagulant used in the blood collection tubes. It can be in a dry format or as a solution. The amount and concentration of EDTA require that blood should be collected up to a specific mark on the tube. If too little blood is collected, dilution of the sample can become an issue with alteration of parameters. Relative excess EDTA in such cases also affects the morphology of blood cells. Transport of specimen should ensure that high temperatures are avoided. Red cell fragmentation is a feature of excess heat [33].

Prolonged storage will result in degenerative changes in the WBCs. This is best illustrated in neutrophils where they have a round pyknotic nucleus. To the casual observer these cells may appear as nucleated red cells. Abnormal lobulation of the lymphocyte nuclei is also another established phenomenon with prolonged storage of blood [29]. These cells may be considered as atypical lymphocytes with an incorrect implication of an underlying lymphoproliferative disorder.

Table 25.1 summarizes sources of laboratory errors in hematology testings.

TABLE 25.1 Sources of laboratory errors in hematology.

FALSELY HIGH HEMOGLOBIN
• Turbid sample (hyperlipidemia, parenteral nutrition, hypergammaglobulinemia, cryoglobulinemia, marked leukocytosis)
• Smokers (high caboxyhemoglobin)
FALSELY LOW HEMOGLOBIN
• Rare
FALSELY HIGH RBC COUNT
• Large platelets
• Red cell fragments
FALSELY LOW RBC COUNT
• Cold agglutinins
FALSELY HIGH MCV
• Cold agglutinins
• Hyperosmolar state (uncontrolled diabetes mellitus)
FALSELY LOW MCV
• Large platelets
• Hpoosmolar state
FALSELY HIGH WBC COUNT
• Nucleatd red cells
• Non-lysi of red cells (due to target cells in hemoglobinopathy)
• Giant pltelets or platelet clumps (due to EDTA)
• Cryolobulins
• Microranisms
FALSELY LOW WBC COUNT
• Leukoagglutination (due to EDTA)
• Cold agglutinins
FALSELY INCREASED LYMPHOCYTE COUNT
• Nucleated red cells
• Non-lysis of red cells (due to target cells in hemoglobinopathy)
• Giant platelets or platelet clumps (due to EDTA)
• Malarial parasites
• Dysplastic neutrophils (hypolobated neutrophils)
• Basophilia

Continued

TABLE 25.1 Sources of laboratory errors in hematology.—cont'd

FALSELY DECREASED LYMPHOCYTE COUNT
• Rare
FALSELY HIGH NEUTROPHIL COUNT
• Rare
FALSELY LOW NEUTROPHIL COUNT
• Neutrophil aggregation • Neutrophil with hemosiderin granules (counted as eosinophils)
FALSELY INCREASED EOSINOPHIL COUNT
• Neutrophils with hemosiderin granules (counted as eosinophils) • Red cells with malarial pigments
FALSELY LOW EOSINOPHIL COUNT
• Hypogranular eosinophils
FALSELY INCREASED MONOCYTE COUNT
• Large reactive lymphocytes • Lymphoblasts • Lymphoma cells • Immature granulocytes
FALSELY LOW MONOCYTE COUNT
• Rare
FALSELY HIGH PLATELET COUNT
• Fragmented red cells (in microangiopathic hemolysis) • Fragmented white cells (in leukemia) • Microorganisms • Cryoglobulin
FALSELY LOW PLATELET COUNTS
• Partial clotting or platelet activation • Giant platelets • Platelet clumps and platelet satellitism (due to EDTA) • GP IIb/IIIa antagonists • Platelet agglutination (due to cold agglutinins)
FALSE-POSITIVE FRAGILITY TEST
• Immunologically-mediated hemolytic anemias

A case study

A cardiac surgeon was following up on his patient during the first week of surgery. A CBC was done to assess current hematologic parameters. The hemoglobin level was acceptable at 12.5 g/dL. However, the RBC count was low at 2.9 million/mm^3 of blood. The values for MCV and MCH were also high. The surgeon was naturally concerned with the low RBC count and found the values for hemoglobin and RBC count discrepant. He called the pathologist to discuss the findings. The pathologist reviewed the smear and found red cell agglutination. Red cell agglutination can be seen in cold hemagglutinin disease. Since hemoglobin levels are measured after lysing red cells, whether the red cells are agglutinated or not does not matter. However, with red cell agglutination the total RBC count would be reduced. The MCV and MCH values would be falsely high. The high MCH value should have acted as a tip off for the lab technologist to preview the smear and to warm the blood prior to a repeat CBC on the hematology analyzer.

CONCLUSIONS

In this chapter common tests that are performed in the hematology section of the laboratory have been discussed. Potentially, sources of errors include all steps involved in testing. These include collection of samples, transportation, storage, and methodology used as well as intercurrent issues of the patients. It is imperative to follow procedures and protocols for all concerned to attempt to obtain meaningful, accurate values. Laboratory technologists and pathologists need to be aware of situations that may provide erroneous results. Correlation with clinical information provided or from the medical records is required in certain situations. Clinicians, if aware of issues related to possible erroneous results, will also contribute to providing appropriate interpretation of laboratory results. In essence, it remains a team effort of laboratory personnel and clinicians to provide an accurate interpretation of laboratory tests for better clinical decisions and patient management.

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CHAPTER

26

Sources of errors in coagulation testing

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INTRODUCTION

Patients with coagulation disorders may either bleed or form thromboses. Hemostasis involves activation of the clotting factors and platelets [1]. Evaluation of platelet events may include a CBC, examination of the peripheral smear, bleeding time (BT), PFA-100 and platelet aggregation tests. Evaluation of the clotting factors is typically done by PT (partial prothrombin time), and aPTT (activated partial thromboplastin time) measurements. Abnormal PT or aPTT will usually lead to mixing studies to determine whether the abnormal result is due to factor deficiency or inhibitors. If there is correction of the prolonged clotting time in the mixing study, then factor assays will be performed to identify the deficient factor(s). Inhibitors include specific clotting factor inhibitors as well as lupus anticoagulants. Inhibitor screen and inhibitor assays or confirmatory tests for lupus anticoagulants will follow. Thromboelastograph (TEG) is a test for global hemostasis. This test is gaining popularity and is being used by many clinicians and laboratories. Blood sample for coagulation tests are typically obtained in tubes with sodium citrate buffer. There are various sources of erroneous test results in coagulation testing. These sources of errors are addressed in this section.

ERRORS IN PT AND PTT MEASUREMENT

The PT measures the time required for a fibrin clot to form after addition of tissue thromboplastin and calcium to platelet-poor plasma collected in a citrated tube. PT measures the activity of factors VII, X, V, II and fibrinogen. If the aPTT is normal, then a prolonged PT is due to factor VII deficiency. The PT is relatively insensitive to minor reductions in the clotting factors. The aPTT is prolonged with deficiencies of factors XII, XI, X, IX, VIII, V, II, and fibrinogen. Just like the PT, aPTT can be normal in minor deficiencies. In general, the deficient factor has to be around 20–40% to cause a prolonged aPTT. Most laboratories use automated method for PT and aPTT measurements. Either optical or mechanical methods are employed to monitor clot formation. If measured with optical methods, shortened times may be seen with turbid plasma (e.g., hyperlipidemia, and hyperbilirubinemia). The aPTT is a test conventionally used to monitor heparin therapy [2]. It is important to properly separate plasma from platelets as soon as possible. Platelet factor 4 can neutralize heparin, thus spuriously reducing aPTT values. Factor VIII levels are reflected in aPTT measurements. Factor VIII is an acute phase reactant. Again if the aPTT is being used to monitor heparin therapy in a patient who has an underlying cause for acute phase reactants to be elevated, aPTT values may be falsely lower than expected.

ERRORS IN THROMBIN TIME MEASUREMENT

The thrombin time (TT) measures the time to convert fibrinogen to fibrin with exogenously added thrombin. Heparin, dysfibrinogenemia, elevated levels of fibrin degradation products, and paraproteins can interfere with fibrin polymerization thus falsely prolonging the TT [3]. Amyloidosis can inhibit the conversion of fibrinogen to fibrin, also prolonging the TT. In certain malignancies heparin-like anticoagulants have been known to be the cause of prolonged TT. In the following section, specific selected test errors that are often seen in coagulation laboratory will be discussed in more detail.

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Incorrectly filled tubes

Citrate tubes for coagulation tests are designed for a 9:1 ratio of blood to citrate buffer. Underfilling or overfilling of the citrate tube results in imbalances in this blood to buffer ratio and produces artificially prolonged or shortened clotting times, respectively [4]. Underfilling or overfilling results in too little or too much blood sample for fixed amount of anticoagulant in the tube. The amount of blood that fills the citrated tubes is controlled by vacuum, which maintains the proper 9:1 ratio of blood to anticoagulant [5]. Underfilling may be caused by air bubbles in the tube, vacuum loss, or by not allowing the tube to completely fill during the blood collection process. If the tube stopper is removed, it also becomes difficult to obtain the correct amount of blood and attain the proper 9:1 ratio. If the patient's hematocrit is known in advance of blood collection to be greater than 55% (such as in patients with polycythemia) or below 21% (patients with severe anemia), the amount of sodium citrate must be adjusted using the following formula:

$$C = 0.00185 \times (100 \times H) \times V$$

where:

C = Volume of 3.2% sodium citrate in milliliters
H = Hematocrit in percent
V = Volume of blood in milliliters

Errors in clotting tests due to hematocrit changes without adjustment in the citrate volume are most significant with elevated hematocrits, as even severe anemia does not significantly change the PT or aPTT.

Dilution or contamination with anticoagulants

Blood collection from indwelling lines or catheters could be a potential source of testing error. Sample dilution from incomplete flushing or hemolysis caused by improper catheter insertion can alter coagulation test results. Heparinized lines should be avoided if blood must be drawn from an indwelling catheter. If using a heparinized line is absolutely necessary, adequate line flushing must be achieved before blood collection. The National Committee for Clinical Laboratory Standards (NCCLS) recommends flushing lines with 5 mL of saline [6]. At least 5 mL or six times the dead space volume of the catheter should be discarded before blood collection. The Intravenous Nursing Standards of Practice recommends that manufacturers' instructions should always be followed on the appropriate discard volume. These guidelines also state that blood should not be acquired from various types of indwelling cannula, venous administration sets and indwelling cardiovascular or umbilical lines [7]. Even when the initial volume drawn is discarded before blood collection according to these guidelines, specimens drawn from a heparinized line are still easily contaminated with heparin. Consequently, blood for coagulation tests should be best drawn directly from a peripheral vein, also avoiding the arm in which heparin, hirudin, or argatroban is being infused for therapy [1]. Before coagulation testing, heparin may be removed or neutralized with polybrene in the coagulation laboratory; however, residual heparin may continue to cause testing interferences [8].

By enhancing antithrombin activity, heparin inhibits activated factors II (thrombin), X, IX, XI, XII, and kallikrein. In contrast, Lepirudin, Danaparoid, and argatroban inhibit only activated factor II [1]. These anticoagulants (heparin, Lepirudin, and argatroban) prolong the aPTT and interfere with coagulation tests such as factor assays and lupus anticoagulant assays. Factor assays may yield falsely-low levels whereas lupus anticoagulant may be falsely-positive.

Traumatic phlebotomy

Traumatic phlebotomy can result in artificially shortened coagulation results such as PT and aPTT. This is due to excessive activation of coagulation factors and platelets by release of tissue thromboplastin from endothelial cells. A proper free-flowing puncture technique will avoid this release of tissue thromboplastin and avoid this artifact.

Fibrinolysis products and rheumatoid factor

Fibrinolysis is mediated by plasmin, which degrades fibrin clots into D-dimers and fibrin degradation products. Plasmin also degrades intact fibrinogen, generating fibrinogen degradation products. Fibrin degradation products and fibrinogen degradation products are collectively known as Fibrin/Fibrinogen Degradation Products (FDP) or Fibrin/Fibrinogen Split Products (FSP). Assays for D-Dimer and FDP are semiquantitative or quantitative immunoassays.

Latex agglutination: Patient plasma is mixed with latex particles that are coated with monoclonal anti-FDP antibodies [9]. If FDP is present in the patient plasma, the latex particles agglutinate as FDP binds to the antibodies on the latex particles. These agglutinated clumps are detected visually. Various dilutions of patient plasma can be tested to provide a semiquantitative result known as FDP titer. Latex agglutination assays are also available for D-dimers. Various automated and quantitative versions of this assay are commercially available for D-dimers in which the agglutination is detected turbidimetrically by a coagulation analyzer rather than visually by a technologist [10,11].

Enzyme-linked immunosorbent (ELISA) assays: Quantitative ELISA assays are also available for FDP and D-dimers. The traditional ELISA method is accurate but is not useful due to long analytical time. An automated, rapid ELISA assay for D-dimers is also available (VIDAS, bioMerieux Inc) [12–14].

One of the most important limitations of D-Dimer and FDP assays is interference by high rheumatoid factor (RF) levels. This may cause false-positive results with almost all available assays. The most useful clue to detect this interference is evaluation of the DIC panel. If all values in this panel (PT, aPTT, Thrombin Time, and Fibrinogen) are normal except for FDP or D-Dimer, the presence of rheumatoid factor is most likely the cause.

PFA-100/200

The PFA-100 is a system for analyzing platelet function in which citrated whole blood is aspirated at high shear rates through disposable cartridges containing an aperture within a membrane coated with either collagen and epinephrine (CEPI) or collagen and ADP (CADP). These agonists induce platelet adhesion, activation and aggregation leading to rapid occlusion of the aperture and cessation of blood flow termed the closure time (CT). The PFA-100 has a high negative predictive value i.e., if the PFA-100 gives a normal result then with some exceptions platelet functional defects can be ruled out. These exceptions are storage pool disorders, type I von Willebrand disease and primary secretion defects. If the PFA-100 is abnormal then formal platelet aggregation testing and platelet nucleotide assays may be required to establish the underlying cause.

PLATELET AGGREGATION TESTING USING LIPEMIC, HEMOLYZED OR SPECIMEN COLLECTED FROM A PETIENT WITH THROMBOCYTOPENIA

Platelet aggregation measures of the ability of platelets to adhere to one another and form the hemostatic plug, which is the key component of primary hemostasis [1]. It can be performed using either platelet-rich plasma (PRP) or whole blood. Substances such as collagen, ristocetin, arachidonic acid, ADP (adenosine 5′-diphosphate), epinephrine, and thrombin can stimulate platelets and hence induce aggregation. Response to these aggregating agents (known as agonists), provide a diagnostic pattern for different disorders of platelet function. Measurement of aggregation response is typically based on changes in the optical density of the sample.

Platelet aggregation is affected by a number of confounding variables. Lipemic and hemolyzed samples complicate aggregation measurements since they obscure spectral changes due to platelet aggregation. Thrombocytopenia also makes platelet aggregation evaluations difficult to interpret since a low platelet count by itself may yield an abnormal aggregation pattern.

THROMBOELASTROGRAPHY

Thromboelastography (TEG) was developed more than 50 years ago. Anticoagulated whole blood is incubated in a heated sample cup in which a suspended pin is connected to a computer. The cup oscillates 4 degrees and 45 s in each direction. In normal anticoagulated blood, the pin is unaffected, but as the blood clots, any impediment in motion of the cup is then transmitted to the pin. Rotational TEG (ROTEG, also known as rotational thromboelastometry [ROTEM]) is an adaptation of the TEG in which the cup is stationary and the pin oscillates. TEG provides various data relating to clot formation and fibrinolysis (the lag time before the clot starts to form, the rate at which clotting occurs, the maximal amplitude of the trace or clot strength, and the extent and rate of amplitude reduction). Another adaption of TEG is rapid TEG. Here the lag time is reduced by addition of tissue factor.

With TEG the effects of antiplatelet medications such as aspirin and P2Y12 inhibitors are not usually detectable. For this another adaption of the TEG, the platelet mapping system, is required.

Heparin, an often used anticoagulant, is known not only to increase the lag time for clot formation but also to artifactually reduce the values of angle alpha and MA (maximum amplitude). This may lead to confusion in choosing blood products to be given to bleeding patients.

Challenges in anticoagulants and lupus anticoagulant tests

The International Society on Thrombosis and Haemostasis Scientific Subcommittee on Lupus Anticoagulant have recommended two sensitive screening tests for LAs that assess different components of the coagulation pathway [15]. These tests are clotting-time based assays, such as the dilute Russell viper venom time (DRVVT), and aPTT-based assays, kaolin clotting time, or dilute prothrombin time (tissue thromboplastin inhibition test). Lupus anticoagulants prolong various phospholipid-dependent clotting times in the laboratory since they bind to phospholipid and thereby interfere with the ability of phospholipid to serve its essential cofactor function in the coagulation cascade. Lupus anticoagulant screening assays usually have a low concentration of phospholipid to enhance sensitivity. Any abnormal (prolonged) screening result typically requires a 1:1 mixing study in which the patient plasma is mixed with one equal volume of normal plasma to

TABLE 26.1 Sources of laboratory errors in coagulation.

FALSELY PROLONGED CLOTTING TIMES
• Underfilling of citrate tube • Polycythemia • Sample from indwelling catheters (dilution or contamination with anticoagulant)
FALSELY SHORTENED CLOTTING TIMES
• Overfilling of citrate tube • Traumatic phlebotomy • Turbid plasma (e.g. hyperlipidemia, hyperbilirubinemia) in optical instruments
FALSELY SHORTENED APTT IN PATIENTS ON HEPARIN
• Delay in separation of plasma from platelets • Elevated Factor VIII (acute phase reactant)
FALSELY PROLONGED TT
• Dysfibrinogenemia, • Elevated levels of FDP and paraproteins • Amyloidosis • Heparin and newer anti-thrombin anticoagulants • Heparin-like anticoagulants (in malignancy)
FALSELY HIGH FDP AND D-DIMER
• Rheumatoid factor
FALSELY ABNORMAL PLATELET FUNCTION
• Lipidemia • Hemolysis • Thrombocytopenia
FALSELY LOW FACTOR LEVELS
• Heparin • Lepirudin • Danaparoid • Argatroban
FALSE-POSITIVE RESULTS OF LUPUS ANTICOAGULANT TESTS
• Heparin • Lepirudin • Danaparoid • Argatroban

demonstrate that the clotting time remains prolonged upon mixing. Confirmatory assays are performed if the screening assay remains abnormal after the 1:1 mixing. Confirmatory assays typically demonstrate that upon addition of excess phospholipid, the clotting time shortens toward normal. The platelet neutralization procedure (PNP) is a confirmatory assay in which the source of the excess phospholipid is freeze-thawed platelets. The hexagonal phospholipid neutralization procedure is also based on the same principle, i.e. the clotting time becomes corrected after addition of phospholipid in hexagonal phase. Note that the aPTT may or may not be prolonged, depending on the amount of phospholipid in the reagent.

In many lupus anticoagulant assays, heparin (including subcutaneous low-dose heparin) may cause false-positive lupus anticoagulant results. By enhancing antithrombin activity, heparin inhibits activated factors II (thrombin), X, IX, XI, XII, and kallikrein. Subsequently, clotting times such as PT and aPTT are prolonged and interfere with lupus anticoagulant assays. Lepirudin, danaparoid, and argatroban inhibit activated factor II and also can prolong clotting times. Before coagulation testing, heparin may be removed or neutralized with polybrene in the coagulation laboratory; however, residual heparin may continue to cause testing interferences [16]. Heparin and newer anticoagulant interferences can be suspected if the thrombin time is prolonged but the reptilase time, which converts fibrinogen to fibrin independently of thrombin, is normal. Results for lupus anticoagulant assays can be interpreted correctly in patients on Coumadin. Table 26.1 summarizes important laboratory errors in coagulation laboratory due to various entities.

A case study

Case one

A 52-year-old female who is a known case of long standing rheumatoid arthritis is under the care of an oncologist due to a recently diagnosed soft tissue sarcoma. The oncologist is concerned for chronic DIC and decides to evaluate her. Her CBC results show thrombocytopenia. Her PT and aPTT results are with normal limits. However, her D-Dimer values are elevated. The oncologist calls the pathologist to discuss the findings in this case. The pathologist reviews her peripheral smear and observes platelet clumping. The pathologist explains that this phenomenon may be seen especially with samples collected in EDTA tubes. Recollection in heparin or sodium-citrate tubes should result in an accurate and a higher platelet count. Rheumatoid factor is one example of a false positive D-Dimer test. This patient was ultimately proved not to have DIC.

CONCLUSIONS

In this chapter common tests that are performed in the coagulation section of the laboratory have been discussed. Potentially, sources of errors include all steps involved in testing. These include collection of samples, transportation, storage, and methodology used as well as intercurrent issues of the patients. It is imperative to follow procedures and protocols for all concerned to attempt to obtain meaningful, accurate values. Laboratory technologists and pathologists need to be aware of situations that may provide erroneous results. Correlation with clinical information provided or from

the medical records is required in certain situations. Clinicians, if aware of issues related to possible erroneous results, will also contribute to providing appropriate interpretation of laboratory results. In essence, it remains a team effort of laboratory personnel and clinicians to provide an accurate interpretation of laboratory tests for better clinical decisions and patient management.

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CHAPTER

27

Sources of errors in flow cytometry

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INTRODUCTION

All you immunologists do is study artifacts of Flow Cytometry.
Anonymous researcher whom nobody likes

While ostensibly wrong, the quote above is very memorable for two very perceptive points. First, the usefulness of Flow Cytometry ("Flow" to its friends) as a high-throughput cell analysis method has generated heavy reliance on it in the biomedical field, especially in Immunology and Hematopathology. Second, the quote highlights the relative ease of generating artifacts by Flow Cytometry. To be fair, all methodologies are vulnerable to artifacts and errors to some extent, however the degree to which this is true is directly proportional to the sample quality requirements, the complexity of the technology involved, and of course the human factor.

Flow Cytometry has a wide variety of applications [1]; the present chapter will focus on various Flow Cytometry errors relating to its main clinical missions of malignant and non-malignant immunophenotyping, cell enumeration and flow crossmatching. Cell sorting, DNA assays, image stream, mass cytometry, microbial flow, Ca++ flux and other exciting or exotic applications are somewhat beyond the routine clinical applications of Flow and the scope of this chapter. The information is organized in a few distinct categories of issues stemming from sample quality, technology, and the human factor, with the aim of improving prevention and recognition of errors.

SPECIMEN QUALITY

Among laboratory techniques, there is a spectrum of dependency on sample quality. On one end are tests focused on single molecules or classes of molecules. For instance, the integrity of the nucleic acids is paramount for molecular testing, but that of the cells is irrelevant. Next step on the spectrum are techniques such as histology, which aim to preserve the integrity of the tissues and cells by fixation, freezing or embedding, although being alive is dispensable. Flow cytometry requires yet a higher standard, where the biochemistry, integrity and viability of the cell have to be preserved for analysis. There is perhaps only one category of assays that requires a higher level of starting material quality, namely functional assays (such as mixed lymphocyte cultures, cytokine release assays, etc), which rely on the cell not only being alive, but also functional.

Cell viability

The most commonly encountered sample issue in any clinical flow laboratory is likely to be cell viability. Attesting this point, are the various clinical standards from regulatory agencies, which address cell viability specifically. The New York State Department of Health has issued standards mandating that clinical Flow Cytometry reports on samples with viability below 80% should be flagged as suboptimal viability as a QC caveat to results interpretation. Furthermore, peripheral blood samples with availability below 50% should be rejected on account of their replaceability [2]. Similarly, while allowing laboratories to determine their own criteria for sample acceptability, the College of American Pathologists regulations also require viability determinations and comments of sample viability [3].

It is incontrovertible that poor sample viability will lead to worse results, but it is surprisingly difficult to ascertain at what level of viability will flow analysis no longer be informative, or even misleading. In reality this is issue is much more layered than a simple cutoff number of overall viability.

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First, the attrition rates of various lineages in a sample are not equal. An overall sample viability determination is not applicable to all lineages present in the sample.

Second, viability determinations may be faulty in many situations. Most viability determinations by flow rely on DNA intercalating, laser-excitable, fluorescent, viability dyes such as 7-Actinomycin D (7-AAD). It is normally membrane-impermeable, but can pass through the membrane tears and pores that form during cell apoptosis allowing nuclear staining of any cell lacking intact membranes. This is an adequate strategy to evaluate viability in a partially compromised sample. It is worth noting however that a severely compromised sample will contain a large fraction of cells in the late stages of apoptosis. A late apoptotic cell will undergo "blebbing" segmenting its bulk into dozens of pieces, of which only one containing the nuclear remnants is likely to yield a 7-AAD$^+$ ("dead event") reading. The rest of the cellular debris, with no or low DNA content, will be 7-AAD-negative ("live") events. Any unlysed RBC will further contribute to this false "alive" population. It is therefore actually difficult to obtain an overall sample viability reading under 50%, as it would require a majority of cells to have died and few of none of them to have broken apart.

Alternatives do exist. Syto-16 is a membrane permeable, DNA binding dye, which is inactivated by Caspase activity in apoptotic cells, thus staining all nucleated cells positive, and apoptotic cells negative [4]. However, it is easy to imagine that it also has "blind spots". Any cells or cellular debris without DNA will read always dead with Syto-16 and any cells dying in a caspase independent manner (i.e. cell necrosis rather than apoptosis) will appear always alive. Fig. 27.1A illustrates co-staining of Syto-16 and 7-AAD, with overall congruent results, and small populations of discrepant interpretation of viability by the two dyes (7AAD$^+$ Syto16$^+$ and 7AAD$^-$ Syto16$^-$).

In the end, viability is an important QC indicator, but it is typically not a clinically-relevant analyte in itself. While acknowledging that the accuracy of viability determinations is imperfect regardless of method, the more important question becomes what is its effect of a significant loss of viability on assay results? Simply put, dead cells and cellular debris stop behaving like their normal, alive counterparts. In some cases, dead events dim the level of expression of some markers. In the example given (Fig. 27.1B) a fraction of granulocytes, which are 7-AAD$^+$, display a stinking reduction of SSC properties (loss of granularity) and concurrent dimming of CD45 expression. The ruptured, convoluted cell membranes and denatured proteins contained within dead cells causes them to become "sticky", gaining a weak and nonspecific affinity for all or most staining antibodies. This feature is easily noticeable, because sticky events appear as a flattened, diagonal (lower left to upper right) population in most two-dimensional dot plots, almost irrespective of the staining antibodies (Fig. 27.1B, right panel). Most cell physiologically normal cell populations appear as round or oval shaped cluster distributions in two dimensional dot plots, a consequence of the fact that the two parameters stained vary independently from each other (i.e. the expression of one marker does not depend on the other). This is patently not true in non-specific adhesion, as the stickier an event is the more staining antibodies it will pick up, hence the diagonal shape. The artifact becomes a particular concern in the evaluation of double positive populations of interest, such as CD34$^+$CD117$^+$ myeloblasts or CD38$^+$CD138$^+$ plasma cells (Fig. 27.1C).

Solution: An ounce of prevention being worth a pound of cure, preservation of sample viability is the obvious best solution to this problem. Keeping an open line of communication with the clinical and transport services, informing them of the best practices for sample collection, preservation, ideal transport times, anti-coagulants, sample storage, etc may improve the quality of material received. However, realistically speaking, these factors lie outside the Flow laboratory's direct control at most institutions. Moreover, a variety of samples have poor viability at collection. In our experience, body fluids and non-lymphoid soft tissue surgical specimens often display a significant amount of necrotic/apoptotic hematopoietic cells. Therefore, a strategy to deal poor viability is necessary.

A first step should be the routine evaluation of overall sample viability, which could immediately give an estimate of artifact likelihood to an experienced flow analyst. While a simple Trypan stain by microscopy would do, the use of a Flow viability dye in combination with CD45 staining allows the evaluation of individual cell lineages, informing the flow analysis which cells are compromised and which are likely to be informative (Fig. 27.1). Elimination of cellular debris from analysis by gating is an easy method of cleaning up the dot plots of artifacts. The forward and side scatter measurements are proportional to the size and granularity of the events analyzed. Cellular debris typically can be assumed to be of lesser size and complexity than the cells which originated it, clustering in the lower left corner of FSC/SSC plots.

When exclusion gating proves insufficient, a second trick can be used. Debris is highly susceptible to permeabilization treatments. Most Flow intracellular staining techniques employ a membrane permeabilization step, which relies on a mild, low concentration amphipathic agent to open transient holes in the cell membranes allowing staining antibodies to enter intracellular space. Live white blood cells are resilient to this treatment and recover membrane integrity, while debris and red blood

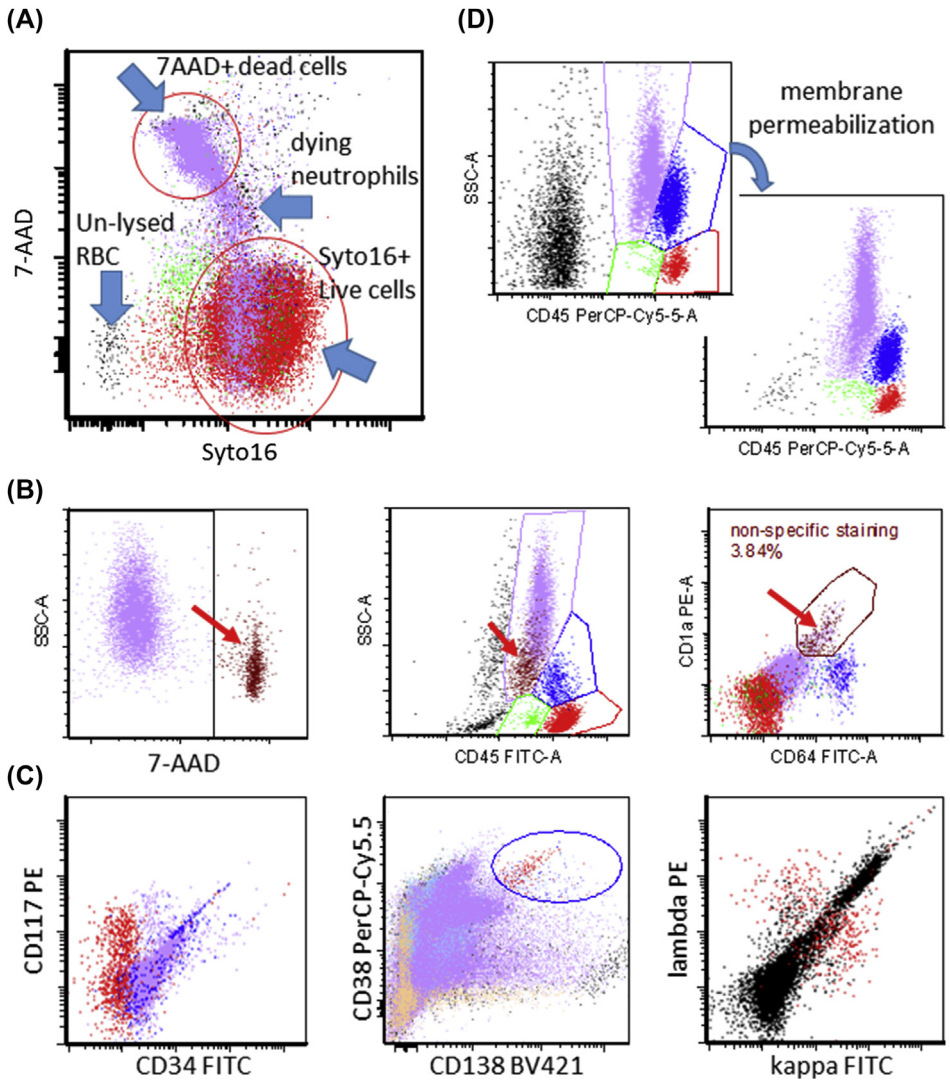


FIG. 27.1 Detection and common immunophenotypic changes of dead cells. (A) 7-AAD and Syto16 co-staining of a BMA sample with a significant fraction of dead and degranulating neutrophils. (B) non-viable neutrophils (in brown) lose granularity by SSC, dim CD45 expression (middle panel), and stain nonspecifically with a variety of markers (right panel). (C) nonspecific staining on dead cells affects all channels and may interfere with the analysis of populations of interest, such as myeloblasts (left panel, overall sample viability 85%), plasma cells (red artifact, middle panel, overall sample viability 77%), and kappa lambda (right panel, sample viability 96%!). (D) Dead cells and cellular debris from a poor quality sample (left panel) disappears after membrane permeabilization was used for intracellular staining.

cells do not and often simply disperse during permeabilization (Fig. 27.1D). Thus permeabilization acts as a second lysing step, and a persistent aberrant population which disappears in the intracellular staining tubes can likely be dismissed as an artifact.

Finally, density gradient centrifugation ("Ficoll") is a very effective method of ridding a sample of dead cells and debris. It utilizes the buoyancy of mononuclear cells to separate them from the heavier granulocytes and red blood cells. Well known to researchers, dead cells and cell debris also lose their buoyancy along with membrane integrity, so ficoll'ed samples tend to have a near perfect viability. Myeloblasts and hematogones (normal or neoplastic) are certainly buoyant (author's unpublished data), mature neutrophils, eosinophils and basophils are not, and the relative densities of other myeloid maturation stages are unknown. Since the myeloid immature stages are of interest in leukemia diagnosis, the technique has a limited use in clinical flow cytometry despite its outstanding effectiveness. Nevertheless, it has niche applications, for example in flow cytometry crossmatch for transplantation. Flow crossmatch is carried out between transplant recipient serum and donor T and B cells. Any monocytes or granulocytes are simply an interfering factor in the assay since they can absorb a large amount of donor specific antibodies from the recipient serum diminishing T and B cell reactivity. Moreover, the donor cellular material

used in crossmatching often consists of cryopreserved samples or cadaveric donor samples, both of which typically display poor viability. Thus, density gradient centrifugation to "clean up" mononuclear cells is not just beneficial, but almost required for flow crossmatch.

Sample transit times

An issue closely related to viability is sample transit time. In our experience, most samples submitted for Flow cytometry analysis arrive in lab the same day of draw or next business day. Occasionally however, the "next business day' is Monday, samples are misrouted, or Flow reflexive testing is added for old samples. Such samples are less than ideal for Flow analysis mainly due to a noticeable loss of cell viability. However, loss of viability is not uniform and several lineage specific effects are recurrently noted.

The earliest effect is the near complete loss of basophil populations in PB samples within 24 h after collection. When very fresh PB samples (<12 h) are analyzed by Flow Cytometry, basophil populations are quite prominent. Basophils have a $CD45^+$ (somewhat dim) expression profile, comparable to the bulk of granulocytes. Puzzlingly, they exhibit a low SSC profile only slightly higher than lymphocytes, despite their notable morphological granularity (the main cellular feature that generates SSC). Thus they lie squarely in the middle of what most labs refer to as "the blast gate", and they can represent a possible confounder for the myeloblast analysis for the inexperienced flow analyst. Basophils express CD9, CD13, CD22, CD25 (dim), CD33, CD36, CD38 (bright), $CD45^+$ (dim), and CD123 (bright), but lack HLA-DR and the typical immaturity markers CD34, CD117 of myeloblasts [5].

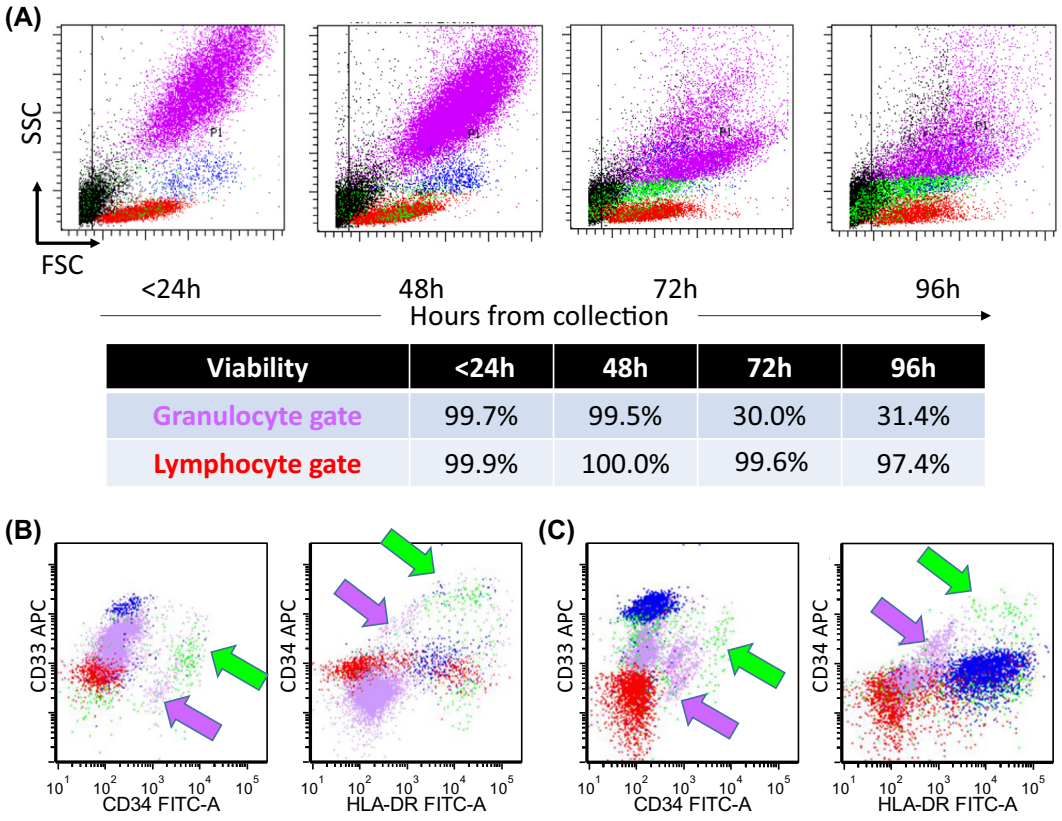
A second lineage-specific effect is the gradual die-off of neutrophils. This is not surprising since neutrophil lifespan has been estimated to be as low as 5.4 days [6]. Therefore, nearly 20% of neutrophils sampled can be assumed to be in the last day of their lifespan, and every 1-day delay in sample delivery may impact the neutrophil population by a 20% hit to its viability. This is of course an oversimplification (since physiological in vivo processes such as degranulation to stimuli and macrophage phagocytosis are unlikely to be recapitulated in a vacutainer), but nevertheless one that roughly matches the viability numbers experimentally obtained in our lab at 96 h in silico.

We investigated how long can a peripheral blood sample remain unprocessed and still yield an informative analysis by Flow Cytometry. A few peripheral blood samples were held at room temperature over 96 h after collection to simulate a late sample delivery, and stained with our clinical leukemia/lymphoma panels at the ~24, 48, 72 and 96-h time points. Comparison of the four time points shows that there is a dramatic loss of granulocyte viability and granularity, which happens quite suddenly after 48 h from collection (Fig. 27.2A). On the CD45-vs-SSC plot, the loss of granularity causes a large fraction of granulocytes to enter the blast gate and the monocytes become difficult to identify due to attrition and granulocyte interference. In contrast, the lymphocytes retain surprisingly good viability over the entire period (24–96 h post collection, Fig. 27.2A), and preserve their original immunophenotypes (data not shown). As an important caveat, these observations are unlikely to extend to samples without serum, which provides a nutrient rich environment for hematopoietic cells and can absorb a large amount of the oxidative and proteolytic stress released by myeloid degranulation. Our experiment also did not investigate any effects of neutrophil extracellular traps (NETs) may have on all cells in the sample.

The risks of analyzing dead granulocytes are real. In the analysis of myeloid leukemia, the loss of granularity could look like a maturation defect, changes in immunophenotype are likely to appear aberrant and may simulate myeloid disease (a false positive or overestimation of existing disease, Fig. 27.2B). Equally worrying, a large dead neutrophil population may overlap and mask a real neoplastic population leading to a false negative call.

A third cell lineage which experiences especially fast attrition is plasma cells. Fully differentiated $CD138^+$ $CD38^+$ plasma cells not only represent a miniscule fraction of bone marrow aspirate samples, but they also appear to be especially labile. Empirically, we observed that plasma cell evaluations reflexed after a weekend consistently yield no informative results. This is generally due to a failure to detect any plasma cells despite a clear population having been present during the initial evaluation, a few days prior.

Some simple statistics can be provided to back up that claim. In the past calendar month, some 84 BMA samples were submitted to our laboratory for Flow Cytometry analysis for leukemia/lymphoma analysis. From these, 8 plasma cell neoplasm cases were censored from the following calculations, and a further 50 revealed no plasma cell populations, typically due to overwhelming burden of acute leukemia or CLL disease. The remainder ($n = 26$) were analyzed for frequency of plasma cells as a function of days spent unprocessed. The samples stained on the same day of collection showed an average of 0.64% plasma cells (±0.96 SD, range 0.1%–4.0%, $n = 15$), while the samples stained the next day showed an average plasma cell content of 0.29% (±0.16 SD, range 0.1%–0.7%, $n = 10$). A single sample stained 3 days after collection showed 0.4% plasma cells. This distribution yields a trend line with a −0.19 slope and a 0.58 intercept, suggesting that approximately



Viability	<24h	48h	72h	96h
Granulocyte gate	99.7%	99.5%	30.0%	31.4%
Lymphocyte gate	99.9%	100.0%	99.6%	97.4%

FIG. 27.2 Loss of sample quality over time. (A) Changes in the flow cytometry profile of myeloid and lymphoid lineages in a peripheral blood sample held at room temperature over 4 days. The dramatic loss of granulocyte viability and granularity interferes with the analysis of the blast gate (green events) and monocytes (blue). The lymphocyte gate (red) remains the least affected population. (B and C) Presence of immunophenotypically aberrant artifacts in old BMA samples on dead granulocytes (*arrow*, in purple) interfering with myeloblast cell analysis (*arrow*, in green) in two cases of MDS. The artifacts display typical features of apoptotic cells: diagonal alignment of staining and loss of some markers (CD33).

−33% (−0.19/0.58) of plasma cells are lost to attrition per day (Fig. 27.3). While the sample size is fairly small and there is little accounting for type and burden of disease, hemodilution, and treatment, the result is remarkably similar to our practical experience. The X-intercept is roughly at day 3, which is just a fancy way to say what flow cytometry laboratories knew all along: that Friday's plasma cells are unlikely to still be there on Monday.

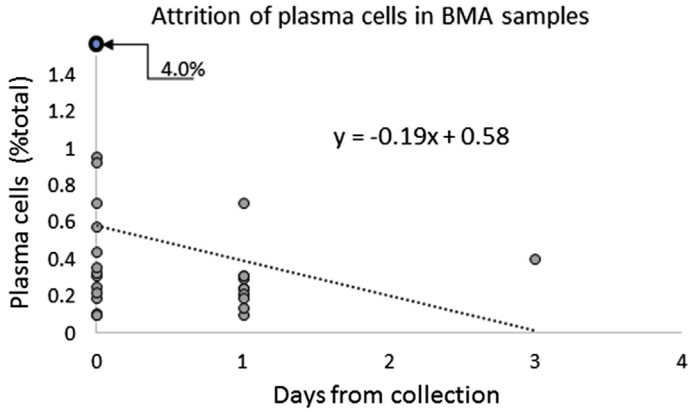


FIG. 27.3 Detection of plasma cells by flow cytometry as a function of sample age at processing; day 0 — sample processed the same day of collection, day 1 — next day.

The labile nature of plasma cells seems to be retained by their neoplastic counterparts. Therefore, this observation has important implications for plasma cell dyscrasia analysis, especially for evaluation of MRD by flow cytometry. The ideal window for accurately detecting, characterizing, and quantifying plasma cells is no longer than 24 h.

Finally, the ex vivo passage of time affects not just cells, but also some cell surface markers. ZAP70 is accepted as a surrogate of IgHV mutation status in CLL, and its expression correlates with short survival. However, objective methods of defining ZAP70 positive results, harmonized across flow laboratories have been somewhat elusive. The task is not made easier by the fact that ZAP70 is the target of some regulatory mechanisms via phosphorylation and ubiquitination [7] and ex vivo its expression in CLL cells dims over time. Sheikholeslami et al. demonstrated that different criteria for positivity apply in fresh versus old samples [8]. Typically the authors used a cutoff of >15% ($ZAP70^+$ CLL cells) to describe the disease as ZAP70 positive, in which case the ZAP70 expression showed the expected correlation with the IGVH status. However, the correlation with IGHV did not hold in samples shipped at room temperature. In these samples, lowering the cutoff to 10% was necessary to reestablish a statistically significant correlation with IGHV mutation status, suggesting loss of ZAP70 phosphorylation or protein over time.

Solution: These observations reinforce that a reliable sample transport system is crucial to proper Flow analysis. A recurrent motif is this section is that a 24 h delay in processing a sample is probably acceptable in most cases; however, bridging the weekend gap is more problematic. A Saturday shift may be the only solution in dealing with late Friday deliveries, which cannot be addressed with overtime, an unpleasant solution for technologists but preferable to sample cancellations and inconclusive results.

Clotting, cell clumping and laminar flow

The accurate data acquisition by flow cytometry is highly contingent on good laminar flow in the instrument capillaries. Flow cytometry instruments ensure that cells flow in single file at a steady rate at the interrogation point (the point where the cell stream meets the laser). The formation of micro clots or cell clumps (another frequent complication of viability loss) can disrupt the laminar flow. Fortunately, a simple tool exists that easily detects problems in the laminar flow and has been long recognized as an important quality control measure [9]; plotting the any fluorescent parameter over time should show the uniform acquisition of that marker over time. Any interruptions suggest the clogging of the capillary lines, and presumably regaining the acquisition indicates

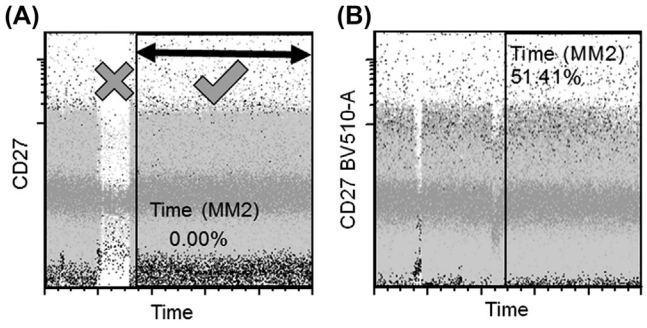


FIG. 27.4 Evidence of laminar flow disruptions during data acquisition in a fluorescence over time plot: (A) acquisition drop outs and (B) dimming of fluorescence, both of which can be excluded by gating.

spontaneous clearing of the blockage (Fig. 27.4A). A subtly different and much more dangerous event is a transient dimming of the fluorescence intensity, which affects all stained channels (Fig. 27.4B). While clearly an artifact of instrument fluidics, a simple clog cannot explain this observation, however, hypothetically turbulent flow, or vibrations defocusing the laser off the interrogation point, could cause temporary and partial loss of the scattered signal affecting all channels.

Regardless of the technical causes of the latter artifact, variability of the signal can have very negative practical consequences on data interpretation. The fluidics problems shown in Fig. 27.4, dimming the expression of CD27 on all cells occurred in concert with CD19 diming (not shown) and were observed in a myeloma MRD case. In the absence of a time plot, there is no clear way to demonstrate that a small $CD19^-$/dim $CD27^-$/dim plasma cell subset is in fact an artifact of fluidics. And it just so happens that loss of CD19 expression is one of the most sensitive indicators of neoplastic plasma cells. Some 95% of myeloma cases are $CD19^-$, albeit it is not very specific as some normal, long-lived plasma cells are also $CD19^-$. Furthermore, the $CD27^-$/dim phenotype is another frequent aberrancy in myeloma: somewhat less sensitive (40%–50% of myelomas are $CD27^-$), but much more specific (virtually never seen in normal plasma cells) [10]. To be fair, in multi tube staining panels, the suspicious population would probably not be trackable across multiple staining, nor would it likely display light chain restriction. Nevertheless, a small risk does exist that instability in the laminar flow can cause not only loss of data but also misinterpretation of the results.

Solution: To obtain quality flow cytometry results, it is imperative to use time plots and eliminate such fluidics artifacts from the analysis, by gating out the regions of non-laminar flow. Elimination of a large fraction of the acquisition will inevitably reduce the assay sensitivity (i.e. the ability to detect small populations of aberrant

cells at low frequency). Yet it is clearly preferable to the risk of generating a false positive artifact, especially in a patient with a medical history, which could reinforce the false observation.

Cell doublets

Cell doublets represent a unique scenario of cell clumping. First, two (or a few) cells can be stuck together for a variety of reasons. In Hodgkin's lymphoma B and T cells rosette together, in the context of normal antigen presentation, in T cells and antigen presenting cells form stable immunological synapses [11], and of course small clumps can form the typical way large clumps form, by aggregation of dead cells. Importantly however, doublets or small clumps tend to not disrupt the laminar flow yielding actual events with a readable immunophenotypes, positive for markers of both/all cells involved in the interaction.

Depending on the scenario, the results could be unexpected if not outright aberrant, and anywhere between informative to a confounding nuisance. Flow cytometry can be adapted to detect and analyze Hodgkin and Reed Sternberg cells in disrupted or intact rosettes, which showed clearly distinct phenotypes [12]. While rosetted Reed-Sternberg cells displayed CD45 positivity and T specific markers (CD3 and CD5), and when free, they showed a loss of CD45 and the expected immunophenotype CD15, CD30, CD40 and CD95.

In the case of physiological T-APC synapsing, cell doublets tend to be a nuisance. They could conceivably form entities with worrisome marker combinations. For instance, a monocyte interacting with a T cell (either synapsing or phagocytosing them) can read as a $CD13^+$ $CD7^+$ event, CD13 identifying it to be of myeloid origin and $CD7^+$ suggesting it to be immunophenotypically aberrant. Fortunately, such events tend to be very rare, and in many cases easily disrupt-able mechanically (vortexing) or chemically (EDTA). The extended phenotype can also easily indicate the low likelihood of a real phenomenon. Such doublets tend to be more common and much more stable in body fluids. The pleural fluid illustrated (Fig. 27.5A) features a small population monocyte/T doublets sharing the immunophenotype of both, closely matching singlet T cells (in red) and monocytes (in blue) in marker expression levels while being clearly distinct from the myeloid neoplasm present in the sample (in green).

Doublets formed by dead cell aggregates are neither informative as HL rosettes, nor as easily recognizable as T-APC interactions.

Technically, the doublets problem has an equally easy solution as the disruption of laminar flow, a graphical tool built into the data. Upon crossing the laser path at the interrogation point, all cells refract a portion of the light forward (forward scatter, FCS) which is picked up by a sensor and converted to a measurable electrical impulse. For a roughly spherical cell, this electrical

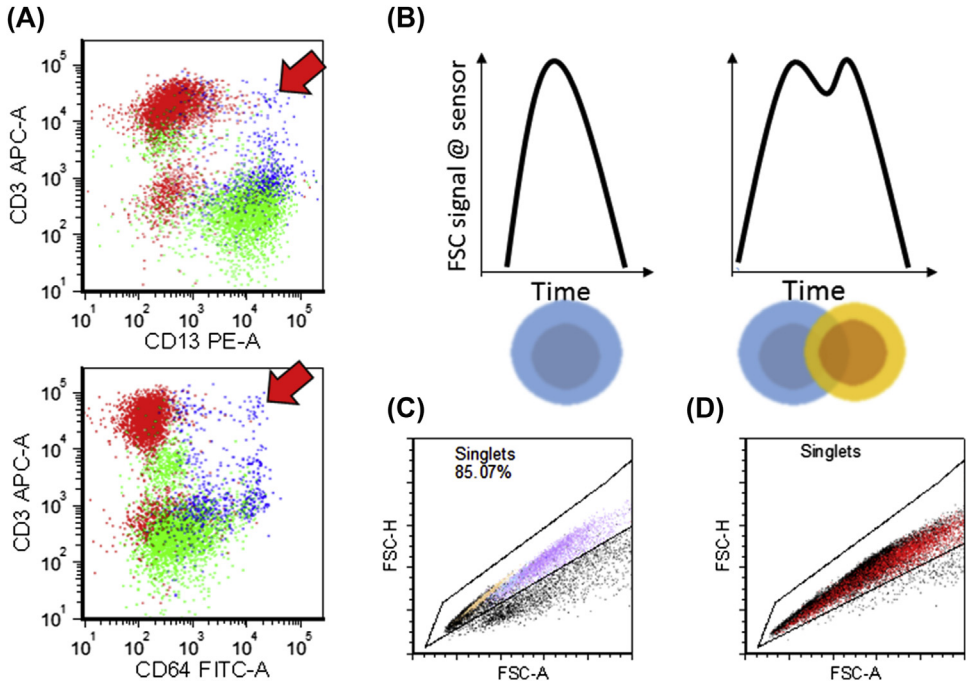


FIG. 27.5 Cell Doublets in Flow cytometry. (A) interacting T cells and monocytes form doublets presenting the immunophenotype of both cells types. (B) Differences in the FSC signal generated by singlet or doublet events. (C) Segregation of singlet events from doublets for flow analysis, using their different FSC-H/A properties. (D) The FSC-H/A profile of WBC singlets (black) and plasma cells (red).

impulse plotted over time has an approximately Gaussian appearance with the amplitude of the signal (termed FCS-H for height) being directly proportional to the bulk of the cell. Also informative is duration of the signal, directly proportional to the width of the cell, so the FSC signal can also be measured as FCS-A (area) which incorporates both the amplitude and duration. While FSC-A incorporates more information, for roughly spherical, "singlet" cells the distinction between FSC-H and FSC-A is academic, the two variables being almost perfectly correlated. However, for doublets the signal is slightly altered in a meaningful way. A cell in a doublet will generate roughly the same FCS-H as it would by itself, as the bulk of it would not change. However, hydrodynamic focusing, the technique that ensures that cells pass single file through the interrogation point, also forces doublets to pass lengthwise though it. This increases the duration of the signal (and hence the FCS-A) significantly (illustrated in Fig. 27.5B). Plotting FCS-H versus FCS-A easily identifies the doublets as the events with a high A/H ratio (Fig. 27.5C). Importantly, plasma cells may also present a slightly elevated FCS-A/H ratio, due to their elongated shape, however this increase is so subtle that they largely overlap the singlet leukocytes and it is unlikely to be picked up by this technique, or to be eliminated with a doublet exclusion gate (Fig. 27.5D).

Because the doublet analysis could be situationally informative, it is advisable that all events be included in the analysis initially, and the option to exclude the doublets can be used only if they prove problematic for the analysis.

Platelet aggregates

Another unique iteration of cell clumping issue is platelet aggregation. Platelets can be easily ignored is Flow Cytometry analysis, mainly owing to their small size which makes them indistinguishable from debris. However, when they aggregate, some clumps can easily achieve sizes comparable with cells which display some reactivity with antibodies, most importantly CD45 and CD34. Needless to say, a CD45dim CD34dim phenotype can interfere with the analysis of myeloblast populations, as well as the enumeration by flow of CD34 stem cell selections for transplantation. This artifact presents a distinctive shape in a CD45-vs-SSC or CD34-vs-SSC plot, resembling an inverted comma (Fig. 27.6). Including the CD34 dim platelet aggregates in the $CD34^+$ gate for enumeration can result in a crass overestimation of stem cell content in the sample, with potentially dire consequences for the stem cell transplant recipient. Fortunately, the CD34 expression is distinctly higher on bona fide stem cells in most samples, so gating the stems cells conservatively is the preferable option in stem cell enumeration tests, even if it does underestimate their count.

Importantly, platelets are very light and usually require hard centrifuge spins to pellet. If this is a recurrent problem, it may be a hint that the laboratory centrifuges the samples too hard or too long, and reducing the spin speed may solve or ameliorate the problem [13]. Validating a different anti-coagulant may also help.

The last few issues discussed (viability, cell clumping, doublets, micro clots, laminar flow) all have a common quality control theme, they are best addressed in the pre-analytical stage. However, should that fail, gating strategies exist which mitigate the impact of patient results. A gating strategy which enables the user to eliminate problem events is highly recommended. Flow analysis templates should be designed for sequential exclusion of debris, doublets, laminar flow problems, and auto-fluorescent events (not necessarily in this order) can make even a severely compromised sample informative once again.

Paraproteins and flow analysis

Analysis of Ig heavy and light chains is of particular interest in flow cytometry because unlike other immunophenotypic markers they can reveal clonality in the B cell lineage. Various neoplastic conditions of the B

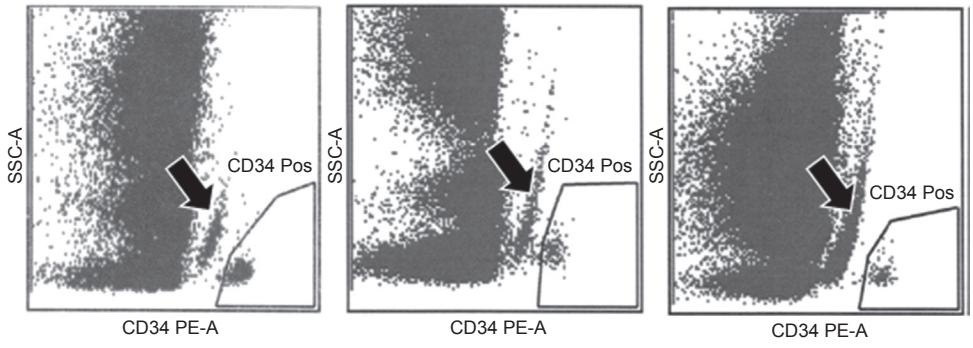


FIG. 27.6 Platelet aggregates forming a distinctive inverted comma streak (*arrows*), display dim CD34 positivity and interfere with stem cell analysis. Three cases are showcased with increasing degrees of severity.

cell lineage, MGUS and myeloma par excellence, can put out an enormous amount of monoclonal antibodies or light chains in circulation, which pose a unique set of challenges for Flow Cytometry.

If the monoclonal antibody or light chain remains in the cell suspension during staining in significant concentration, it acts a competitive inhibitor of heavy and/or light chain staining of the sample. To illustrate the point let's perform the following thought experiment. Let us assume that the total amount of cell surface bound light chain in a 100 µL volume of bone marrow aspirate from a patient is 1 unit (1U). Specifically, 1 U may represent as little as 1000 B cells × 100,000 molecules/cell, or 1 × 1E8 molecules or 1.3 × 1E-16 mol. The light chain content of the serum in the same volume of sample will far exceed the cell bound 1 U of light chain. Worse yet, in a hypothetical myeloma patient with 1.5 g/dL monoclonal Ig in circulation it translates to approximately 1E-8 mol per 100 µL, in other words 100,000,000 U of soluble and 1 U of cell bound light chain protein. If the sample is washed two times prior to flow cytometry staining, each wash diluting the serum content by 1000×, the serum monoclonal light chain content becomes 100,000 U after the first wash, and 100 U after the second. Since all B cells pellet during washes, the cell-bound light chain remains 1 U. Thus, for the purposes of flow cytometry staining for kappa or lambda light chain, that means that there is an amount of "decoy" for the anti-light chain staining antibody that exceeds the intended, cell-bound target by orders of magnitude! The problem is made worse by skipping a wash, in which case the decoy outnumbers the target by an even higher margin. A third wash would reduce the interference to negligible levels.

In the clinical case shown in Fig. 27.7A, a myeloma population is secreting lambda Ig. Insufficient washing is causing the mature B cell population in the same sample to appear unusually dim or negative for one light chain. Since the interfering paraprotein is monoclonal, it interferes with the staining of one light chain, but not the other. Thus the, normal mature $CD20^+$ B cells in the sample appear lambda negative/indistinct (staining competitively inhibited), while kappa staining appears polytypic and normal (Fig. 27.7A and B). Another clue to monoclonal interference is the perfectly normal (polytypic) staining pattern of B cells in cytoplasmic light chain staining. If the interfering antibody is soluble, the solution may be as easy as additional sample pre-washes before commencing staining.

Excess monoclonal antibodies in circulation can produce a second and distinct artifact. Fc-receptor bearing cells will adsorb the monoclonal Ab on their surface, causing them to stain like they express a monoclonal BCR. In Fig. 27.7A, a population of non-B lymphocytes clearly display lambda staining. Ironically, the staining is not necessarily a false positive nor artifact; these are

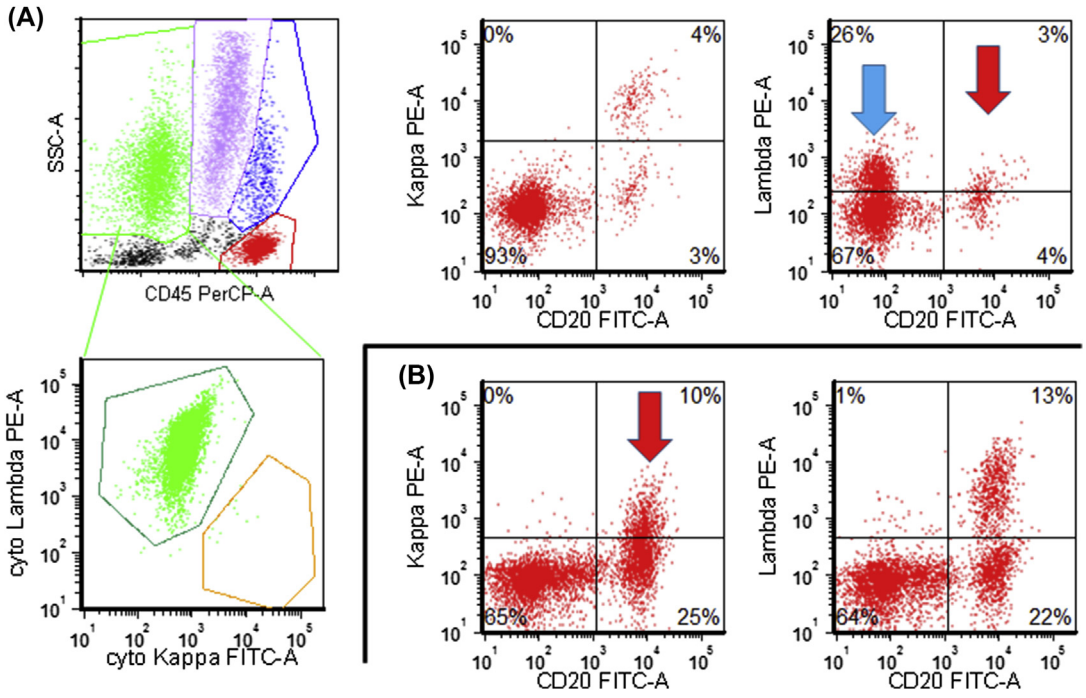


FIG. 27.7 Artifacts in samples with paraproteins. (A) In a BMA sample with a significant Ig lambda light chain restricted myeloma population (in green), the serum monoclonal paraprotein affects the lambda light chain staining of B lymphocytes (*red arrow*) and non-B lymphocytes (*blue arrow*, presumably $FcgRIII^+$ NK and T cells). (B) A similar case showing kappa light chain staining interference, but no Fc capture of paraprotein.

presumably CD16 (FcgRIII)$^+$ NK cells which have adsorbed a significant amount of the myeloma-secreted monoclonal antibody. Additional circumstantial evidence supporting this notion is the percentage of NK cells (28% of lymphocytes) roughly equal to the lambda + non-B lymphocytes. The phenomenon is also note restricted to NK cells, depending on the nature of the monoclonal Ig, CD16$^+$ NKT, CD16B$^+$ neutrophils, CD32$^+$ B, CD64$^+$ monocytes, and FcμR$^+$ cells may also display unusually high levels of heavy and light chain staining. Additional washes are unlikely to disengage captured antibodies from Fc receptors, however, our laboratory has had mixed results with the "warm-up protocol". A washed sample is warmed up to 37°C for 30 min, spun down and restrained. The results can be anywhere between marginally better and spectacularly better. This staining pattern can even be informative about the nature of the monoclonal paraprotein, since antibodies can be captured on Fc receptors (Fig. 27.7A), while free light chains cannot (Fig. 27.7B).

Excess monoclonal antibodies or light chains in circulation represent the extreme of the competitive inhibition concept and highlight the importance of adequately washing the samples. However, the problem is not unique to myeloma/MGUS samples: the normal Ig content of human serum can interfere with flow staining in exactly the same manner if not washed properly, and theoretically so can any cell surface receptor that generates soluble isoforms by alternative splicing, cleavage, or shedding, forming its own competitive inhibitor. Extensive washing is the obvious solution, however recognizing this interference pattern can also be a powerful tool in recognizing the effects of neoplastic disease even in cases when the culprit population is too miniscule and risks being missed.

Mab therapies

Monoclonal humanized antibody therapies represent a novel strategy of targeting hematological neoplasms with exquisite specificity. Fig. 27.8 illustrates the logical nomenclature of therapeutic monoclonal antibodies (based on WHO guidelines [14] and adapted from Baumgart et al. [15]), which can originate from murine, human, humanized, chimeric, Fab' fragments of Fc-containing Abs, and have tumors cytolytic, immunomodulatory, and many other functions. The difficulties posed by these are varied depending on their target, structure, effects, and half-life in circulation. The discussion here will focus on Rituximab and Daratumumab, two frequently encountered mAb therapies in leukemia/lymphoma/myeloma diagnostic work.

As the name suggests, Rituximab is a tumor-targeted ["tu"], chimeric ["xi"], monoclonal antibody ["mab"] directed against human CD20 molecules. The rituximab heavy chain is gamma1 and its light chain is kappa. Rituximab demonstrates intense cytotoxic activity, completely depleting the mature B cells within days [16], and remaining at detectable levels in circulation for up to 6 months [17]. Its target, CD20, is largely B lineage marker present on B cells from the late hematogones stages to plasmablast stage. Plasma cells completely lose CD20 expression, however it can be retained in about 30% of myeloma cases [9]. Partial, dim CD20 expression is observed on a subset of human T cells.

The immediate flow cytometric effect noticed with Rituximab during the first few days of treatment is a complete lack of CD20 on the remaining circulating B cells (Fig. 27.9A). A possible explanation is that the cytolytic activity has ceased due to complement depletion [18]. However, it is puzzling that the kappa/lambda

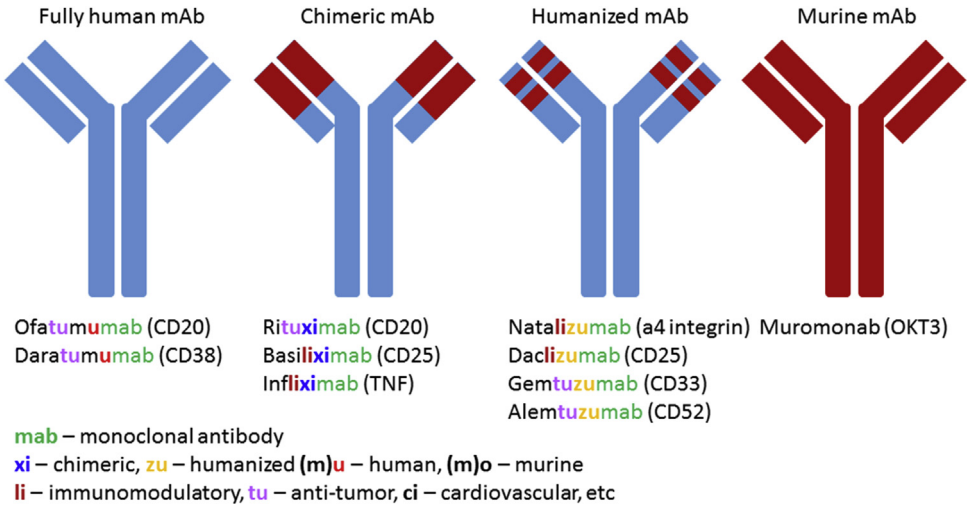


FIG. 27.8 The nomenclature of therapeutic monoclonal antibodies.

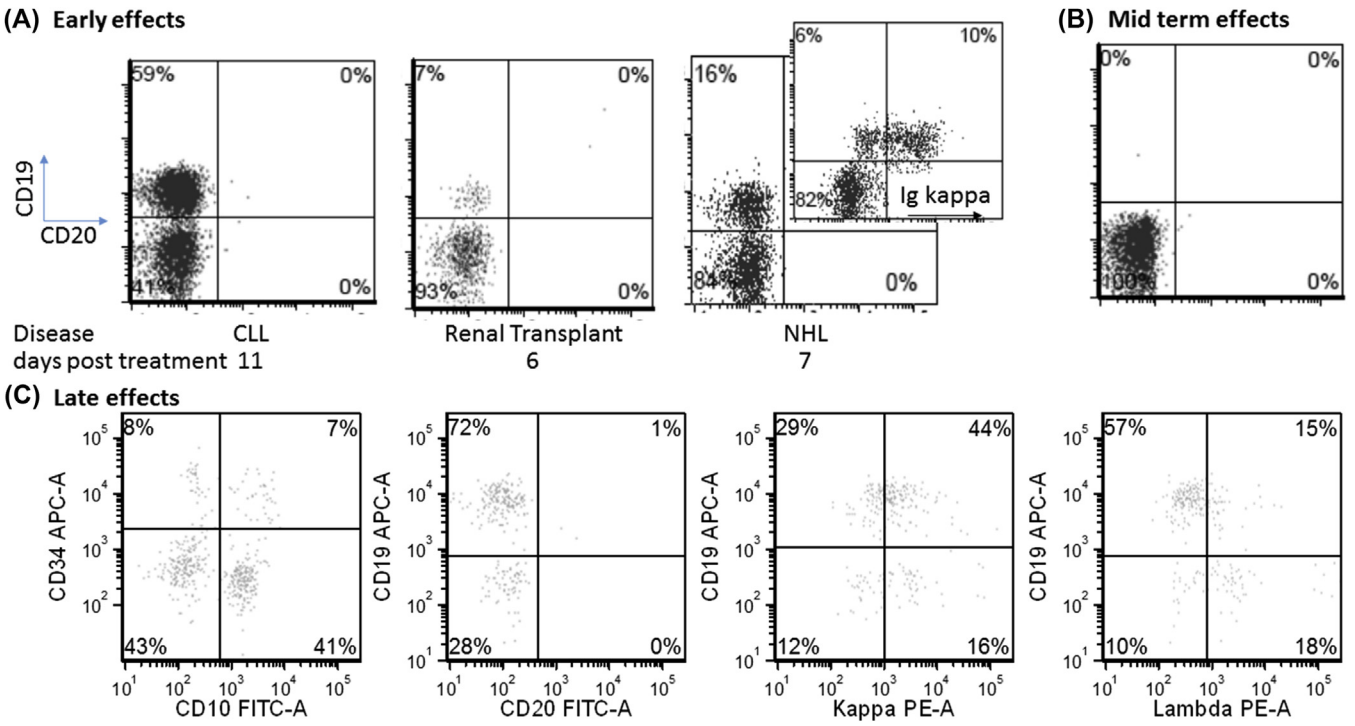


FIG. 27.9 Anti-CD20 mAb therapy effect on Flow Cytometry analysis of B cells: (A) early effects (first two weeks) — interference/blocking of CD20 staining, to (B) mid range (1–6 months) — disappearance of the B cell lineage and (C) late effects — opsonization without killing on the resurgent B cell lineage.

staining on the CD20$^-$non-malignant B cells at this stage is usually polytypic, suggesting that the Rituximab antibody is not opsonized on the surface (which would generate a significant kappa bias due to Rituximab's own light chain). Therefore, the absence of CD20 is not due to Rituximab blocking of the receptor. Alternative explanations proposed are receptor internalization or even trogocytosis [19], both of which would be consistent with the staining pattern observed. From a flow cytometric point of view, the salient message is that CD20 ceases to be a useful diagnostic marker at this treatment stage, while light chain staining may still be informative.

Following the initial few weeks, Rituximab treatment becomes spectacularly effective at removing CD20$^+$ cells from circulation. There is frankly nothing interesting about the intermediate-term effect of Rituximab, a complete depletion of B cells being its well-known hallmark (Fig. 27.9B). However, months after the last treatment cycle has been completed, we have observed a rare effect. Recovering B cell compartment consisting of small hematogone and/or mature B populations seen in bone marrow aspirate or peripheral blood samples appears to be CD20$^-$cleanly negative and kappa restricted! In the case presented in Fig. 27.9C, 4 months after the last Rituximab cycle, a population of B cells is observed with the CD45 and SSC characteristics of pre-B cells (not shown) which displays a picture-perfect B maturation pattern with respect to CD19, CD10, and CD34. Strangely, it completely lacks the upregulation of CD20 typically seen in late hematogones, but rather resemble the CD20$^-$ B populations from recently treated patients. The light chain staining on these cells, shows little to no lambda staining, but a positive shift in kappa. A relapse of the patient's follicular lymphoma is not supported by the immunophenotype of the cells, nor any other diagnostic testing performed. An alternate explanation exists: residual levels of Rituximab become sufficiently low a few months after treatment to allow the recovery of the B cell compartment; however, they are still sufficiently high to interfere with CD20 staining by flow. Unlike in the early stages of treatment, kappa light chain staining can be attributed to Rituximab (a human IgG1 kappa antibody) that is actually opsonizing cells without killing them. Needless to say, if this iatrogenic effect occurs on mature B cells, in a patient with historically kappa restricted disease, it can represent a significant confounder to flow results interpretation and carries a high risk of false positive call. Just like in the case of paraproteins, the most relevant clue in this case may be the staining pattern of lambda light chain, which should be normal and polytypic on mature B cells in the absence of disease.

In the last 10 years, Daratumumab, a fully human IgG1 kappa anti-CD38 antibody, has been proven to be an

effective therapy for treatment of plasma cell neoplasias, alone or in combination with traditional treatments [20]. Much like Rituximab, it employs a variety of killing mechanisms against $CD38^{+bri}$ plasma cells: complement-mediated cytotoxicity, antibody-dependent cellular cytotoxicity, and antibody-mediated phagocytosis. Unlike Rituximab, the expression of its target is ubiquitous on hematopoietic cells: plasma cells, hematogones, NK cells, monocytes, and activated lymphocytes (Fig. 27.10). Of clinical concern, though of minimal impact in the flow lab, CD38 is also expressed on other tissues such as neuronal cells and glial cells of the central nervous system, peripheral nerves, pancreas islets cells, osteoclasts, skeletal muscle cells, cardiac muscle cells, and bronchial epithelium [18]. Following treatment the expression of CD38 disappears by flow on all hematopoietic cells (Fig. 27.11). This is partially due to endocytosis, exocytosis or "trogocytosis" of the target receptor [21], as seen with Rituximab. While cytotoxicity plays a role, Daratumumab is not nearly as acerbically cytotoxic as Rituximab, a fact easily evidenced by flow cytometry, as a myriad of typically $CD38^{+}$ cell types, including sometimes neoplastic plasma cells, can be documented in the patient's circulation despite treatment by any other phenotype. In the case illustrated in Fig. 27.11B and C, after sustained 10 and 14 monthly doses of Daratumumab respectively, CD38 expression is uniformly negative, yet enduring $CD38^{+}$ NK, T, monocyte and unfortunately neoplastic plasma cell populations are clearly identifiable. As before, the monoclonal antibody treatment invalidates the use of its target marker as a useful diagnostic tool by flow cytometry. Worse yet, while many alternative markers are available for tracking B cell populations, gating becomes extremely difficult for plasma cell analysis. Even in cases where a CD138 population is particularly bright, it cannot be assumed to uniquely define plasma cells. In Fig. 27.11C, some 30% of the CD138 gated events lie in the granulocyte gate. Without redesigning and validating a new myeloma panel, our laboratory has had some success using either CD45 or CD19 against CD138 to remove some of the contamination in the plasma cell gate observed with CD38 alone (Fig. 27.11C). Admittedly, this strategy might vary significantly from lab to lab (depending on the myeloma panel marker composition) and from case to case (as each disease tends to be immunophenotypically unique). Several viable alternatives to CD38 have been suggested as gating markers for plasma cells, including

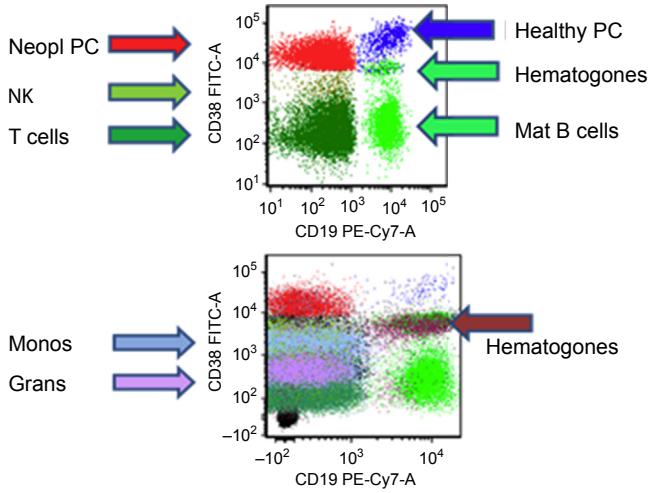


FIG. 27.10 Expression distribution of CD38 on various lymphoid and myeloid cell subsets in bone marrow cells. PC — plasma cells.

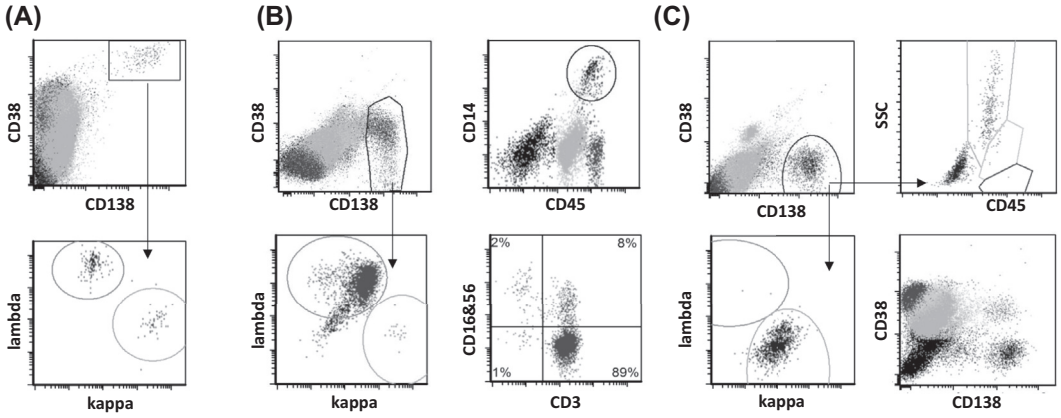


FIG. 27.11 Effects of Daratumumab on CD38 detection by flow cytometry. (A) Typical CD38 and light chain expression by Flow Cytometry on plasma cells (in a BMA sample not treated with Daratumumab). (B and C) Lack of CD38 expression and persistent lymphoid, myeloid and plasma cell populations in myeloma patients treated for 10 (B) or 14 (C) consecutive months with Daratumumab.

non-competing CD38 clones [22], CD229(LY9) [23], CD269(BMCA), and CD319(SLAMF7) [24].

Plasma cells show extraordinarily high levels of light chain expression, which appears to dim significantly with Daratumumab treatment. Fortunately, while dim, the staining does not appear to be obstructed in any significant way by the kappa light chain of the therapeutic antibody.

Flow cytometry analysis of fluids

Fluids represent an especially challenging sample class submitted for flow cytometry, which includes cerebrospinal, pleural, ascites and pericardial fluids. Poor cell quality, large amounts of cellular debris, viscosity and inflammation related changes are recurrent motifs seen in these samples. The most typical problems observed are failure to stain light chains on B cells and occasional dimming of CD7 on (non-neoplastic) T cells (Fig. 27.12). The "warmup protocol" discussed earlier for disengagement of antibodies from Fc receptors, does occasionally improve the light chain staining suggesting interference. The CD7dim pattern observed on normal T cells in some fluids appears to be an intrinsic characteristic of the sample. The mechanisms underlying this partial dimming are unknown, but the pattern clearly does not match the $CD7^{+}$ level of circulating T cells nor the $CD7^{-}$ (clean negative) T cell subset described in the acquisition of memory function [25].

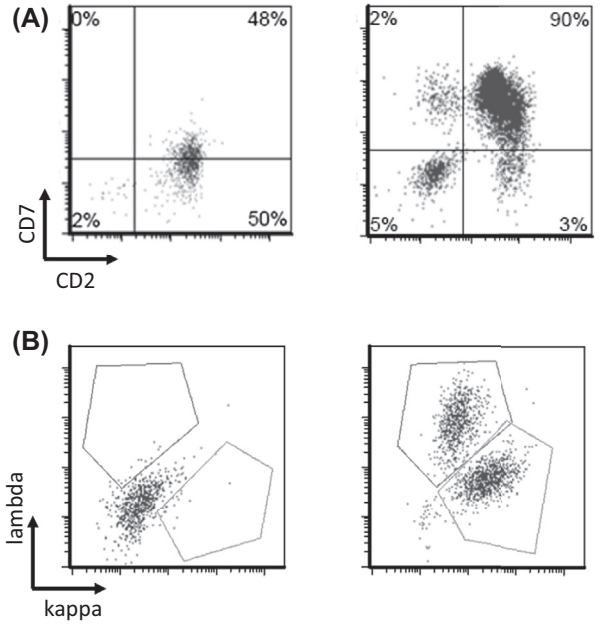


FIG. 27.12 Variable staining of markers in body fluids versus normal counterparts: (A) CD7 on T lymphocytes and (B) light chains on mature B cells.

TECHNOLOGICAL CHALLENGES IN FLOW CYTOMETRY

As a high complexity testing method, flow cytometry has a particularly heavy reliance on technology. It is therefore not surprising that a myriad of errors encountered are related to failures of improper application of technology. The scenarios discussed below are by no means comprehensive, but are a collection of the more important and interesting technology faults we have encountered in our laboratory.

Compensation

Any discussion of the technological pitfalls of Flow should start with proper instrument calibration. Compensation is a critical concept in Flow taught as one of the most basic prerequisites to the most novice of students. It will be used here as the prototype of poor instrument calibration, but it is certainly not the only one. To understand the concept one must peel back the basic optical workings of a flow cytometer. In brief, a laser is used to transfer energy to fluorophores that pass in its path. The fluorophores absorb this energy and according to physical chemistry laws, they must release it back, which they do also in the form of light. While the incoming energy is a pure light of a single wavelength, the emitted light is not; it has a wide spectral footprint covering large segments of the visible spectrum. Detection of any band of this emission is a straight forward proposition in single color flow cytometry. The problem arises when multiple fluorophores bound to the same cell are excited by the same laser, at the same time, each producing overlapping emission spectra. The overlap means that each fluorophore will generate a fraction of its signal interpreted by the flow cytometer as a different fluorophore's emission. The extent of that cross-spectral spillover can be measured by analyzing each fluorophore's emission by itself, while recording the signal leaked in all other fluorophores' detectors at the same time. Then a mathematical transform can be applied to the data to delete superfluous signal. The measurement of cross-spectral spillover and the mathematical transformation to correct it is essentially the compensation, and its importance cannot be underestimated. The reason is simple: choose a result, any result, and one can probably achieve it with a failure in compensation.

To illustrate how compensation can generate aberrant results, a sample was stained with CD3, CD4, CD8 and CD45 markers, a common T cell marker combination for analysis of malignant or non-malignant immunophenotypes. The lymphocyte analysis is displayed with correct compensation (Fig. 27.13A) and then re-plotted with deliberately forced compensation values between

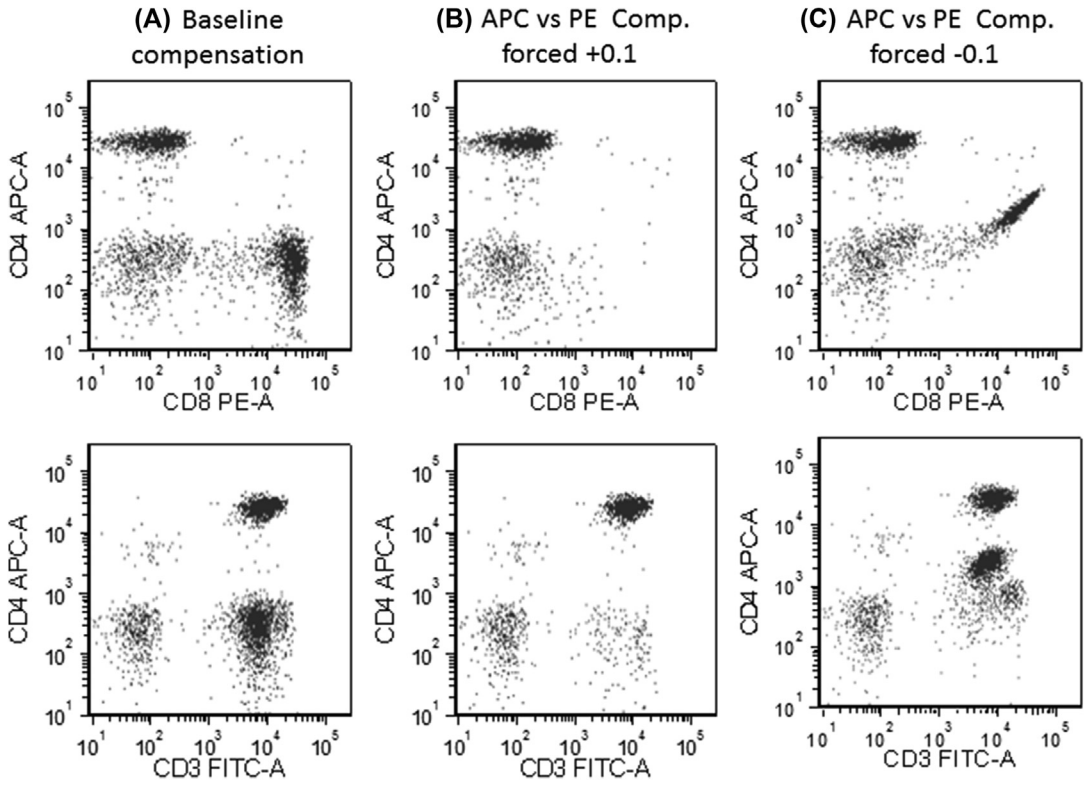


FIG. 27.13 Relatively small errors is compensation can have dramatic effects on data. A population of lymphocytes is stained with CD3, CD4, and CD8. The same data is displayed with (A) adequate APC-PE compensation values, (B) significantly overcompensated causing the $CD8^+$ T cells to disappear from the draw space (B), and significantly undercompensated, causing an aberrant $CD3^+$ CD4dim population.

the APC and PE channels (Fig. 27.13B and C). Overcompensating the APC-PE spillover, results in abnormally low APC values for all PE positive events. As a result the CD8 population "hides" under the axis of the logarithmic scale which stops at 10. Incidentally, the events have not disappeared and switching to a biexponential axis display may show the missing CD8 population. Undercompensating the APC-PE value, makes the $CD8^+$ T cells aberrantly bright in APC and apparently CD4 partially positive. Since the APC-PE correction addresses how much APC-signal PE fluorescence generates, the $CD4^+$ T cell population is unaffected.

The experiment teaches another two essential lessons. First, poor compensation between two channels can be recognized when the two channels involved are plotted against each other. When two independently variable markers are plotted against each other (like CD4 and CD8), they will form roughly circular or oval clusters by flow cytometry. Over/under-compensation changes this paradigm, because the two markers plotted are no longer independent, but rather linked by a mathematical formula set by the (bad) compensation. The result is an "unnatural" flattened shape similar to the problem of non-specific staining. The second lesson is more unnerving: bad compensation is extremely difficult to recognize on any other plot (than the two mis-compensated channels against each other). In Fig. 27.13 (lower panels), plotting CD3 against CD4 the result went from a normal T cell distribution, to an apparent aberrantly high CD4:CD8 ratio, to a prominent and aberrant $CD3^+$ CD4dim population, without any hint that it is all accomplished though an artifice of compensation.

Compensation problems are best addressed preemptively through good training and practices on instrument calibration. Retroactively, compensation issues can still be addressed after data acquisition on digital instruments and the first step is recognition.

Spillover beyond compensation

Unfortunately, in some circumstances the signal spillover between two channels, has little to do with compensation. The demand to squeeze as much information from as little material as possible has driven the development of newer generations of fluorophores. These new developments have overcrowded the visible and near infrared spectrum typically used in flow cytometry detection, and have given rise to new problems. Fluorophores have a tendency to increase their distribution or spread into other detectors proportionally

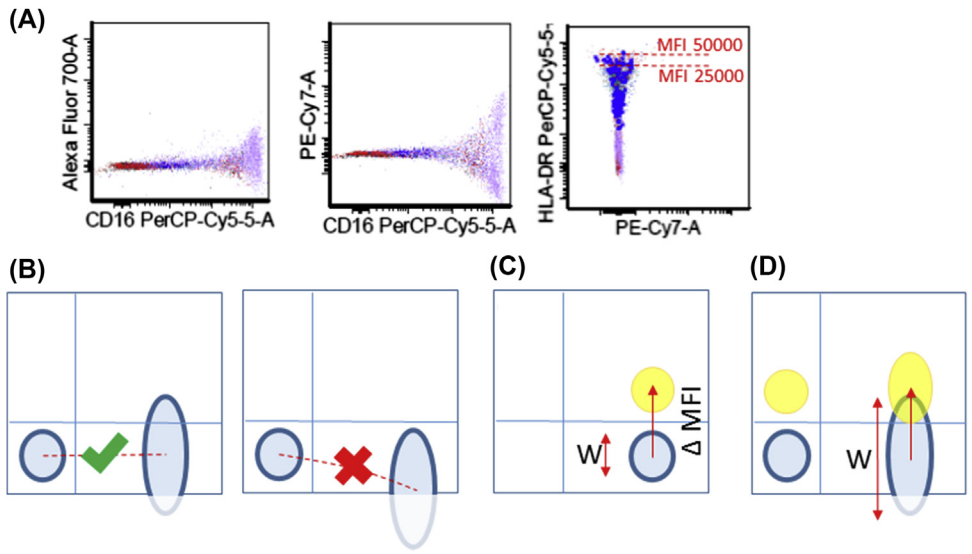


FIG. 27.14 Spillover between fluorophore signals. (A) Spillover of PerCP-Cy5.5 signal into other (empty) channels. The amplitude of spillover is proportional to the signal intensity in the source channel and it varies in different target channels. (B) Correct application of compensation aligns the mean fluorescent intensities (MFIs) of positive and negative populations, rather than the peak signal. (C) The shift in MFI of a positive population in the target channel (on the y axis) is obscured by spillover from the source channel (x axis).

with their signal intensity (Fig. 27.14A). For the purposes of this discussion, the fluorophore generating the signal spillover will be termed "source" and the split-into detector will be termed "target".

The spillover is a characteristic of all fluorophores, albeit the classical organic and phycobilliprotein dyes, such as FITC, PE, APC, were minimally affected. Incidentally, in their case, old school flow cytometry taught to adjust compensation to line up the leading edge of the positive population with the negative (Fig. 27.14B), an approach which neatly hides the spillover effect, but also unknowingly or deliberately sets the wrong compensation value with all the pitfalls discussed earlier. Ideal compensation values are derived from lining up the mean or median fluorescence intensities of negative and positive populations rather than the top most-positive edges of the populations. Since the spillover is minimal with these old fluorophores the compensation errors were also likely minor, but likely many an old flow result was erroneous because of it.

The problem is not so subtle with the newer fluorophores, with the most severe issues being encountered between the tandem dyes PerCP-Cy5.5 and PE-Cy7 (Fig. 27.14). So two questions arise (i) What are the consequences and (ii) how to deal with fluorophore spread and spillover.

The first and most obvious consequence is that quadrants set using a double-negative population or isotype controls are no longer relevant for positive populations that display spillover (Fig. 27.14). Such quadrants would invariably generate a false positive subset, proportional to the amount of spread into the target channel. The_undertruly appropriate negative control for spillover/spread is "fluorescence minus one", a control tube in which the entire staining is repeated, except the spillover target channel which would contain only isotype control antibody. Since the spillover is present in both staining and control tube, the signal difference between them is fully attributable to the actual staining and not interference. Fluorescence minus one is an essential step during panel validation; unfortunately, it cannot be implemented as routine part of clinical flow cytometry testing without significantly scaling up the costs.

A second consequence is loss of resolution. An important parameter quantifying how well flow cytometry resolves a positive population is the "staining index" (SI), which is the forward shift in fluorescence of a positive population (ΔMFI) normalized by the distribution of the negative population ("w") (Fig. 27.14C). If two fluorescence channels experience spread, the ΔMFI is unaffected, but the w-distribution of the negative population is increased, lowering the SI. If the expression of the marker analyzed in the target channel is positive but dim, the increase in w can easily obscure the MFI shift (Fig. 27.14D), even though the shift would distinctly bimodal in the absence of any signal spillover (Fig. 27.14C).

The bad news is that data acquired with spillover are unfixable. However, effective panel design strategies largely avoid the spillover problem or render it irrelevant:

1. Empty channel. Leaving the source channel empty would prevent the spillover and leaving the target channel unstained would make the spill over

irrelevant. Pros: While a little bit of a wimpy choice, it is very effective and surely nobody would pass moral judgment. Cons: giving up a channel diminishes the information gained per stain.

2. Exclusion channel. In a variant of strategy 1 above, the spillover source channel is stained with "Lin-" markers, i.e. lineage markers for populations to be excluded from the analysis. For example, CD71 can be stained in B-ALL analysis to remove unlysed erythrocytes from the CD45 negative area where B-ALL often lies. Alternatively, an NK exclusion marker can be used in the same way, since reports exist that $CD19^+$ NK cells can be a confounder in the analysis of B-ALL MRD [26]. Pros: The spillover source is effectively used as a quality control measure to clean up the analysis of a population of interest. Cons: Channel not directly informative on the population of interest.
3. Mutually exclusive markers. Placing mutually exclusive markers in the source and target channels (i.e. CD3 vs. CD19, or kappa vs. lambda) allows the user to safely ignore the spillover. Pros: providing that the two markers make sense together in the same staining tube, this strategy makes full use of the cytometer capabilities. Cons: Mutual exclusiveness can often not be guaranteed in malignant immunophenotyping, making this approach more effective for normal cells, where markers have very defined patterns of expression. For example, CD13 and CD7 are mutually exclusive in normal WBC defining myeloid and T/NK lymphoid populations, but are occasionally co-expressed in myeloid neoplasms.
4. Dim markers in source. It is readily apparent that even in cases of spillover, the source and target channels function appropriately for most of their fluorescence range. In the example shown (Fig. 27.14A), spread becomes an exponentially-increasing problem in the last decade at MFIs >10E4, but is acceptable at lower values. Therefore, a simple solution is to swap-out the culprit source marker and swap-in a dimmer marker of interest, whose intensity does not surpass the 10E4 threshold. Pros: Manageable spillover. Cons: The user forfeits making use of the full brightness of the source channel. Since not all markers are commercially available in all fluorophores, this a dimmer marker solution may not always be available.
5. Quenching. When a dimmer marker cannot be swapped into the source channel, an unconventional solution exists to make a bright marker dimmer. Diluting a fluorophore-labeled antibody with unlabeled antibody of the same clone will cause less fluorophore opsonization on the cells. Importantly, down titrating the fluorophore conjugated antibody with the aim of under-saturating the target marker may accomplish the same effect, but it is unadvisable. The target antigen in clinical samples could be highly variable, either due to paucicellularity or decreased expression target marker. In such samples a high fluorophore-to-cell ratio and saturation is reestablished, yielding high source signal and significant spillover once again. Pros: Quenching can solve the spillover problem without drastic panel redesign. In our experience, 1:1 mix with unlabeled antibody can reliably reduce the MFI of staining by about 50% if the signal is within the linear range of detection. Cons: A two-fold reduction of fluorescence intensity is a relatively subtle change of a log-10 scale (Fig. 27.14A). It is unlikely to be effective when the MFI of staining has to be reduced by 10-fold (see the CD16-PerCP-Cy5.5 example, Fig. 27.14A). It entails additional expense (the cost of unlabeled quenching antibody) without gain of additional information.

The problem of spillover is not an easy one. First, none of the solutions suggested above fix the problem, but rather are just creative ways of circumventing it. Second, spillover has to be accounted for from the ground up in panel design or else it carries the potential of continuously producing erroneous flow results. Lastly, while a number of (imperfect) solutions exist, ignoring the phenomenon is the only one that's unacceptable.

Tandem dyes

Flow cytometers are not the only sophisticated pieces of machinery in a flow lab. The fluorophore conjugated antibodies are amazing little biochemically- and biophysically-engineered nanobots, which hardly garner a thought the clinical day-to day operations. Tandem dyes represent an innovative solution for expanding the multiparametric flow cytometry repertoire. In brief, a traditional flow fluorophore (for example PE) is covalently bound to a second fluorophore (Cy7). The combined (PE-Cy7) tandem molecule retains the absorption spectrum of the base dye (PE) and thus and accepts the same laser excitation already implemented in existing flow instruments, but emits in a distinct spectrum determined by the second fluorophore (Cy7 in our example). The base dye transfers the energy to the emitter fluorophore, through a process termed "FRET" (Förster resonance energy transfer) [27]. To most flow users, the biophysics of FRET might as well be magic. (It is modeled using "virtual photons", which would violate the laws of physics had they been actual photons.) From a flow cytometry point of view, it suffices to understand just a few facts about FRET: (i) the energy transfer is very efficient so long the base dye and emitter are in low nm range proximity (an issue already

addressed at antibody manufacture by covalently binding the two), (ii) the efficiency is not 100% and thus base dye emission can be observed, and (iii) the excited emitter radiates energy at wavelengths distinct from those of the base dye, thus the base dye and the tandem dye can be used side-by side.

Unfortunately, they also present a number of unique challenges. First, a labile nature and a propensity for photobleaching result in a very short shelf life. Second, performance wise, they tend exhibit significant spillover and compensation problems with the base dye, problems amplified by their brightness. Lastly, some tandems exhibit stability and cross-reactivity issues and as such they cannot be cocktailed with the base dye or with other derivatives of the base dye. These problems and their solutions are generally well known in flow cytometry laboratories. For example, stocking and handling tandem dyes have to take into account their short shelf life (exposure to light, warm storage, freezing). Antibody mixes prepared in lab can be formulated without tandem dye conjugated antibodies, which can be "spiked in" individually during staining. Spillover has a number of solutions discussed in more detail in the previous section. Typical problems associated with tandem dyes are listed in Table 27.1.

TABLE 27.1 Typical problems encountered with tandem dyes.

Issue	Solution
Photobleaching – Light can irreversibly lower FRET efficiency.	Protection from light at all times, during storage and staining.
Freezing – Freezing can denature tandem dyes.	Tandem dye conjugated antibodies should not be frozen. Proteins are especially vulnerable to denaturation by freezing [29].
Fixation – The brightness and/or the FRET efficiency of tandem dyes may be reduced by fixation.	If a fixation step is required, various fixatives should be validated for best results. Permeabilization protocols often rely on fixatives.
Batch-to-batch variability. Tandem dyes have a complex manufacturing process and can display significant variability between manufacturers and even lot-to lot.	Each batch in clinical use has to be quality controlled and re-optimized as needed.
Spillover and Compensation	If FRET is <100%, some signal in the base dye channel will be observed. Good panel design to address spillover, thorough compensation versus. the base dye, appropriate controls, FMO controls.

Beyond the usual suspects listed in Table 27.1, we observed an additional specific failure of tandem dyes which received little attention in flow cytometry circles: oxidation. Our laboratory validated an 8-color flow panel for T/NK cell neoplasms staining CD45 with an APC-H7 conjugated antibody (an APC-Cy7 analog), while the APC channel was used for isotope control, CD19, CD25, CD26 or CD30. T cell analysis worked as intended yielding congruent results with older T cell panels. However, a problem was noted in the granulocyte gate, which appeared to occasionally show significant fluorescence in the APC (donor) channel in all tubes in the panel including IgG isotype control (Fig. 27.15A). Nonspecific binding, a common problem with granulocytes, did not seem a likely explanation because the signal was too intense and affected the APC channel specifically much more than other detectors. APC fluorescence was retained when the staining did not include any APC antibodies, but disappeared when CD45-FITC was used instead of CD45-APC-H7. Since the problem also seemed granulocyte lineage specific, thus ruling out photobleaching or fixation of the antibody stock. Another significant clue was the tendency of phenomenon to be more pronounced in older samples. If the granulocytes tend to degranulate after a few days of storage as exemplified in Fig. 27.2, the hypothesis emerged that the oxidative stress released by these are somehow affecting the tandem dye.

To test this hypothesis we oxidized CD45 APC-H7 antibody directly with various concentrations of H_2O_2 (0%–1.5%). In one control, equal amounts of CD45 antibody, fetal bovine serum, and 3% H_2O_2 were mixed (in that order, for a final concentration of 1% H_2O_2 and 33% FCS). After 15 min of oxidation at room temperature in the dark, the CD45 antibodies was added to 50 μl of peripheral blood and after 15 more minutes of incubation the data was acquired. It is readily apparent that the signal in the APC channel (although unstained) increases with the oxidative stress applied to the APC-H7 antibody (Fig. 27.15B). Importantly, in this experimental setup it occurs not only on the granulocytes, but also on lymphocytes. Adding a large amount of FCS to the antibody before H_2O_2, protects the antibody from oxidation and the lymphocytes stain negative in the donor channel once again. The APC bleed though remains a problem on Granulocytes; they presumably provide their own oxidative stress in a gradient with the highest concentration proximal to the cell membrane where the opsonized antibody can be found. This model fits the observation that the granulocytes can alter the tandem dye antibody opsonized on themselves, but are unable to affect the tandem dye at a distance opsonized on other cells. A few conclusions can be drawn from this experience. First oxidation is a mechanism affecting tandem dyes. Second, the staining pattern

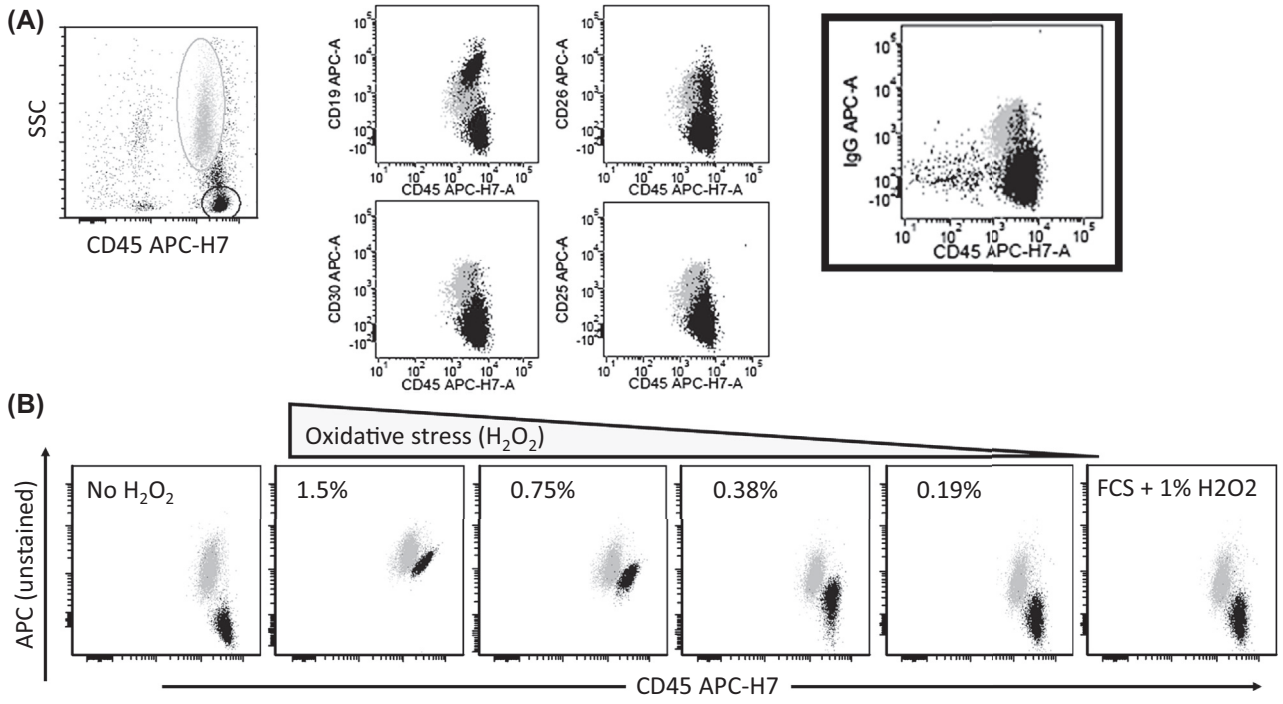


FIG. 27.15 Oxidative stress effect on tandem dye APC-H7. (A) Granulocyte lineage specific signal leakage from APC-H7 to APC. (Granulocytes in grey) (B) Hydrogen peroxide oxidation of APC-H7 increases base dye APC signal.

strongly suggests that oxidation does not affect the excitation of the base dye, but reduces FRET efficiency. A third conclusion is that this phenomenon occurs naturally in vitro in granulocytes, and it is not quenchable in high serum concentration, but it is relatively short ranged. And finally, the phenomenon is sufficiently frequent and strong with APC tandem derivatives, that it is probably wise not to include myeloid markers in that channel without a thorough validation to rule our this effect.

The strange case of calcium oxalate and CSF

Calcium oxalate is common crystal in nature, which features a prominent role in kidney stones. Admittedly, it is a very surprising and unlikely source of flow cytometry errors, as it is not present in blood or tissues typically analyzed by flow. Unfortunately, it can be introduced through the fetal bovine serum (FBS) used to prepare wash buffers.

The anecdote that brought it to fore in our laboratory started with populations of strange events in our CSF samples submitted for leukemia/lymphoma analysis. Since CSF's are almost invariably paucicellular a clear phenotype could not be established, but they appeared to be recurrently CD45dim with intermediate SSC. The CD45dim phenotype is always a source of alarm in leukemia/lymphoma analysis, so the CSF samples were investigated further by light microscopy. Surprisingly this revealed no cells that could account for the CD45 dim population. The scrutiny was then extended to the reagents, starting with the wash buffer. A small number of beautiful octahedral crystals conspicuously stood out on the otherwise clear background. They were then easily traced back to the FBS. A quick literature search shows that this is a fairly common and well described problem: Pedraza et al. [28] suggest calcium oxalate dihydrate crystals (consistent with the octahedral/bi-pyramidal shape observed in our laboratory) were found in all of the several FCS sources they investigated. More worrisome they demonstrated that Ca oxalate are not inert bystanders in biological assays. The crystals can reach sizes of 5+μm, close to that of a lymphocyte. They avidly adsorb proteins and DNA and are readily adhered to or phagocytosed by cells, inducing either apoptosis or proliferation. Since most FCS stocks are filtered by manufacturers, it is likely that the crystals form after distribution during freezing and storage. The crystals disappear at 37°C in cell culture medium within 24 h.

In our experience, the presence of these crystals is not nearly as common as reported by Pedraza et al., due perhaps to the fact that our laboratory screens FBS lots for purchase by their ability to support T cell proliferation in mixed lymphocyte reactions. However, their size and protein adsorption characteristics make them

highly worrisome in flow assays because they can appear as cells and might exhibit fluorescence by adsorbing staining antibodies. Killing cells may prove an additional challenge for samples with low cellularity. Warming up the FCS for 24 h, seems impractical for flow laboratories, but a simple re-filtering or at least a quick microscopy analysis may prevent a lot of anxiety down the road.

THE HUMAN FACTOR

All laboratory errors invariably have one thing in common: human input. Even in cases where the problem can be traced directly to samples, technology, equipment, or reagents, the laboratory personnel should ultimately be in control of the issue, so in sense humans are always part of the root cause. The ways personnel can contribute to errors is highly complex, but can be described as either lack of knowledge (education, training, experience) or inability/unwillingness to effectively apply that knowledge (distractions, lack of focus, disinterest, work overload).

As expected, the former issue can be more readily identified and easily addressed. Appropriate education can be simply screened in an interview during the hiring process. A vital part of the knowledge needed for laboratory operations is imparted during the initial training. Finally, practical experience builds naturally thereafter, on the job. Constant employee development through continuing education, professional conferences, competency evaluations, participation in proficiency testing and open discussion in laboratory meetings can ensure that the errors traceable to knowledge gaps are kept to a minimum. In fact, these represent a very small minority of failures in a clinical lab, simply because the lack of knowledge is testable and has an obvious solution in more training.

Unfortunately, even employees who are fully trained and knowledgeable make mistakes. The reasons why knowledge fails to translate into satisfactory performance are immensely intricate, and often obscure. Work overload is perhaps the simplest of these. In a clinical laboratory, the incoming sample volume is never constant, nor is the urgency with which it has to be processed. Staffing a laboratory for the highest demand is obviously not a proper solution, as inefficient most of the time and possibly insolvent. Typically, most labs are staffed for average volume, which inevitably creates a labor shortage during peak times, holidays, overlapping vacations/sick leaves, or sudden personnel departures. Some short-term relief for spiking work volumes can come from purposely delaying non-critical samples to be processed in overtime or next day. A worse turn-around-time is the trade off to ensure quality laboratory results. From our own experience, introducing an afternoon shift increased instrument usage, thus reducing backlog caused by equipment bottlenecks during peak times. Incidentally, this measure also relieves overcrowding, ensuring that the space is never shared by the full complement of staff. Relief may come from ironing out any inefficiencies in the laboratory workflows. Automation of staining, if practicable, provides significant labor savings over manual flow cytometry staining. Similarly, barcoding data entry is "hands-down" superior in efficiency and accuracy to manual entry. Pre-formulating reagents (antibody cocktails, buffers) may also save time. A useful pre-emptive policy in any laboratory is limiting the number of overlapping vacation requests allowed at any given time, safeguarding that any random increase in sample volume does not coincide with an inversely proportional drop in available labor. So while work load is to some extent unpredictable, it can be at least mitigated by good practices.

Lack of focus either due to distraction or disinterest may be the most insidious error factors in the laboratory environment. They are more difficult to detect by far than any of the previous issues, often requiring the laboratory management playing amateur psychologist. Perplexingly, disinterest in the work can be a preexisting condition, with a job applicant successfully hiding it during interviews to secure a steady paycheck. Contacting former employers and references proves key in these cases, because while job interest can be feigned, past job performance cannot. Alternatively, losing interest can happen after hiring due to repetitive work, lack of recognition or mistreatment (whether real or perceived). Distractions are sometimes identifiable: noisy or poorly designed work environment, listening to music, podcasts, phone conversations while on the job. In contrast, personal issues such as health, family, lack of sleep, etc can be extremely disruptive to work performance and the employee is under no legal or contractual obligation to disclose them. Irrespective of the source, lack of focus will inevitably result in decreased work performance. At first, it will manifest itself as small slip-ups. These should be treated as an opportunity to discuss the employee performance, and should never be ignored as they are likely to be the only warnings of bigger problems to come.

Different case scenarios will be discussed which showcase how a variety of these issues can translate into egregious errors to in the laboratory. All are based on real events, minimally fictionalized.

Case scenario 1

At the expiry of grant funding, a research worker "employee X" is offered and accepts a clinical lab position. He is extremely smart, graduate level educated,

skilled in research wetlab work, with a great analytical mind, and whose initiative has helped the clinical operation in the past. As a clinical lab employee his performance is lackluster, and has a glaring problem with chronic tardiness, which doesn't abate when moved to the afternoon shift. His coworkers and direct supervisors notice repeated small mistakes in his work, which they choose to address at least initially by fixing themselves and covering for him. When confronted with these issues the employee states he wishes to continue in his position and intends to improve. A serious red flag surfaces when he states the work he is performing is beneath him.

While the employee X was on vacation, a strange problem started to occur. Over the course of two days, three CSF samples showed a positive result for mature B cell leukemia/lymphoma. Since CSFs are typically paucicellular and contained only trace amounts of neoplastic B cells, the disease could not be characterized in detail, however they appeared to be recurrently $CD5^+$, CD20dim, and kappa light chain restricted, a phenotype suggestive of CLL. The collection of unlikely results, the consistent phenotype across samples, the frequency of unlikely CLL^+ CSF samples, including in patients without prior history, suggested a technical error, most likely a contamination. All work in the lab was stopped to elucidate the mystery. Reviewing all CSF samples processed lately, two more similar cases were found with the same immunophenotype, including one with an unlikely demographic: a female in her 20s. The puzzling aspect was that other than being CSFs and containing the same aberrant population, the samples apparently had nothing in common. They arrived in lab at different times, were processed by different techs, and the data was acquired on different instruments. They shared the same sample-accessioning center, but they originated from different operating rooms and clinics. Although it looked increasingly likely that a contamination had occurred, it did not appear to have occurred in the lab. Small aliquots of the reagents, antibody cocktails and wash buffers were "flowed" by themselves as the only other possible commonality between them. The wash buffer bottle in use for the questionable CSF samples had been finished and discarded, but still contained enough liquid material to investigate. To everyone's shock, it showed extremely rare cells, which - when pelleted and stained - displayed the familiar immunophenotype: $CD5^+$, CD20dim, kappa.

Next, the sample record was reviewed for the identity of the contaminant. A single sample matched the immunophenotypic pattern: a bone marrow aspirate, with 90% CLL cells, received, stained and acquired late on the prior Friday evening, by employee X. It was the last sample the employee processed that night, before he went on an extended vacation to an exotic location. No other CSF samples stained before displayed contamination, while all the CSF samples processed after did. The sequence of events became clear. The problem had nothing to do with the CSFs, but rather the wash buffer had become contaminated with CLL cells from Friday and subsequently all samples processed in the flow laboratory had been contaminated. The contamination in each stained tube amounted to no more than 1–2 dozen cells, but figured prominently in low cellularity CSF samples which generally lack any B cells. By contrast, peripheral bloods, bone marrow aspirates or lymph node samples are highly cellular, and a few scattered contaminating events were easily masked by large B cell populations in these samples.

In the aftermath, the lab had to cancel numerous results, contact clinicians before the results reached the bedside and rescind a few molecular tests reflexed on flow samples. A vast array of reagents had to be qc'd. A policy to perform data acquisition on plain wash buffer with every CSF was instituted to monitor the buffer quality. The employee was unreachable by email due to the remoteness of his vacation destination, but upon his return he could offer no explanation. He offered a guess that the wash buffer bottle was treated as waste bottle and some material was discarded in it. He was removed from clinical duties. As a precaution, any bench top waste containers were labeled with bright orange labels, indicating "WASTE", and all buffer bottles were fitted with dispensers to prevent any material going in. As a new policy, the laboratory extracts 90% of the sample material from all original tubes and vacutainers using a sterile transfer pipette, recapping the tube and saving the remaining 10% essentially untouched in the original container to serve as a quality control backup and source material for reflex testing, should it be needed.

Most importantly, the root cause analysis became apparent after the sequence of events was pieced together. A number of factors came together for this to occur: a routine lack of focus and interest on the part of the employee was exacerbated by a high volume of work, late on Friday, before a presumably long-anticipated vacation. There was a failure on the side of management as well; there were minor but numerous signs that the employee was not cut out for clinical work, which in hindsight should have been acted on (in a different manner than giving another chance to improve). Finally, the protective attitude of his coworkers was misplaced; and open and transparent discussion of the employee's shortcomings might have signaled the need for retraining or reassignment.

Case scenario #2

Staining light chains by flow cytometry plays an important role in the diagnosis of lymphoid neoplasms, Firstly, because the relatively high frequency of B neoplasms and secondly because light chains provide a powerful clue about the clonal status of mature B cell populations. An issue arose in the laboratory, where the kappa/lambda light chains would recurrently have poor definition. Specifically, staining would not show a typical bimodal kappa-lambda cluster distribution, but rather weak positivity for either light chain, (which did not separate from the negative cells) or near complete lack of staining. Occasionally, such results are expected, for example on CLL, which often exhibits low light chain expression, or in fluids where nonspecific staining "noise" obscures positive results. However, these were not the only cases with substandard staining, samples with polytypic B cell populations, with clear normal light chain distribution by cytoplasmic staining, would fail to stain kappa/lambda on the cell surface. Cytoplasmic staining seemed to work with no problems. Several such cases were observed in a short span of time, and restraining frequently fixed the problem, suggesting that a technical failure was somehow at work.

The investigation took the obvious route: investigating samples, reagents, equipment and personnel. Reagents could quickly be ruled out as the culprit; the problem was spread out over a few lot numbers, all of which had been properly qc'd and yielded perfectly normal results on numerous samples. Equipment failures were also highly unlikely since they performed satisfactorily for other samples. Furthermore, the channels used for kappa/lambda detection had performed well for other markers in the very samples where kappa/lambda failed. The failures were also not associated with a single flow cytometer instrument. All instruments were properly maintained, and calibrated, and yielded proper light chain data most of the time. The problem seemed to occur exclusively in bone marrow aspirate and peripheral blood samples, albeit in a minority of cases, and it never appeared in lymph nodes or soft tissues. The association with serum containing samples suggested that this problem was traceable to insufficient sample washing. If enough free antibody is present in the supernatant at the time of staining it can act as a competitive inhibitor to the staining antibody, as discussed in this chapter in the context of myeloma (Fig. 27.7).

When the audit trails for these samples were reviewed, it revealed a strong association of these staining problems with a particular technician, "Y". The employee was invited for a discussion and presented with the problem. She could offer no explanation initially. However, reviewing the standard operating procedure for staining, she admitted that she washed all samples only once before adding antibodies, a significant deviation from SOP. Despite her extensive experience in the lab, retraining was the obvious recourse.

The section on personnel contributions to laboratory errors would be remiss without a salient point on corrective actions. It is obvious that many people being investigated as a root cause for a laboratory error become very defensive, and covering up is instinctively a mechanism of not getting in trouble. From the point of view of management, there is hardly a more frustrating exercise than to try to piece together a mishap when the personnel actively hinder the effort. Therefore, it is paramount to impress on the employees the notion that root cause analysis is not a process to assign blame, but rather an effort to understand how a problem has come to pass and change the practice so it does not become a recurrent issue.

CONCLUSIONS

Clinical flow cytometry is in many ways similar to all other laboratory medicine specialties: it is procedurally a high complexity discipline and errors can crop up in any aspect of the operation. The classification of errors in this chapter into sample, technological and personnel issues is not incidental. It constitutes a very effective way to investigate an incident and find the root cause, eliminating all the functioning components of a system until only the faulty one(s) remain. When dealing with samples or technology, the issues can be extremely difficult, but there are no egos to bruise. When a person is at fault — and until the AI overlords take over, a person is always at fault — the issue becomes thornier. To enlist the employees cooperation in resolving the issue, they must understand that personal infallibility is not expected, nor reasonable to expect. Instead, a realistic and constructive goal is to eliminate risks by learning from every misstep and instituting robust preventive policies going forward.

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CHAPTER

28

Interferences in blood bank testing

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INTRODUCTION

Transfusion of blood products requires pre-transfusion testing in order to select and issue safe blood. Pre-transfusion evaluation for non-emergent blood transfusion includes a review of the patient's medical history and pre-transfusion testing. Medical history review should include clinical diagnosis, medication, potential sources for allo-immunization such as prior blood transfusion, pregnancy, hematopoietic progenitor cell (HPC) or organ transplantation, and prior blood bank (BB) history including records for clinically significant antibodies (Abs), adverse effects to blood transfusions and special transfusion requirements. The pretransfusion testing includes (1) ABO/RhD typing and antibody screen (Ab screen) on recipient's blood sample; (2) confirmation of the ABO typing on all donor units and of the RhD type in the units labeled RhD-negative; (3) selection of appropriate ABO/Rh compatible units from the BB inventory; (4) in vitro compatibility testing or crossmatch (XM); and (5) labeling the red blood cell (RBC) units with at least two independent patient identifiers, donor unit number, and compatibility test results. The basics of BB tests and possible interferences are reviewed within this chapter and relevant clinical case-scenarios are presented.

Type and screen (T&S) is performed routinely prior to any transfusion and includes ABO/RhD typing and Ab screen. Electronic or serologic XM is performed prior to any RBC transfusion as part of the pre-transfusion testing. Additional common blood bank (BB) tests reviewed here are the direct anti-human globulin test (DAT), autocontrol (AC) and serologic RBC phenotype.

The basis of most BB tests is the reaction between RBC antigens (Ags) and corresponding Abs. This reaction is visualized by RBC agglutination; however, hemolysis, if present, may also indicate a positive reaction. In addition to a true Ag-Ab reaction, a positive reaction may be due to other causes, including sample error, contamination, and clerical or technical errors, or true interferences. The results of the BB tests should always be interpreted within the patient's specific clinical context.

ABO TYPING

The ABO blood typing is performed in two steps, a forward and a reverse reaction. In the forward reaction, the presence of the A and B Ags is determined by testing the patient's RBCs with licensed anti-A and anti-B commercially available Abs. In the reverse reaction, the patient's plasma is tested against reagent RBCs of A1 and B types to detect the presence of anti-A1 and anti-B Abs. A positive reaction is indicated by RBC agglutination graded from 1+ to 4+ with 4+ being the expected reactivity strength for naturally occurring ABO Abs. An ABO discrepancy work-up is initiated when the current blood type does not match the historical blood type, when there is a mismatch between the forward and reverse typing interpretation, and when mixed field (mf) or weaker than expected reaction strength is detected. ABO discrepancies should be resolved prior to blood release unless there is an emergent need for transfusion, in which case universal blood products are issued.

AABB Standards for Blood Banks and Transfusion Services require two blood samples for initial blood ABO/RhD typing. The first blood type determination shall be performed on a current sample, and the second by one of the following: testing a second current sample, comparison with previous record, retesting the same sample if patient identification was verified using

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an electronic identification system or another process validated to reduce the risk of misidentification.

RhD typing

RhD typing is performed by mixing the patient's RBCs with anti-D reagents. Results must be interpreted in reference to appropriate positive or negative controls which, in many cases, may be provided by a negative or positive reaction in the concurrent reverse ABO typing. If the D reaction appears positive in an AB patient, in whom the reverse reactions is also positive for both anti-A1 and anti-B, typing should be repeated with an inert control reagent (such as bovine serum albumin) to confirm a true D-positive reaction. If the patient's D type appears negative, further testing is performed to detect the presence of so-called "weak D antigen" (Du), where the D Ag is present but not detected by routing RhD typing. Weak D testing is performed by adding the patient's RBCs to the anti-D reagent Ab in the presence of anti-human globulin (AHG). If the weak D testing is negative, the RhD type is interpreted as a true RhD negative. A parallel test with a negative diluent control is always included for the weak D test. All RBC units labeled RhD-negative are re-tested for the D Ag as part of pre-transfusion testing within the hospital BB using blood from a segment attached to the donor unit. Repeat testing of donor units by the transfusion service for weak D is not required by AABB Standards. Weak D testing is also performed in certain circumstances such as in fetus/newborns of RhD-negative mothers or when there is a suspicion that the patient possesses a partial D Ag and is at risk of allo-immunization.

Antibody screen and extended panel

The Ab screen is performed by mixing patient's plasma with two or three reagent RBCs of known phenotype prepared from donors of type O so that the naturally occurring anti-A or anti-B Abs would not interfere with the testing. For routine pre-transfusion testing, it is required that Ab screen reagents should contain at least one reagent with homozygous RBCs positive for the following 18 major RBC Ags (C, c, D, E, e, Fy^a, Fy^b, Jk^a, Jk^b, K, k, Le^a, Le^b, P_1, M, N, S, and s).

If present in patient's plasma, allo-Abs against major RBC Ags react with the reagent RBCs resulting in a positive Ab screen. This prompts additional testing with an extended panel of 10–12 reagent RBCs of varying phenotypes also obtained from group O donors. This testing aims at identifying whether the Ab detected in the patient's plasma has specificity against major RBC Ags, and is potentially of clinically significance. Clinically significant Abs are known to cause hemolysis such as in acute hemolytic transfusion reactions (HTR) and hemolytic disease of the fetus and newborn (HDFN). If they are present, Ag-negative RBCs are provided indefinitely to prevent HTR. If the plasma reactivity is due to clinically insignificant allo-Abs XM-compatible RBC units can be released, and transfusion of Ag-negative units is not necessary.

Plasma reactivity detected in the Ab screen/panel may be due to various causes, immune or non-immune, and should be distinguished from false positive reactions and sample errors. The reaction conditions modulate this reactivity and play a significant role in interpretation of BB testing [1–3]. The presence of false negative reactions and technical errors should also be ruled out. The Ab identification (AbID) is the post-testing interpretation of the various reactions performed and is covered elsewhere [4]. The Ab reactivity is not always typical and testing additional reagent RBCs may be needed to achieve an acceptable level of confidence that the reactivity seen is due to a true RBC Ag-Ab reaction. Even after ruling out Abs against major RBC Ags, a weak reactivity with some reagent RBCs may persist, which is commonly interpreted as the result of an Ab of unknown significance (AUS) [5]. The Ab ID process cannot be performed if the patient's plasma reacts with all reagent RBCs (panagglutinin).

The direct human antiglobulin test (DAT) and/or autocontrol (AC) are required to be part of the Ab ID process. These tests are discussed in the next section.

Autocontrol and direct anti-human globulin test

The autocontrol (AC) is performed by mixing the patient's plasma with his own RBCs. A negative AC implies that the reactivity detected in plasma is due to an allo-Ab, while a positive result suggests an auto-Ab, but does not rule out the presence of allo-Ab if the patient has a history of recent RBC transfusions. A positive AC should prompt performing the direct antiglobulin test (DAT) and, if positive, the RBC eluate should be tested against the Ab screen reagent RBCs. Both auto- and allo-Abs might be present in plasma or bind the patient's RBCs, and their reactivity can overlap.

The DAT or direct Coombs' test detects the presence of Ab or complement fraction bound to RBCs *in vivo*. The test is simply performed by adding AHG or Coombs' serum to washed patient's RBCs. The RBCs agglutinate if they are coated *in vivo* with immunoglobulin G (IgG) or the complement fractions, indicating a positive reaction. A positive DAT is not always associated with decreased RBC survival and/or hemolysis. As many as 15% of hospitalized patients and between 1 in 1000 to 1 in 9000 blood donors can have a positive DAT [6]. When hemolysis is present, a positive DAT indicates an immune cause for RBC destruction. Common cases of hemolysis associated with a positive DAT are HTRs (recipient allo-Abs coat transfused donor RBCs),

HDFN (maternal Abs cross the placenta and bind fetal RBCs), autoimmune hemolytic anemia (auto-Abs bind the patient's own RBCs), drug-induced immune hemolytic anemia, and passenger lymphocyte syndrome (Abs produced by lymphocytes from a transplanted organ bind recipient RBCs). Hypergammaglobulinemia may be also associated with a positive DAT due to non-specific passive adsorption of immunoglobulins (Ig) onto circulating RBCs. A negative DAT does not totally exclude the above conditions due to limitations of the test. The routine DAT detects only IgG Abs and complement fractions C3d and C3b if more than ~200 molecules are coating the RBCs. Abs belonging to other Ig subclasses (IgA, IgD, IgE) are not usually detected. In the case of a positive DAT with a prior RBC exposure via transfusion or pregnancy, an RBC eluate is performed and tested against the Ab screen panel to identify if any antigen specificity of the eluted antibodies. Correct DAT interpretation also requires knowledge of the reagents used, as polyclonal Abs have been reported to give false positive DAT results [6,7]. Similarly, knowledge of the reaction conditions is critical for correct interpretation of a positive RBC eluate [8,9]. A positive DAT may also be caused by certain drugs [10,11].

Although the DAT and AC are often used interchangeably, they are not the same test. DAT detects the *in vivo* coating of patient's RBCs whereas the AC indicates the *in vivo* and *in vitro* plasma reactivity.

In vitro compatibility testing or crossmatch

According to the AABB Standards, XM testing shall use methods that demonstrate ABO compatibility. In a general sense, compatibility testing refers to the entire quality process that ensures that the safest blood products are issued. This process includes numerous steps from product ordering to blood testing, product issue, record keeping, and documentation. In a more restricted sense, compatibility testing may refer just to the *in vitro* assay historically known as a serologic XM. In the *major XM,* the patient's plasma is tested against donor RBCs obtained from a segment of the RBC donor unit. The major XM aims to detect whether the transfused RBCs will survive in a patient's blood; it also detects the presence of plasma reactivity that might not have been identified by the Ab screen, for example with Abs against low incidence (LI) Ags. The *minor XM,* in which the patient's RBCs are tested against donor's plasma, was largely replaced by routine performance of the Ab screen, and was discontinued altogether in 1976.

The method of performing the major XM depends on the results of the Ab screen. If the Ab screen is negative and the patient does not have a history of clinically significant Abs, the XM is performed in the immediate spin (IS) phase, otherwise known as the incomplete XM. Alternatively, an electronic or computer XM wherein the ABO types of both donor and recipient are electronically confirmed and assessed for ABO compatibility by AABB-approved computer software is also acceptable. The XM performed at 37 °C and in AHG phase has been historically discontinued for cases with negative Ab screen due to significant savings in labor and cost. If the Ab screen is positive, the major XM is performed in AHG phase with selected Ag-negative RBC units. This serologic XM test serves also as a final checkpoint for preventing ABO typing errors.

RBC phenotype

The RBC phenotype is not performed for routine transfusions, but used when identifying the presence of Ags is essential to the AbID or selection of compatible RBC units, for frequently transfused patients, detection of rare phenotypes in donors, or as part of paternity or forensic investigations. Ideally, the RBC phenotype of frequently transfused patients should be performed prior to initiation of transfusion therapy since this category of patients has a much higher risk of alloimmunization. The test is performed serologically by mixing the patient's RBCs with commercially available reagent antisera against common RBC Ags (CcEe, MNSs, Kell, Duffy, Kidd, etc.) or by molecular methods.

INTERFERENCES IN BASIC BLOOD BANK TESTING

Interferences that alter the expected reactivity pattern leading to misidentification or misinterpretation of significant findings may occur in the routine BB tests described in the previous section. True interferences should always be distinguished from errors occurring in the process of blood testing and administration, although they may appear similar at the time of initial interpretation. If suspected, potential errors, including technical and clerical, should be ruled out for correct test interpretation. A major source of error can occur with deviations from protocols for patient identification and sample collection. These errors may lead to blood drawn from a patient different than the one intended, blood drawn from the correct patient into a mislabeled tube, or general sample mix-ups, leading to so-called wrong blood in tube (WBIT). WBIT does not represent a true interference, but an error in the quality process. Early recognition, further investigation, and increased adherence or alterations to processes are required to prevent such events from reoccurring. Two blood samples from the same patient are required for ABO/Rh determination prior to transfusion in order to prevent clerical errors or detect WBIT. If WBIT is suspected, it should be ruled out by repeating the test on a different sample collected from the same patient.

True interferences are classified as pre-analytical, analytical and post-analytical. Pre-analytical variables are those that influence test results prior to specimen collection and are related to patient characteristics, sample collection, and the specimen itself. Pre-analytic variables are grouped in three categories: related to specimen collection, handling and processing, and related to physiologic factors and patient's endogenous variables, including diseases, circulating antibodies, and drug therapy. Patient-related pre-analytical variables, such as demographic data (age, sex, race), clinical diagnosis, and past and current medication, are critical in interpretation and drugs may directly interfere with testing results. Physiologic variables (age, sex, time, season, altitude, conditions such as menstruation and pregnancy) and other variables such as subject preparation (fasting), prior exercise, and posture during blood drawing are not known to interfere with BB tests. Factors related to specimen collection, such as effects of the duration of tourniquet application, the anticoagulant/blood ratio, specimen handling, and processing steps in the preparation of serum/plasma and RBC separation, can introduce a pre-analytic variable when they result in hemolysis or fibrin clots present in the sample that may be misinterpreted as a positive reaction.

Methodology used for testing plays a definite role in test interpretation. In general, microcolumn gel technology is known to be more sensitive than tube testing and less sensitive than solid phase adherence technology for detection of clinically significant allo-Abs. The advantage of using a methodology with increased sensitivity is better identification of patterns of reactivity specific to Abs that might not be elucidated by a lesser sensitive methodology. Studies showed that clinically significant allo-Abs identified by solid phase technology displayed a non-specific pattern in gel testing. Few studies reported, however, that gel technology is less sensitive than the tube test methodology for detecting ABO isohemagglutinins and that expected anti-A or anti-B may be missed. Reagents and chemicals present in the reaction environment of various testing kits may also result in a positive reaction and initiate extensive testing to rule out the presence of allo-Abs. Similarly, the patient's auto-Abs can react with reagent RBCs present in the Ab screen and panel, and consequently mask possible allo-Abs.

Interferences in ABO/Rh typing

ABO discrepancies include disagreement between historical and current blood type, mismatch between forward and reverse reactions, reactivity weaker than expected (4+) between the RBC Ags and corresponding Abs or detection of mixed field type of reactivity. ABO discrepancies detected in patients to be transfused must be resolved before any blood component is transfused unless blood is urgently needed, in which case group O RBCs are issued. Discrepancies occurring in donor samples must be resolved before blood is labeled with a blood type. At the initial read of an ABO discrepancy, it is not known which of the typing reactions, the forward or the reverse, reflects the patient or donor's true ABO type. Additional testing and knowing patient's age, clinical diagnosis, historical blood type and transfusion history are critical for final interpretation.

Clerical and technical errors must be first ruled out since they are the most common causes for ABO discrepancies. Clerical errors are responsible for more than 95% of fatal transfusion reactions and imply patient or specimen misidentification or blood sample mix-up. Causes of technical errors include failure to follow manufacturer's instruction, failure to add cells or reagents, incorrect test cell preparation such that the pipettor dispenses a lower amount of A or B reagent cells into the well, improper centrifugation or incubation conditions, use of contaminated reagents, or defective equipment. Since clerical and technical errors are not uncommon, the best course of action is to repeat testing on a better washed RBC sample and using plasma from the original specimen to see if the discrepancy persists. Repeat blood drawing might be needed, especially if sample mix-up is a concern.

True ABO discrepancies (not due to clerical and technical errors) are generally grouped in four categories: weak expression or loss of expected RBC Ag; presence of unexpected RBC Ag-like reactivity; weak or loss of expected Ab; and presence of unexpected Ab reactivity.

Weak or absent reactivity of expected antigen

Weak A or B Ags are seen in ABO subgroups, age extremes (newborns and elderly), or cancers. In certain hematological malignancies (leukemia, Hodgkin's lymphoma) aberrant ABO glycosyltransferase formation has been described that leads to less Ag being formed on the red cell membrane, whereas in solid organ cancers an excess of soluble substance similar to A or B Ags can neutralize the typing reagents and result in a false negative reaction.

Unexpected RBC antigen-like reactivity

Interferences causing RBC agglutination such as *rouleaux* formation, polyagglutinable RBCs, or agglutinating substances such as Wharton's jelly may lead to misidentification of unexpected RBC antigens. Autoagglutination due to cold agglutinins often causes unexpected reactivity (Ag-like in the ABO/RhD typing, but also Ab-like reactivity in the Ab screen). Abs directed against chemicals or drugs present in the reaction microenvironment, hematopoietic progenitor cell (HPC) transplant, fetomaternal hemorrhage, and B(A) phenomenon are other known causes. mf agglutination is

usually noted in chimeras indicating a mixed RBC population (such as in post-transfusion, massive transfusion of another blood group, and HPC transplant). Polyagglutination is a phenomenon suspected when RBCs react with most normal adult sera but not with autologous serum. It may be due to exposure of a cryptic Ag on the RBC surface due to action of an enzyme associated with a micro-organism (acquired microbial polyagglutination), by passive adsorption of microbial structures with antigenic structures similar to A, B, H, T and Tn Ags, or Ags acquired due to a somatic or inherited stem cell mutation.

Weak or loss of expected antibody

The most frequent cause of this discrepancy type is failure to detect anti-B in A or O plasma samples. This can readily be corrected by tube testing, usually performed at room temperature (RT). Weak or absent Ab reactivity is seen in newborns, elderly, immunosuppressed patients, post HPC transplant, or in severe immunodeficiencies.

Unexpected antibody reactivity

Detection of unexpected reactivity may be due to passively acquired Abs (via transfusions, administration of Rh immunoglobulin, intravenous Ig, or other drugs) and the passenger lymphocyte syndrome. *Rouleaux* formation may mimic the presence of agglutination. Individuals with ABO subgroups can develop allo-Abs that react with reagent RBCs in the reverse typing. The classic example is a patient in the A2 subgroup with anti-A1 Ab. Positive reaction can result also from cold agglutinins and true allo-Abs reacting with Ags present on the surface RBCs used for testing (such as anti-M, anti-N, anti-P1, anti-c). A citrate-dependent auto-Ab causing errors in blood grouping was also described [12].

Interferences in the antibody identification

The Ab identification process is not always straightforward. If clerical and technical errors are ruled out, the reactivity observed *in vitro* may be due to (1) false positives, (2) real Ag-Ab reactions and (3) non-immune interactions. False positive reactions or pseudoagglutination were described above. Common causes of interferences in this category include the presence of fibrin clots, *rouleaux* formation and agglutination due to albumin and plasma expanders. Real Ag-Ab reactions can result from the presence of allo- or auto-Abs directed against specific RBC Ags alone or in combination.

Auto-Abs typically react with a patient's own cells and all reagent RBCs present in the Ab screen and panel in a non-specific manner. Cold auto-Abs (CAA) and warm auto-Abs (WAA) are typically pan-reactive and, if present, they interfere with the detection of allo-Abs. In such cases, further work-up is needed to rule out the presence of underlying allo-Abs against major RBC Ags. Distinguishing between auto-Abs versus allo-Abs is essential and the reactivity pattern suggests the type of auto-Ab. CAA react only at cold temperatures, including at RT, as with standard *in vitro* testing, but not at 37 °C, and are generally considered clinically insignificant. The typical CAA is 2 + or mf equally pan-reactive with reagent RBCs present in the Ab screen/panel. WAA react at 37 °C or in the presence of AHG and the typical WAA is strong (3+ or 4+) and equally pan-reactive. In addition to auto-Abs, passively transfused Abs originate from plasma products containing allo-Abs, or intravenous Ig, or from patients in post organ or HPC transplant status.

Interferences due to Abs present in the patient's plasma were described for a variety of chemicals, antibiotics, potentiators (low ionic strength solution − LISS or polyethylene glycol - PEG), or other substances (lactose, lactate, meibiose, phenol, saccharose, thrombin) present in the testing environment or commercial antisera [13–15]. Chemicals that may lead to generation of antibodies reacting with RBCs are Paraben, thimerosal, sugars, EDTA, inosine, citrate, acriflavine, sodium caprylate, yellow #5, and tartrazine [14].

Case studies

The following case studies are meant to provide a basic understanding of the most common interferences seen in BB tests. The emphasis is on building a stepwise approach to final interpretation and correlation with clinical history.

Case study #1

Clinical summary and initial testing

A type and cross request for one RBC unit is received for a 74 year old male with active gastro-intestinal bleeding and a historical blood type of A RhD-negative. The patient has a history of massive transfusion six years prior to admission after a pedestrian accident led to multiple traumatic injuries. The ABO/RhD typing is presented below:

Forward			Reverse	
Anti-A	Anti-B	Anti-D	A1 RBCs	B RBCs
3+	0	0	2+	4+

Initial interpretation and further testing

The forward reaction shows a blood type A with a weaker than expected reaction with anti-A, and the

reverse reaction shows a strong reaction (4+) with B reagent RBCs indicating the presence of normal anti-B in patient's plasma, thus a blood type A. In addition, reactivity with A1 RBCs is detected. Correlation with patient's known historical blood type, clinical history, Ab screen and testing for cold agglutinins is required in cases with extra-Ab reactivity. Most likely, this is a case of A subgroup with anti-A1 Ab. Testing of the patient's RBCs with *Dolichos biflorus*, a lectin that agglutinates A1 Ag, is negative. In general, ABO subgroups present with weaker than expected reactivity in forward typing. The most common A phenotypes are A_1 (80%) and A_2 (less than 20%). The A^1 and A^2 genes code for different A transferases, and the A_2 transferase is less efficient in converting H to A substance, resulting in red cells that have approximately 20–25% less A antigen than A_1 cells. In addition to this quantitative difference, A_1 and A_2 antigens have a different carbohydrate composition. This biochemical difference explains why 1–8% of A_2 individuals and 22–35% of A_2B individuals produce anti-A_1 Ab. The anti-A1 Ab causes discrepancy between the forward and the reverse typing because A1 reagent RBCs are used for reverse testing. The anti-A1 is not usually clinically significant since it does not usually react at 37 °C, therefore release of A blood group is safe for the patient. However, a mini-thermal amplitude test should be performed to rule out cold Abs, which may give a similar ABO discrepancy and also prove that the anti-A_1 does not react at body temperature. The results of the mini-thermal amplitude test are shown below.

	IS	RT	37 °C	AHG
A1 cells	2+	2+	2+	2+
A2 cells	0	0	0	0
B cells	4+	4+	4+	4+
I	0	0	0	0
II	0	0	0	0
III	0	0	0	0
Autocontrol	0	0	0	0

Final interpretation

The anti-A1 reactivity is maintained at 37 °C and in AHG phase. The finding is critical because, unlike typical cases of anti-A1 Ab, this suggests a clinically significant Ab and A blood type should not be transfused. Only crossmatch compatible O RhD-negative units are safe for this patient.

Case study #2

Clinical summary and initial testing

A request for T&S is made for a 24 year old pregnant female as part of her routine prenatal testing. The patient's historical blood type is AB RhD-positive. The patient's current ABO typing results are presented below:

Forward			Reverse	
Anti-A	Anti-B	Anti-D	A1* RBCs	B RBCs
4+	4+	4+	2+	0

Results of the thermal amplitude test are shown here:

	IS	RT	37 °C	AHG
A1 cells	0	0	0	0
A2* cells	2+	2+	0	0
B cells	4+	2+	0	0
I	0	0	0	0
II*	2+	2+	0	0
III	0	0	0	0
Cord RBCs	0	0	0	0
Autocontrol	0	0	0	0

Initial interpretation and further testing

This patient's plasma reacts with A1 RBCs as in the A2 subtype cases described before, however this reaction is likely not due to an anti-A1 Ab because no reactivity is seen with other A1 cells. The patient's RBCs types positively with *D. biflorus* indicating that patient's true type is A1. In addition, the patient's plasma reacted with A2 cells and one screening cells. As in any case of a positive Ab screen, further testing was performed and showed an anti-M Ab. All the clinically significant allo-Abs were ruled out and the patient's RBCs typed negative for the M Ag. To further prove that the reactivity seen in the ABO typing and mini-thermal amplitude test was due to the anti-M antibody, the reacting (*) RBCs were tested for M Ag. Results of the M Ag typing are presented here:

Reagent RBC tested	Patient's plasma	Anti-M reagent
A1 RBC*	2+	4+
A2*	2+	4+
II*	2+	4+
B cells	2+	2+

Final interpretation

This interference is explained by the presence of alloanti M antibody in the patient's plasma.

Case study #3

Clinical summary and initial testing

Two RBC units are requested for a 54 year old male admitted for melena who was found to have a hemoglobin (Hb) level of 6 g/dL. His previous blood type

is unknown. Results of the ABO/Rh typing are presented below. What ABO type of RBC units should be issued for this patient?

Forward			Reverse	
Anti-A	Anti-B	Anti-D	A1 RBCs	B RBCs
4+	3+	0	0	4+

Initial interpretation and further testing

Current blood type reveals an ABO discrepancy between the forward and reverse reaction. His RhD type is negative. The forward type appears to be AB with a weaker B Ag expression and the reverse type reveals blood type A. Based on reactions pattern and strength, it is most likely that the blood type is A with unexpected B Ag present. This pattern has been described in acquired B phenomenon. This occurs in certain clinical setting (patients with carcinoma of colon or rectum, intestinal obstruction, septicemia or wound infections) when microorganisms (*Proteus vulgaris, Clostridium* and *Escherichia coli 086*) release bacterial enzymes that deacetylate the true A antigen (N-acetyl galactosamine) to galactosamine, which cross-reacts with the anti-B reagent as the true B Ag (galactose). The discrepancy becomes evident in the reverse typing when strong anti-B Abs are identified.

Distinction of true B versus acquired B requires knowledge of clinical conditions and extra-testing. In the acquired B phenomenon, although the patient's own RBCs appear to type as B, they do not agglutinate with his own serum containing anti-B Abs, whereas other reagent RBCs of B type give a positive reaction. Repeat forward reaction after acidifying the anti-B reagent to pH 6 or after addition of galactosamine will maintain reactivity only in the case of true B Ag but not with the acquired B Ag. Other methods used to distinguish the true B Ag from the acquired B are typing with *Griffonia simplicifolia* (former *Bandeira simplicifolia*) or *Phaseolus lunatus* lectins, which will agglutinate only the true B Ag. Genotyping is also helpful, as well as detection of the B Ag in secretions if the patient has a secretor status.

Final interpretation

The patient's true blood type is A RhD-negative. Early recognition of the acquired B phenomenon is critical for cases with weak anti-B Ab that might be mistaken as AB type. Transfusion with type specific RBCs in such cases may result in an acute hemolytic reaction. As in any case of ABO discrepancy, if emergent RBC transfusion is needed, universal RBCs of O type are issued.

Case study #4

Clinical summary and initial testing

A 2-year-old female with acute intestinal obstruction and a hematocrit of 21% undergoes emergent surgery to remove necrotic bowel segments. During the surgery she received 250 mL fresh frozen plasma (FFP). Immediately post-surgery, she developed fever, hemoglobin/hematocrit drop, hemoglobinemia, and hemoglobinuria. Repeat of her pre-transfusion Ab screen was negative. Repeat XM was found to be compatible, as it was prior to transfusion. The DAT of the post-transfusion sample was positive (1+) with complement fraction detected on the RBC surface. The RBC eluate was non-reactive with reagent RBCs present in the Ab screen. The DAT of the pre-transfusion sample was negative.

Initial interpretation and further testing

This patient experienced DAT conversion to positive after transfusion of FFP. The RBC eluate is negative and repeated Ab screens and XM are negative. The clinical summary and BB testing are suggestive of polyagglutination due to the *T activation phenomenon.*

T activation**,** also called Hubener-Thomsen-Friedenreich phenomenon, refers to alterations of the RBC membrane Ags, usually by microbial enzymatic action, with subsequent exposure of previously hidden (cryptic) Ags that will react with most adult normal sera. The enzyme responsible for the reaction is a neuraminidinase removing N-acetylneuraminic acid (sialic acid) residue from glycophorin A and B exposing the cryptantigen (or T Ag). This is accompanied often by decreased expression of M and N antigens normally situated on glycophorin molecules. The exposed T Ag reacts with the anti-T Ab found in normal sera. The anti-T Ab is a naturally occurring IgM antibody formed as a result of stimulation with T-like Ags present on bacteria and viruses.

Typical clinical scenarios accompanied by T cell activation include children with bacterial infections with *Streptococcus pneumonia*, *Clostridium perfringens*, *Vibrio cholera* or influenza virus. These patients have positive DAT and hemolysis often after exposure to FFP. This is a transient phenomenon lasting weeks to months and, if suspected, should be proven by further testing with a lectin panel. Lectins are extracts from plant seeds used in BB testing for their property to agglutinate certain RBC Ags. Further testing revealed that patient's RBCs strongly reacted with *Arachis hypogea* (peanut lectin) and *Glycine soja* (soya bean). No reactions are detected with *Salvia sclarea, Salvia horminum* and *D. biflorus.*

Final interpretation

Additional testing with specific lectins identified a T activation phenomenon. Recognition of this phenomenon is important to explain ABO discrepancies, incompatible XM, or DAT conversion with complement coating the patient's RBCs, and post-transfusion hemolysis with negative work-up since these patients undergo immune hemolysis, but not due to allo-Abs against specific RBC Ags [16–19]. Especially if detected within the context of a transfusion reaction work-up, the presence of acute hemolytic transfusion reactions, clerical errors, and allo-Abs against major RBC Ags have to be ruled out. The DAT is usually negative in patients with T-activated RBCs, but it may be positive with complement such as in this case. As these patients have hemolysis and usually blood products are requested for them, early identification and testing is critical in order to recommend avoidance of transfusion with FFP since it may lead to further hemolysis. If RBC transfusions are needed, they should be washed and if platelets are transfused, plasma should be removed first and platelets resuspended in 5% albumin.

Case study #5

Clinical summary and initial testing

The patient is a 55 year old female with a history of coronary artery disease and back pain who was admitted for fatigue and was found to have a creatinine of 2 mg/dL and a Hb level of 8 g/dL. A request for one RBC unit is made for this patient. Her historical blood type is A RhD-negative with a negative Ab screen. She does not have a history of blood transfusion, but is a mother of two children and had three abortions. Her current blood typing and Ab screen results are illustrated below:

Forward			Reverse	
Anti-A	Anti-B	Anti-D	A1 RBCs	B RBCs
4+	0	0	1+	4+

Reagent RBC	AHG
I	1+
II	1+
III	1+
Cord RBCs	1+
Autocontrol	1+

Initial interpretation and further testing

Pan-reactivity with mild reactivity may be due to multiple causes. Before embarking in additional time-consuming testing, a clinical history that may be suggestive of multiple myeloma should promptly raise the question of pseudoagglutination. Pseudoagglutination is due to *rouleaux* formation of RBCs and can be mistaken as true agglutination. When viewed microscopically on peripheral blood smear or microscopic examination of the reaction tube, the RBCs have the appearance of a "stacks of coins". If *rouleaux* phenomenon is suspected, the first step is to repeat testing after adding 0.9% normal saline and resuspending the RBCs or wash patient's RBCs. Repeat ABO typing after washing patient's RBCs yielded the following results:

Forward			Reverse	
Anti-A	Anti-B	Anti-D	A1 RBCs	B RBCs
4+	0	0	0	4+

A peripheral blood smear revealed the characteristic *rouleaux* formation. Serum protein electrophoresis and immunofixation studies revealed the presence of a monoclonal gammopathy of IgG lambda. The patient was scheduled for a bone marrow biopsy.

Final interpretation

The ABO discrepancy was resolved and patient serology is most likely explained by the high level of IgG present in her plasma. Pseudoagglutination is a common occurrence for a category of patients with hypergammaglobulinemia with or without monoclonal gammopathies (multiple myeloma, macroglobulinemia, Waldenstrom's macroglobulinemia, hyperviscosity syndrome, and liver diseases). It may be also caused by hyperfibrinogenemia or exogeneous factors, such as infusion of high molecular weight plasma expanders like dextran, polyvinyl-pyrolidon, or some intravenous X-ray contrast materials that circumvent the natural influence of the ζ potential to keep RBCs apart.

Rouleaux phenomenon may cause ABO discrepancies, but also incompatible XM, positive Ab screen, and positive extended panel, but it does not usually interfere in the AHG phase because the recipient's plasma is washed away prior to addition of AHG. Review of patient clinical and transfusion history is thus essential for interpretation.

Abs to high-incidence (HI) RBC antigens, such as k, Kpb, Jsb and Lub, may give a similar pattern, panreactive (1+), but the AC is negative. Also, being true Ag-Ab interactions, they will not disappear after saline replacement. These Abs are rare and difficult to identify

due to lack of negative panel cells for other HI Ags. Further testing in a reference BB laboratory is often needed.

Case study #6

Clinical summary and initial testing

A newborn male was delivered to a 28-year-old mother via vaginal delivery at 39 weeks gestation. The mother's blood type is O RhD-negative with anti-D antibody detected in her serum early in the pregnancy. The newborn has been monitored for HDFN and he has not required any interventions, throughout his fetal life. His bilirubin level is 12 mg/dL and the forward typing shows the following results:

Forward		
Anti-A	Anti-B	Anti-D
3+	4+	0

Initial interpretation and further testing

The newborn forward typing suggests that his RBCs are of AB type. Reverse typing is not performed on newborns since they do not produce Abs until the age of six months at least. His RhD type is negative, thus HDFN due to anti-D antibody is ruled out. As his mother is blood type O and she does not have other allo-Abs that can explain the baby's high bilirubin, a possible extra-reactivity due to sample contamination with Wharton's jelly should be suspected. Spontaneous agglutination of samples contaminated with Wharton jelly was described in textbooks, but is not very common. It represents a non-specific agglutination due to hyaluronic acid and albumin found in cord blood. Repeated testing with baby's washed three times with 0.9% saline yielded the following results:

Forward		
Anti-A	Anti-B	Anti-D
0	4+	0

The DAT performed on the baby's RBCs was positive (2+) with IgG and no complement fixation noted. An anti-B Ab was eluted from the baby's RBCs.

Final interpretation

The baby's true blood type is B RhD-negative. Initial reactivity was most likely due to Wharton's jelly coating the patient's RBCs and interfering with the RBC testing. He has hemolytic disease of the newborn due to IgG anti-B Ab present in his mother's blood and phototherapy is indicated due to his high bilirubin, as well as monitoring for clinical and laboratory indicators of hemolysis.

Case study #7

Clinical summary and initial testing

The patient is a 36 year old male who was admitted for multiple abdominal gun-shut wounds status-post massive transfusion for life-threatening bleeding four days prior to the current testing. At that time the patient received six O RhD-negative RBC units, six FFP units and one apheresis platelets. His blood type was retrospectively found to be B RhD-negative. The patient underwent successful surgical repair but he remained critically ill and his Hb levels dropped from 9 to 7 g/dL over the past few days. Two RBC units are requested in preparation of surgical re-exploration. Current ABO typing is shown below. His Ab screen is negative.

Forward			Reverse	
Anti-A	Anti-B	Anti-D	A1 RBCs	B RBCs
0	2+ mf	0	3+	0

Interpretation

The patient's initial blood typed as O RhD-negative. He now has an ABO discrepancy with weaker than expected B Ag with mf reaction and weaker than expected anti-A Ab. The history of massive transfusion is essential for interpretation and explains the current findings due to dilution of patient's own B cells by transfused RBCs of O type. Additional testing is not required in this case. The patient's true blood type is B RhD-negative and XM ABO compatible or specific RBC units can be released.

Case study #8

Clinical summary and initial testing

One RBC unit is requested for a 55 year old female with a history of HPC cell transplant three weeks ago for recurrent acute myelogenous leukemia. The patient was O RhD-negative and the HPC donor was B RhD-negative. Her current ABO/Rh typing results are shown below:

Forward			Reverse	
Anti-A	Anti-B	Anti-D	A1 RBCs	B RBCs
0	2+ mf	0	3+	0

Interpretation

This case is serologically similar to Case Study #7, however, the patient's clinical history is different and critical for recommendation regarding transfusions. ABO mismatched HPC transplants are routinely performed and during the period of engraftment the recipient ABO type gradually switches to the donor type and during this time, an ABO discrepancy often with mf reactivity is noted. The ABO discrepancies are explained by the patient's history and no further testing is required. In this case the patient is transitioning to a blood type B reflected by detection of a B Ab in the forward typing and the presence of anti-A Ab in the reverse typing. These findings are expected for such a transition and reflect good HPC engraftment. Most important for these cases is to transfuse with blood products compatible with both recipient and donor blood type until engraftment is complete.

Case study #9

Clinical summary and initial testing

A request for type and screen is made for a 30 year old female pregnant at 28 weeks of gestation. The patient has another living child and a history of two abortions. She has never received a transfusion. Her blood type is found to be A RhD-negative and she has a positive Ab screen. Additional testing reveals the presence of (4+) anti-D reactivity. In addition, an anti-C Ab cannot be ruled out. The patient does not remember if she was administered Rh Ig during her first pregnancy.

Initial interpretation and further testing

Since allo-anti-C Ab could not be ruled out, the patient's plasma was tested with C-positive reagent RBCs in the presence of ficin and an anti-C allo-Ab was demonstrated. The patient's RBCs were tested for C Ag and found to be negative. When plasma reactivity is suggestive of both anti-D and anti-C, additional testing performed at a reference laboratory should be performed to rule out anti-G Ab. This is an allo-Ab directed against G antigen, an antigen that is part of the Rh blood group, formed when C and/or D are present. Anti-G Ab cannot be detected by routine methods because reagent RBCs are not profiled for this Ag. In this case, additional testing revealed the presence of an anti-G Ab in the patient's plasma.

Final interpretation and recommendations

The patient has an anti-G allo-Ab present in her plasma. This antibody may cross the placenta and cause HDFN. Since she does not have a true anti-D allo-Ab, she is still eligible for prophylaxis of HDFN due to anti-D. Should she require further transfusions, RBC units negative for both C and D Ags should be issued. The same transfusion requirements are made if the anti-G is detected in male plasma except that distinguishing between anti-D versus anti-G status is neither required nor necessary. This distinction is useful only to capture the true RhD-negative females who have not formed anti-D Abs and thus are eligible for RhIg administration.

Case study #10

Clinical summary and initial testing

One RBC unit was requested for a 9-year-old male with a history of bilateral renal dysplasia status post renal transplant who was diagnosed with graft rejection. His Hb level is 7 g/dL. The patient has a history of blood transfusion when he was 7 years old during the peri-transplant period, but not recently. His blood type is known to be O RhD-positive and his Ab screen was negative at prior testing. The current ABO/Rh type is the same, but the Ab screen is positive and additional testing reveals the presence of anti-D and anti-c antibodies.

Interpretation

A careful review of medical and drug history reveals that this patient was administered intravenous iimmunoglobulin (IVIg) to treat his rejection. His newly identified anti-D and anti-c allo-Abs are likely due to passive transfer from the IVIg preparation. Passive transfer of allo-Abs is commonly seen after administration of IVIg [20–22]. These preparations are extracted by cold fractionation from large pools of volunteer plasma donors and they contain a broad spectrum of Abs to viral, bacterial, and other microorganisms. Isohemagglutinins and allo-Abs against RBC Ags may be present in up to 17–50% of these preparations and continue to be present in patients' plasma several weeks to months post administration. These antibodies are usually associated with self-limited hemolysis. Early recognition of this typical clinical scenario may prevent additional unnecessary testing and transfusion delays.

Case study #11

Clinical summary and initial testing

A request for T&S is made for a 56 year old female with coronary artery disease who is scheduled for coronary artery bypass grafting. Her Hb level is 10 g/dL and

she is currently asymptomatic. Her ABO/Rh test results are shown below. The Ab screen is equally pan-reactive (2+). The extended panel also shows 2+ pan-reactivity and the AC is also 2 + positive.

Forward			Reverse	
Anti-A	Anti-B	Anti-D	A1 RBCs	B RBCs
4+	0	0	2+	4+

Initial interpretation and further testing

The initial presentation is identical to that of Case Study #1 (A2 subgroup with anti-A1 antibody) and Case Study #2 (anti-M allo-antibody). Unlike Case #2 where the Ab screen was positive with one cell, in this case (2+) pan-reactive plasma is seen. This pattern is typical of a CAA. Underlying allo-Abs against major RBC Ags cannot be ruled out.

CAA are antibodies against I/i Ags of the Ii blood group collection. They are associated with cold agglutinin disease (CAD) if the thermal amplitude extends up to the lower body temperature (35 °C). These Abs cause hemolysis via complement fixation. Even if they elute from RBCs at core body temperature, the attached complement causes hemolysis. The presence of CAA is demonstrated by thermal amplitude tests, cold auto-adsorption, or rabbit erythrocyte stromal test (RESt). In this case RESt was performed. The patient's plasma is incubated with rabbit erythrocytes, which are rich in I Ag and absorb anti-I Ab if present. The plasma incubated one or several times with RESt is then run against the usual Ab screen for detection of any leftover reactivity. A negative Ab screen after RESt is suggestive of anti-I, anti-IH or anti-H cold agglutinins. The RESt technique can be used if the patient has been recently transfused. The results should be interpreted with caution as RESt also can remove the reactivity of clinically significant allo-Abs such as anti-B, anti-D, and anti-E. Re-testing after washing cells 3–4 times with warm (37 °C) saline and/or performing serum testing at 37 °C may also be useful (pre-warm technique). Alternatively, allogeneic cold adsorption may be performed with phenotypically similar allogeneic cell lines if the patient's full phenotype is known or with selected reagent RBCs (usually R1, R2, and rr) negative for K, Jka, Jkb, Fya, Fyb, and S, so that potential clinically significant allo-Abs can be detected in the adsorbed plasma. If the patient has not been transfused within the past three months, a cold auto-adsorption technique may be used. In this method, the patient's own RBCs treated with a proteolytic enzyme solution to remove Ab binding sites are subsequently exposed to the patient's serum/plasma at 4 °C. Results of the thermal amplitude test and RESt are shown here:

Antibody screen	IS	RT	37 °C	AHG	CC	RESt x 2
I	2+	2+	0	0	4+	0
II	2+	2+	0	0	4+	0
III	2+	2+	0	0	4+	0
Autocontrol	2+	2+	0	0	4+	0
Cord blood	0	0	0	0	4+	NT

Final interpretation

Plasma reactivity is due to CAA. These are not considered clinically significant unless patients are exposed to cold temperature, such as in induced hypothermia during surgery. Measures to warm the blood transfused are required for patients with CAA and ABO and XM compatible RBC units can be released.

Case study #12

Clinical summary and initial testing

A 72 year old female with known chronic lymphocytic lymphoma and an indolent course of disease not requiring chemotherapy is evaluated for fatigue. During initial investigations starting two month ago, she was found to have a Hb level of 7 g/dL and received two RBC units at a different hospital. Her current Hb level is 8 g/dL and she is still weak. An additional RBC unit is requested. The patient's ABO/Rh typing results show B RhD-positive blood type. The Ab screen and extended panel show that her plasma equally and strongly (4+) reacts with all reagent RBCs tested. The DAT is positive with the same strength (4+) with IgG and mild (1+) complement fixation.

Forward			Reverse	
Anti-A	Anti-B	Anti-D	A1 RBCs	B RBCs
0	4+	4+	4+	0

Initial interpretation and further testing

The presentation suggests the presence of WAA. Clinically significant allo-Abs cannot be ruled out. Since the patient received two RBC units, her phenotype cannot be performed. The clinician was contacted to elucidate how urgent the need for blood was, and he stated that the patient was clinically stable. Phenotype prediction

by molecular methods revealed that the patient's RBC phenotype was negative for C, D and Jk(b) antigens. Allogeneic adsorption with phenomatched cells showed that she had an anti-Jk(b) Ab in her plasma and RBC eluate.

Final interpretation

This patient has WAA with underlying anti-Jk(b) allo-Ab in her plasma and RBC eluate. These findings are consistent with a delayed hemolytic reaction with possibly hemolytic WAA. Auto-immune hemolytic anemia (AIHA) caused by WAA is mostly due to IgG Abs with no RBC specificity; however, WAA with apparent specificity for Rh, Kell, Kidd, MNSs, ABO, Vel, and LW Ags have also been reported. The clinical significance of WAA is related to their propensity to cause hemolysis, which cannot be determined from the immunohematology testing, but from results of an *in vivo* hemolysis panel (hemoglobin, hematocrit, bilirubin, LDH, haptoglobin). Blood transfusions are usually avoided in warm AIHA, but if necessary, transfusion of phenomatched least incompatible blood is recommended.

Case study #13

Clinical summary and initial testing

One RBC unit is requested for a 54 year old female with relapsed acute myeloid leukemia who presents with fatigue and shortness of breath and was found to have a Hb level of 5.3 g/dL. The patient's ABO/Rh typing results are illustrated below. The Ab screen and extended panel show that her plasma variably reacts (1+ to 4+) with all reagent RBCs tested, and is not removed with DTT- or ficin-treated red cells, and after cold auto-, cold allo-, or rabbit erythrocyte stroma (RES)-adsorption. The DAT and AC are negative. Review of her medical history reveals that she is enrolled in a novel clinical trial with anti-CD47 therapy. She has not received prior transfusions in the past three months. Historical blood type is A RhD-positive. Her RBC phenotype is not known.

Forward			Reverse	
Anti-A	Anti-B	Anti-D	A1 RBCs	B RBCs
4+	0	4+	3+	3+

Initial interpretation and further testing

The ABO typing currently shows a discrepancy between the forward typing (4 + with anti-A, nonreactive with anti-B) and the reverse typing (3 + with both A_1 cells and B cells). RhD typing is positive.

The presentation suggests the presence of a panagglutinin. The presence of clinically significant allo-Abs cannot be ruled out. Repeat Ab screen and extended panel testing with LISS and PEG enhancements shows non-reactive plasma in the AHG phase, ruling out clinically significant allo-Abs directed toward major RBC Ags. One Kell-negative RBC unit XM compatible at 37 °C AHG phase was issued.

Final interpretation

Anti-CD47, currently in clinical trials for patients with acute myeloid leukemia, non-Hodgkin lymphoma, and solid tumors, may interfere with BB testing. Unlike interferences with anti-CD38 therapy, the anti-CD47 interference appears as an ABO discrepancy with unexpected reactivity in reverse typing and plasma panagglutination [23,24]. An RBC phenotype should be performed prior to commencing therapy.

CONCLUSIONS

The reactivity detected in BB testing can result from various causes. Discrimination of clinically significant allo-Abs is critical for issuing the right type of RBCs and preventing HTR. Clerical and technical errors, spurious results, false positive and false negative reactions are discerned after careful consideration of the whole case, including the patient's clinical and blood bank history, type of test, reaction conditions, and technology used. Along this process, typical clinical scenarios should be considered. A summary of the basic transfusion process, routine tests, and typical case studies are presented in this chapter with the scope to aid in rapid recognition of some of the most common interferences.

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CHAPTER

29

Errors and adverse effects of blood transfusion

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INTRODUCTION

Blood component transfusions are therapeutic and may be life-saving treatments for patients with a variety of conditions. Blood components (red blood cells, plasma, platelets and cryoprecipitate) are obtained by whole blood donation or by apheresis collection. In 2015, 11,349,000 units of red blood cell, 1,983,000 units of platelets, and 2,727,000 units of plasma were transfused in the United States [1].

Blood transfusions, although mostly safe, are associated with some risks and may lead to significant morbidity and mortality. The International Society of Blood Transfusion (ISBT) defines a transfusion related adverse reaction as a response or effect in a patient temporally associated with the administration of blood or blood components [2]. Blood transfusion associated adverse reactions are tracked through the National Healthcare Safety Network (NHSN) Hemovigilance Module of the Centers for Disease Control and Prevention [3]. The Food and Drug Administration (FDA)'s Code of Federal Regulations (21 CFR 606.170(b)) requires that facilities notify the FDA, Center for Biologics Evaluation and Research (CBER), Office of Compliance and Biologics Quality (OCBQ), as soon as possible after confirming a complication of blood collection or transfusion to be fatal [4].

In order to minimize the adverse effects and errors of transfusion, transfusion services should have standard operating procedures (SOPs) that define when, what and how to perform compatibility testing between the donor and recipient blood to ensure the safety of the recipient. Hospitals should also have SOPs that define how blood products should be infused and which safeguards need to be performed prior to transfusions.

Acute transfusion reactions (ATRs) occur within 24 h of blood component administration and often during the transfusion itself [5]. ATRs include acute hemolytic transfusion reaction (AHTR), sepsis, transfusion related acute lung injury (TRALI), anaphylaxis, and transfusion associated circulatory overload (TACO). Delayed transfusion reactions (DTRs) occur days, months or years after transfusion [5]. DTRs include delayed hemolytic transfusion reaction (DHTR), transfusion-associated graft versus host disease (TA-GVHD), post-transfusion purpura, and iron overload.

This chapter will discuss factors that may lead to errors in blood component selection, testing and administration. It will also describe various aspects of reactions that may occur with blood transfusion.

ERRORS IN TRANSFUSION

Prior to issuing blood for transfusion, a transfusion service/blood bank performs a type and screen test to determine the recipient's blood type and to detect the presence of any red blood cell (RBC) alloantibodies. Blood is crossmatched, either electronically when no alloantibodies are present or manually when alloantibodies are present, to ensure compatibility between the recipient and donor blood. At the bedside, a two-person three-way check is performed to ensure that the proper blood component is transfused to the intended recipient.

The most common causes of mistransfusion involve clerical and human errors in identifying the proper patient, sample and/or blood unit [5]. Supporting data reveal a mislabeling rate of 1:71 to 1:165 with a wrong-blood-in-tube rate of 1:2000 to 1:2800 [6]. Available technologies such as radiofrequency identification chips, handheld barcode scanners, and "smart" refrigerators

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similar to systems used for pharmacologic agents can be leveraged to reduce error rates.

Improper testing or interpretation of tests in the blood bank may fail to identify or misidentify RBC alloantibodies. In turn, patients can develop life-threatening hemolytic transfusion reactions if transfused. In order to prevent such errors in the blood bank, policies and procedures are in place and technologists are trained rigorously on proper testing techniques. Quality assurance programs that include policies and procedures for corrective and preventive action to address errors help ensure that error-prone processes are revised.

ADVERSE EFFECTS OF TRANSFUSION

When a transfusion reaction is suspected, several steps need to be taken (Table 29.1). A post-transfusion sample from the transfusion recipient must be obtained and sent to the blood bank/transfusion service for further work-up (Table 29.2). The purpose of the work-up is to ensure that the right blood was issued to the right patient and that no alloantibodies were missed that may have potentially caused a hemolytic transfusion reaction (HTR).

TABLE 29.1 Suspected transfusion reaction work-up.

Action	Rationale
1. STOP the transfusion!	Main indicator of survival in the event of hemolytic transfusion reactions (HTRs) is the amount of incompatible blood transfused.
2. Keep the vein open	Use normal saline to keep the line open to facilitate medication administration, if necessary.
3. Document patient vital signs (pre- and post-transfusion) and signs and symptoms post-transfusion.	A description of the patient's condition is necessary to determine the type of transfusion reaction the patient may have experienced.
4. Draw a post-transfusion blood sample in a tube with EDTA anticoagulant (i.e. pink or purple top tube)	A post-transfusion blood sample from the patient is necessary for the blood bank to work up a potential HTR.
5. Send a post-transfusion urine sample to the laboratory for urinalysis	To detect hemolysis.
6. Send a CBC and serum sample to the laboratory for blood counts, bilirubin level and other tests of hemolysis (haptoglobin, lactate dehydrogenase) as appropriate	To detect hemolysis.
7. Treat the patient with the appropriate medications/fluids, if necessary.	To stabilize the patient.

TABLE 29.2 Blood bank work-up of suspected transfusion reactions.

Item	Test
Documentation	- Clerical check to ensure the right unit was transfused into the intended recipient
Blood component bag	- Visual check of bag label to ensure the right unit was transfused into the intended recipient - Visual check of product appearance to determine the presence of hemolysis or contamination
Post-Transfusion Blood Sample	Required (pRBC transfusion only) - Visual check for hemoglobinemia or icterus, (Detects as little as 2.5–5 mL of hemolysis) - Direct antiglobulin test (DAT) - ABO type As needed - Antibody screen - Elution study - Crossmatch - Culture
Pre-Transfusion Blood Sample (only if post-transfusion blood sample shows positive results)	Required (pRBC transfusion only) - Visual check for hemoglobinemia or icterus, (Detects as little as 2.5–5 mL of hemolysis) - Direct antiglobulin test (DAT) - ABO type

Acute hemolytic transfusion reaction

Acute hemolytic transfusion reactions (AHTRs) occur due to a reaction between preformed antibodies and red blood cell antigens. The most severe reactions involve ABO antigens and recipient isohemagglutinins which result in rapid intravascular hemolysis, but may also involve other red blood cell antigens and alloantibodies which result in extravascular hemolysis. Rapid hemolysis of as little as 10 mL of incompatible blood can produce symptoms of an AHTR [5]. AHTR occurs with an incidence of about 1 in 76,000 and results in fatality in about 1 in 1.8 million red cell transfusions [5].

Signs and symptoms of AHTRs vary in severity and include fever, chills, hemoglobinuria, hypotension, renal failure, disseminated intravascular coagulation,

back pain, pain at the infusion site and anxiety. The severity of symptoms correlates with the amount of incompatible blood transfused. Immediate recognition and cessation of the transfusion can prevent dire consequences [5].

AHTRs should be managed with saline infusions or pressors to treat the hypotension and ensure adequate renal blood flow (goal: >1 mL/kg/h) as well as to maintain venous access. Diuretics may be used to promote increased urine output and enhance renal cortical blood flow [5,7]. Disseminated intravascular coagulation (DIC) is treated by supportive care with platelet, plasma and cryoprecipitate transfusions. In addition, analgesics may be needed as well [5].

Delayed serologic and hemolytic transfusion reaction

Delayed transfusion reactions (DTRs) are due to an incompatibility between donor red blood cell antigens and the recipient's alloantibodies formed as a result of active immunization from a prior transfusion. DTRs may or may not result in hemolysis. DTRs that do not result in hemolysis are referred to as delayed serological transfusion reactions (DSTRs) while DTRs that result in hemolysis are referred to as delayed hemolytic transfusion reactions (DHTRs).

DSTRs or alloimmunization occurs with an incidence of 1 in 100 [5]. Alloantibodies form due to an immune response to foreign antigens on the red blood cell surface. As of 2017, there are 36 blood group systems including ABO. Blood group antigens can be carbohydrates or proteins. The antigens of the ABO blood group are carbohydrates. They are produced by a series of enzyme catalyzed reactions whereby various sugars are added to a growing chain [5]. In addition to carbohydrate antigens, the RBC membrane contains three types of proteins that carry blood group antigens: single-pass proteins, multi-pass proteins, and glycosyl-phosphatidyl-inositol (GPI)-linked proteins [8].

The RBC membrane contains millions of antigens. A person's own antigens are invisible to their own immune system. However, foreign antigens (i.e. antigens that are not found on one's own RBCs) will stimulate the immune system to generate alloantibodies. Some antigens are highly immunogenic while others are less so. RBC alloimmunization is primarily induced by blood transfusion or pregnancy. The conditions that stimulate production of alloantibodies are complex and not completely understood but may involve donor characteristics, blood component characteristics and processing, as well as recipient characteristics [9]. Antigens may be presented indirectly or directly. Indirect antigen presentation occurs when the donor's antigen presenting cells (e.g., dendritic cells, B lymphocytes) are infused in non- or poorly-leukoreduced blood components. Direct antigen presentation depends on recipient characteristics. Some recipients are considered "good responders or presenters" if they have human leukocyte antigen (HLA) moieties that can present peptides antigenic to T lymphocytes very efficiently [9]. Inflammatory environments have also been found to be necessary for coactivating the antigen presenting cell and the responding antigen-reactive or specific T lymphocyte to rescue the activated T lymphocyte from either apoptosis or tolerance [9,10]. Patients with certain diseases such as sickle cell disease are at high risk of alloimmunization due to chronic transfusions, pro-inflammatory state, and red blood cell antigen mismatches between the typical donor and recipient due to racial disparities.

DSTRs result in a positive antibody screen. Unnecessary transfusions should be avoided to prevent further alloimmunization which would complicate compatibility testing and cause delays in finding compatible blood in the future.

DHTRs occur with an incidence of 1 in 2500 to 11,000 [5]. Hemolysis ensues several days to a month after transfusion. Extravascular hemolysis is more common than intravascular hemolysis caused by complement-fixing antibodies (e.g., anti-Kidd alloantibodies). It may be due to an anamnestic response or primary response to a RBC antigen. It is unknown why certain antigens lead to the formation of alloantibodies that can cause hemolysis while others do not. It is also unknown why certain IgG1/IgG3 antibodies to specific epitopes are more lytic than antibodies of the same isotypes but directed against different epitopes [9]. An antibody screen of the recipient's blood should be performed within 72 h prior to any transfusions and on average 3 weeks after a RBC transfusion to detect and/or monitor alloantibodies [9]. A full crossmatch is necessary when an alloantibody has been identified or is previously known from the recipient's history. A patient with DHTR is often asymptomatic but may present with fever, new positive antibody screening test and mild jaundice. Evidence of hemolysis includes decreased hemoglobin, increased direct bilirubin, low haptoglobin, and increased lactate dehydrogenase. Patients with DHTRs are managed with supportive care [5].

Febrile nonhemolytic transfusion reaction

Febrile nonhemolytic transfusion reactions (FNHTRs) are one of the most common acute reactions that are characterized by fever unrelated to the underlying disease. They are considered to be benign events free from any long term side effects. A sudden rise in temperature of 1−2 °C accompanied by chills, discomfort and rigors are indicative of a possible FNHTR [11]. FNHTR may

be due to recipient antibody-donor leukocyte interactions or from the accumulation of inflammatory mediators produced by leukocytes during storage [12]. The incidence of FNHTR is about 0.1%–1% with universal leukocyte reduction (LR) [5]. The incidence varies greatly according to the extent of reporting, age and type of blood product, use of LR, presensitization of recipients, and premedication of the recipient with antipyretics [11,13,14]. FNHTRs are more common from platelet (PLT) and non-LR blood products than from RBC and LR products [11–13].

FNHTR are treated with antipyretics. Because antipyretics are effective therapies for FNHTRs, it makes biological sense to premedicate patients prior to a blood transfusion. However, studies have not shown that they prevent transfusion reactions [13]. Premedicating with antipyretics simply mask the presence of fever which could be a harbinger of more serious reactions such as a HTR, septic transfusion reaction or transfusion related acute lung injury (TRALI).

Allergic transfusion reaction

Allergic transfusion reactions manifest during or within 4 h of transfusion. They can occur with any blood product transfusion but are most commonly encountered with platelet and plasma components [15]. Allergic transfusion reactions are typically due to interactions between an allergen in the donor blood with preformed antibodies in the recipient's blood. In some cases, infusion of antibodies from the donor blood may lead to an allergic reaction. Plasma proteins, chemicals, and potentially food may all serve as allergens in the blood [15].

Allergic transfusion reactions range in severity from mild to anaphylactic. Mild allergic reactions occur with an incidence of 1–3% and present with urticaria, pruritus, and flushing [5]. The symptoms are attributed to activation of sensitized mast cells and basophils secondary to transfused plasma proteins, leading to release of histamine [15]. These signs and symptoms may be managed by antihistamines and corticosteroids [5]. In some mild cases, if symptoms resolve after treatment, the transfusion may be restarted slowly.

Anaphylactic transfusion reactions occur with an incidence of 1 in 20,000 to 1 in 50,000 [5]. IgA and haptoglobin are plasma proteins that may be implicated in anaphylactic transfusion reactions. Most IgA-related anaphylactic reactions occur in people who are IgA-deficient (i.e., serum IgA<0.5 mg/L) and who possess serum class-specific IgA antibodies [15]. However, severe acute reactions to blood transfusions have been reported to occur in patients with normal serum concentrations of IgA and subclass IgA1-or IgA2-or allotype IgA2m(1)- or IgA2m(2)-specific IgA antibodies [16]. Racial differences in the incidence of IgA- and haptoglobin-deficiency and consequent racial differences in the prevalence of anaphylactic shock mediated by these antibodies have been observed [15]. Haptoglobin deficiency and haptoglobin antibodies should be considered primarily among Eastern Asians, while IgA deficiency and IgA antibodies should be first considered among Europeans, as the cause of transfusion related anaphylactic reactions [15].

Methylene blue, a reagent used for pathogen inactivation of fresh frozen plasma, has been reported to cause anaphylactic shock after plasma transfusion [17,18]. Peanut allergens have also been reported as a potential cause of anaphylaxis in a 6 year old boy who had a history of a serious peanut allergy and IgE antibodies specific to a major peanut allergen in the serum [19]. However, subsequent studies have not been able to demonstrate the presence of peanut allergens in volunteer donors' blood after very large consumption of peanuts [20]. Until concrete evidence is provided and the true incidence reported, caution must be observed in attributing an anaphylactic reaction to peanut allergens.

Presenting signs and symptoms of an anaphylactic reaction may include hypotension, urticaria, bronchospasm, respiratory distress, wheezing, local edema and anxiety [5]. Management approaches include fluids, epinephrine, antihistamines, corticosteroids, and beta-2 agonists [5].

Following an anaphylactic transfusion reaction, recipient workup for immunoglobulin A and haptoglobin deficiencies is advised to guide future transfusion management. Special components such as washed blood products or rare components donated by immunoglobulin A or haptoglobin deficient donors may be warranted for future transfusions. These investigations, however, are often negative, and anaphylactic transfusion reactions tend to be isolated events [21]. Patients with a history of multiple allergic transfusion reactions are often premedicated with antihistamines and/or glucocorticoids prior to transfusion. However, there is limited evidence for this practice [22].

Septic transfusion reaction

Septic transfusion reactions occur at an estimated rate of 0.3–3.3 per 100,000 transfusions, but the incidence is dependent on the type of component transfused. Symptoms include fever, rigors, and hypotension, and usually occur during or shortly after transfusion [23]. Because platelets are stored at room temperature, they are the component most prone to bacterial growth and subsequent septic reactions in recipients upon transfusion (approximately 1 in 5000 units contain bacteria).

With suspicion of septic transfusion reactions, all recently transfused units must be evaluated for

contamination by Gram stain and culture. Clinical suspicion of septic transfusion reactions should be confirmed by isolating the same organism in culture from both the residual blood product (or segment if completely transfused) and recipient [24]. In addition, the transfusion service should urgently and carefully investigate any blood components transfused to other patients from the same donor, and the blood supplier should be immediately notified to recall any blood components from the same donation. Recipients should be started on broad-spectrum antibiotics immediately following culture collection until bacterial contamination is confirmed.

There are several strategies to reduce the risk of bacterial contamination of blood components. The first line of defense starts at collection from donors and includes screening questions to ensure the donor is well and healthy, and vital signs to ensure the donor is afebrile and normotensive. Skin decontamination is performed and diversion pouches are used at the time of collection to discard the first 10–50 mL collected, which may contain commensal skin flora. Before distribution to hospitals, platelets are cultured to ensure they are free from significant bacterial growth [25]. In hospital blood banks, components are visually inspected at the time of issue to assess for changes consistent with bacterial contamination (e.g., color changes such as white stranding in platelet units). Lastly, pathogen reduction technologies are now in clinical use for plasma and platelets to inactivate viruses and bacteria, and may provide a safeguard against emerging pathogens.

Transfusion associated circulatory overload (TACO)

TACO affects approximately 1–8% of transfused patients and is caused by an excessive amount of blood transfused to a patient rapidly. Risk factors include older age, renal and cardiac disease, and baseline fluid overload, including recent administration of multiple blood products or other fluids [26]. Diagnosis requires new onset or exacerbation of 3 of the following within 6 h of transfusion [1]: acute respiratory distress [2], elevated brain natriuretic peptide [3], elevated central venous pressure [4], evidence of left heart failure [5], evidence of positive fluid balance [6], radiographic evidence of pulmonary edema [27]. Treatment is mostly supportive and may include supplemental oxygen and diuretics. At risk patients, including those with a history of TACO, may benefit from pre-transfusion administration of diuretics and a slowed rate of transfusion.

Transfusion-related acute lung injury (TRALI)

TRALI is a form of acute lung injury that occurs with transfusion of plasma-containing components and is currently the leading cause of transfusion-associated fatalities in the United States. Diagnosis of TRALI requires no evidence of acute lung injury prior to transfusion, with onset of hypoxemia (PaO_2/FiO_2 <300 mmHg, oxygen saturation <90% on room air, or other clinical evidence) during or within 6 h [27]. Besides hypoxemia, other symptoms include tachypnea, dyspnea, and sometimes fever. TRALI is thought to occur through a two event model; the first event includes an inflammatory clinical risk factor leading to activation of the pulmonary endothelium and subsequent sensitization of lung neutrophils and the second event is transfusion of anti-human leukocyte antigen (anti-HLA) or anti-human neutrophil antigen (anti-HNA) antibodies. Acute lung injury results from transfer of antibodies (anti-HLA or anti-HNA) or other inflammatory mediators during transfusion, resulting in activation of sensitized neutrophils [28].

The risk of TRALI has been reduced with the use of male-only plasma, and by screening female plasma and platelet donors for anti-HLA and anti-HNA antibodies [29]. The risk of TRALI following implementation of these mitigation strategies is now estimated to be 0.4 per 100,000 plasma transfusions, 1 per 100,000 platelet transfusions, and 0.5 per 100,00 red blood cell transfusions [30].

Post-transfusion purpura

Post-transfusion purpura is defined as a swift decrease in platelet count to less than 20% of a recipient's pre-transfusion count, 5–12 days following red blood cell or platelet transfusion. Associated symptoms include skin purpura and mucosal membrane bleeding, and life-threatening cases may involve intracranial hemorrhage [27,31]. Post-transfusion purpura most commonly occurs in human platelet antigen (HPA)-1a negative patients who have developed anti-HPA-1a from prior pregnancy or transfusion, but other HPA antigens have been implicated. Interestingly, both transfused and the patient's own platelets are destroyed, even though the patient's own platelets lack the implicated antigen.

First line therapy includes intravenous immunoglobulin in most cases, and steroids and therapeutic plasma exchange on a case-by-case basis [32,33]. Random donor platelets or platelets from antigen-negative donors may be used for transfusion; however antigen-negative platelets may not provide a better increment during the acute phase. After the acute phase is complete, future red blood cell and platelet transfusions should be autologous, from antigen-negative allogeneic donors, or washed [34].

Transfusion-associated graft versus host disease

Transfusion-associated graft versus host disease (TA-GVHD) is a rare but lethal complication of

blood transfusion. It occurs as a result of differences in histocompatibility between the blood component recipient and donor, presence of immunocompetent T lymphocytes in the cellular blood components and the inability of the recipient to reject the immunocompetent cells [35]. When immunocompetent or immunocompromised hosts are exposed to viable donor lymphocytes in transfused blood components and their own immune systems do not recognize these donor lymphocytes as foreign, the donor lymphocytes are able to respond against the recipient, resulting in TA-GVHD [36].

Signs and symptoms of TA-GVHD typically occur 8–10 days after transfusion and may include cutaneous eruptions, watery diarrhea, liver function test abnormalities, fever, marrow aplasia and pancytopenia [35,37]. The diagnosis of TA-GVHD can be made by the presence of characteristic clinical findings and a tissue biopsy consistent with GVHD (demonstration of the presence of leukocyte chimerism, specifically donor T lymphocytes in recipient tissue) according to criteria established by the National Health and Safety Network (NHSN) [35,36].

The prognosis for TA-GVHD is almost always fatal because the treatment for TA-GVHD is rarely effective. Hence, prevention of the disease with cellular blood component irradiation to inactivate viable lymphocytes is paramount [38]. Recent reports have demonstrated that pathogen-reduction of platelet components with amotosalen and UVA light can also be used to prevent TA-GVHD [35].

Hypotension

Diagnosis of a hypotensive transfusion reaction involves exclusion of all other causes of low blood pressure related to transfusion (e.g., sepsis, anaphylaxis, hemolysis), and in adults is defined as a drop in systolic blood pressure >30 mmHg within 1 h of transfusion, with an overall systolic blood pressure < 80 mm Hg [27]. Bradykinin generation is thought to play a major role; besides hypotension, patients have also experienced facial flushing, and angiotensin-converting enzyme (ACE) inhibitor therapy is an important risk factor [39,40]. Besides discontinuing the transfusion, some patients may require other supportive measures. Hypotension usually quickly resolves upon transfusion cessation.

Iron overload

Each unit of RBCs contains approximately 200–250 mg of iron (1 mg/mL of RBCs) [5]. Frequent blood transfusions may lead to iron overload because there is no physiologic means of excreting excess iron from the body. Patients with chronic anemia due to underlying conditions such as aplastic anemia, thalassemia syndromes, myeloproliferative neoplasms and myelodysplastic syndromes often require regular RBC transfusions [41]. A patient is considered transfusion dependent when they receive an average of 2 RBC units per month [42].

Iron overload develops when excess iron from frequent RBC transfusions accumulates in tissues and organs such as the liver, pancreas, heart, bone marrow, joints and endocrine glands, which leads to organ injury and dysfunction [41]. As a result of iron accumulation in these organs, cirrhosis, diabetes, heart failure, arthritis and an increased risk of infections may occur [41,43–45]. (Table 29.3).

In order to prevent the development of iron overload in chronically transfused patients, the number of RBC units transfused, serum ferritin levels and iron liver concentration may be monitored. Measurement of serum ferritin levels is the most widely available and used, inexpensive, and non-invasive indirect method of estimating the degree of iron overload and monitoring its progression [41]. The main limitation of measuring serum ferritin levels is that the predictive ability of the test may be affected by inflammation, metastatic disease and chronic diseases. However, this limitation can be overcome by repeated measurements over a long period of time [41,46,47]. Magnetic resonance imaging of the liver and heart may also be used to assess iron overload in these tissues [48]. Hepatic iron is the best measure of total body iron loading [48,49]. MRI has been successfully applied to measure iron content in the heart. The correlation between cardiac T2*, cardiac iron loading and its utility in predicting cardiac events has been validated [50,51].

TABLE 29.3 Clinical manifestations of iron overload [45].

Organ involved	Effect
Liver	Enlarged liver Elevated serum aminotransferase level Cirrhosis
Endocrine	Diabetes Hypogonadism Thyroid dysfunction (hypo- and hyperthyroidism)
Joint	Arthropathy
Heart	Cardiomyopathy (restrictive and dilated) Arrhythmias (sick sinus syndrome, atrial fibrillation) Heart failure Sudden death
Skin	Hyperpigmentation

TABLE 29.4 Iron chelators [54].

Drug	Route of administration	Dosage	Adverse effect
Desferrioxamine	Intravenous or Subcutaneous	30–60 mg/kg	Local skin reactions Infections Ototoxicity Nephrotoxicity Visual impairment (optic neuritis)
Deferasirox	Oral	10–40 mg/kg	Transient skin rashes Gastrointestinal upset Hepatic toxicity Renal toxicity Fanconi-like syndrome of hypophosphatemia Cataracts Proteinuria
Deferiprone	Oral	75 mg/kg	Neutropenia Agranulocytosis Arthropathy Gastrointestinal upset Hepatotoxicity

Iron chelation therapy (Table 29.4) should be considered for patients requiring chronic RBC transfusions, especially if they have been transfused with more than 10–20 units of RBCs and if the serum ferritin level exceeds 1000 ng/mL [41,52–54].

Case study

A 30 year old female Gravida 5 Para 2 but without any significant past medical history presented to the hospital with heavy active vaginal bleeding at 13 weeks gestation. This was consistent with inevitable abortion so she was taken to the operating room for a dilation and curettage procedure. The patient had an extensive blood loss of >1000 mL during her procedure. She received 2 units of packed red blood cells, 2 units of fresh frozen plasma and 1 dose of cryoprecipitate. While in the post-anesthesia care unit (PACU), the patient was hemodynamically stable but complained of itching and was found to have urticaria on her thighs, chest and back. The patient denied any respiratory distress chest pain, dizziness or swelling in her arms and legs.

Vital signs

	Temperature (°C)	Heart rate (beats per minute)	Blood pressure (mm Hg)	Respiratory rate (breaths per minute)
Pre-transfusion	36.3	88	113/61	18
Post-transfusion	36.2	91	111/61	19

Laboratory results

	White blood cell ($\times 10^3/\mu L$)	Hemoglobin (g/dL)	Hematocrit (%)	Platelets ($\times 10^3/\mu L$)	Prothrombin time (s)
Pre-transfusion	8.17	8.2	25.2	275	27.1
Post-transfusion	10.85	10.5	31.4	209	14.3

Blood bank workup

The clerical check showed that the correct product was issued to the patient. A visual check of a post-transfusion sample was negative for hemolysis or icterus. A direct antiglobulin test was performed and was negative for polyspecific reagent, anti-IgG and anti-C3.

Interpretation

The patient's symptoms are most likely due to a mild allergic transfusion reaction. The presenting signs and symptoms of a mild allergic transfusion reaction may include pruritus, urticaria, flushing, and wheezing. These reactions are usually due to an idiosyncratic reaction to plasma proteins. Premedication with diphenhydramine prior to subsequent blood component therapy may prevent these signs and symptoms.

CONCLUSIONS

Blood transfusions are life-saving but not benign treatments. Despite comprehensive SOPs, extensive staff

training and education, and meticulous testing, errors that may lead to adverse events may still occur. Multiple sources of error are possible beginning with blood collection in donors, and pre-transfusion testing to blood product issue and transfusion at the bedside for recipients. Adverse events ranging from mild to severe and acute to delayed may also occur, although they are relatively rare. Transfusion reactions should be assumed to be immune-mediated hemolytic transfusion reactions until they are proven otherwise by the blood bank work-up.

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CHAPTER

30

Methodological issues in point of care testing devices

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INTRODUCTION

Point-of-care (POC) testing, also known as on-site or near-patient testing has enjoyed explosive growth during the last two decades because they are easy to use, require no sample processing, can deliver test results at patient bedside in a short time, and can be connected to Laboratory Information Systems (LIS). The POC tests have been used in testing for various disease states, involving chemistry as well as immunoassays. In 2011, POC market was estimated to be 30% of all diagnostic market. However, POC testing suffers from interferences the same way, if not more, as the Central Laboratory (CL) assays do. There are also controversies about whether or not using POC testing contributes to increased cost due to inadequate test utilization. In this chapter, several of the factors contributing to the methodological issues regarding POC testing will be covered. Whether POC devices are used in a clinical laboratory, emergency department, home testing, pharmacies, at accident scene, disaster recovery, physician's office, nursing homes, elder care centers, or a pain management clinic, understanding their performance and the nuances that can lead to serious errors is paramount for successful patient management.

DESIGN OF POC DEVICES

Most POC devices use the same reaction modes as used in the central laboratory assays. However, unlike the central laboratory assays, they must use uncentrifuged whole blood, urine, and other body fluids as samples. Often they have chromatographic paper, which serves the same role as reaction flow cells; hematocrit is filtered, and the serum is drawn by capillary action, reacting with immobilized reagents and generating the signal, which is calibrated to give the analyte concentration. These signals can be electrical (e.g., glucose) or colorimetric (e.g., gold sol), and the results are mostly qualitative.

Current POC immunoassay devices use a solid phase competitive assay in a lateral flow format. They are often available as strips, cups or cassettes that can be read manually or using a reader [1,2]. Close to the sample application line, there are labeled conjugate, and labeled control antigen pads. Up in the strip, further away from the application line, there are test and control lines. The test line contains detection antibodies against the target analyte or labeled conjugate. The control line contains antibodies against the control antigens. As the sample migrates from the application line to the labeled conjugate and control antigen pads, it carries its contents to the test and control lines. If the concentration of the target analyte in the sample is below the cut-off, a color line will appear because the labeled conjugate will bind to the immobilized detection antibodies. On the contrary, if the target analyte is above the cut-off, it will compete with the labeled conjugate and inhibit color formation. Results from strips, cups, and cassettes can be evaluated visually by inspecting the presence or absence of color. Conversely, a separate reader unit can be used to interpret and report the result qualitatively or semiquantitatively.

The POC testing can be done for a single analyte or as a panel. Some strips and cassettes can also be read by semi-automated or automated endpoint readers [3]. In general, all these devices are easy to use, easy to read, and often, CLIA waived. Some users have found difficulties in reading and interpreting competitive IA's in the POC settings and reported that it was unacceptable to use these devices at their institutions [4].

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METHODOLOGICAL ISSUES OF POC DEVICES

Among the Chemistry assays, precision, accuracy and interferences present the biggest challenges. Overall, all POC devices have poorer precision and sensitivity than CL systems.

Glucose

Glucose is probably the most common POC analyte. POC assays for glucose mostly use the enzyme glucose oxidase, employing the following reaction:

$$\text{Glucose} + O_2 \xrightarrow{-(\text{Glucose oxidase})} \text{Gluconic acid} + H_2O_2$$

Electrical signals are derived from the decomposition of H_2O_2. Pitkin and Rice reviewed the glucometers, noting the reduction in accuracy of glucose measurement, especially in the hypoglycemic range, compared to the CL glucose assays [5]. Another paper compared the performances of five commonly available glucometers [6]; the authors concluded that all five POC devices have comparable precision and accuracy; but the major difference was in interferences − from hematocrit, maltose, ascorbic acid, acetaminophen, galactose, dopamine and uric acid. Sartor et al. found that POC glucose values were falsely elevated in burn patients undergoing vitamin C therapy; the authors observed a bias of 10−200 mg/dL in seven such patients, probably caused by ascorbic acid interference with the POC glucose assay [7]. Underestimation of blood glucose (regression slopes of 0.78−0.91) was found in four POC devices when compared with CL plasma hexokinase glucose methods; the POC device glucose results showed significant interference from variations in hematocrit and ascorbic acid [8].

Metabolites

POC assays for creatinine and bilirubin have been available for several decades. POC measurement of creatinine can quickly identify patients at risk for contrast induced nephropathy before any contrast imaging is performed. The POC creatinine assays use enzymatic methods with redox outputs that are displayed electronically; they can even be converted into estimated glomerular filtration rate (eGFR) as the output. Straseki et al. compared POC with CL enzymatic creatinine assays [9] and found poor result concordance, mostly because of higher standard errors and imprecision of the POC assay. There were more discrepant results among renal impaired than in the normal populations. By univariate analysis, they found no interference in either method from bilirubin, pO2 and pH. By multivariate analysis, however, variations in red blood cell counts, plasma water fraction, and hematocrit explained 91.8% of variance in POC creatinine results.

Radiological contrast agents have been reported to interfere in POC creatinine assays, resulting in false positive results and incorrect nephrological therapy [10]. Compared to CL assays, POC creatinine assays had 1.5−6 times poorer precision (mostly because of unit to unit differences) and increased standard error. POC testing of lactate, magnesium, ionized calcium, sodium, potassium, chloride, and bicarbonate are done with ion-sensitive electrodes. However, discordance between the POC results (using whole blood) and CL results (using serum or plasma) is often seen − probably because of hematocrit interference. Dashevsky et al. were, however, pleased with a good agreement between POC and CL electrolytes (sodium, potassium), blood urea nitrogen, creatinine and hematocrit in the emergency center of a large hospital [11].

Urinary microalbumin is another kidney health marker where POC assays are available. The specimen quality is important to get correct results (avoid turbid or bloody urine).

Drugs of abuse

The reliability and effectiveness of drugs-of-abuse POC devices have been challenged by several studies [4,12−20]. The main issues described in the literature are due to the specificity of the antibodies used in manufacturing POC devices, the inconsistency of cut-offs among reagent lots of the same device, the nomenclature used to name drugs or drug classes, and the difficulty of interpreting the device reporting lines depending on the read time used on the device [21].

Accuracy issues reported due to antibody specificity are not inherent to POC devices but rather a characteristic of immunoassays. Results obtained with immunoassays, whether they are from a laboratory instrument or a POC device, often need confirmation with a more specific chemical method (for example, liquid chromatography combined with mass spectrometry or tandem mass spectrometry). Good laboratory practice recommends that before a patient management decision is made, confirmation of the presence of the drug should be performed [21]. Immunoassays in general use the binding properties of antibodies to capture or detect a molecule, but because antibodies used in clinical assays may be different, drug-of-abuse POC devices may or may not cross-react with related drugs.

In 2002, George and Braithwaite [20] eloquently described some of the potential risks of using POC devices to measure drugs of abuse. Some of the issues they described then still persist today despite the tremendous growth we have seen in this industry.

One of the first methods described in the literature in 1988 used paper chromatography to detect cocaine. It was reported that when three trained individuals read 100 urine tests results, they only correctly identified the presence of cocaine 50% of the time [14]. Several studies performed in the '90s listed different sensitivity and specificity for POC drugs-of-abuse assays when compared with other laboratory methods such as Syva EMIT, or gas chromatography/mass spectrometry (GCMS) [15,21]. In all these studies the performance of the assays was highly impacted by factors such as the use of incorrect nomenclature, turbidity of the specimen, reading time, testing settings, and personnel training. For example, a cartridge using a latex agglutination inhibition method intended to detect morphine also detected other opiates such as codeine and dihydrocodeine [4]. A competitive immunoassay for cannabinoids (accuPINCH) provided negative results in cannabinoid positive turbid specimens. Moreover, if the reading time is longer than recommended 5 min, it may increase rate of false positive test results [14]. Testing settings may also impact accuracy if the personnel reading the device were multitasking, trying to perform other patient care activities at the same time. In one particular report, an essential buffer bead used in a device was prone to loss if personnel not properly trained used the device [15].

False negative and false positive findings have been previously described in the literature when drugs of abuse are measured using immunoassays in automated analyzers. Several reviews list the names of different substances interfering with immunoassays, and even the concentrations at which these substances may interfere [13,22,23]. Since POC devices use immunoassay methodology and the format of the assay in the device may be similar to that of the laboratory immunoassay, interpreting results when using POC devices for drugs of abuse is just as important as when immunoassays are used in the laboratory. Providing interference information to ordering providers is important for understanding what may be the cause of discrepancies between expected results and findings. The National Academy of Clinical Biochemistry Laboratory Medicine Practice Guidelines Recommendations "For the use of laboratory tests to support poisoned patients who present to the emergency department" published in 2003, emphasized the importance of providing information about assay limitations to the ordering providers [24]. Ideally, this information is also available when drugs of abuse are measured at the POC. The untrained user may contribute to inaccurate results, and in particular this issue becomes very evident when the personnel using the POC devices are also tasked with other nontechnical duties such as patient care. Training personnel and validating the devices in similar settings to those that are going to be routinely used is very important and have been cited in the literature as contributing to decrease in errors [25].

POC devices report qualitative results, positive or negative, based on a pre-established cut-off. In comparison with automated chemistry assays, the cut-offs used in POC devices are very similar. However, during evaluation of a POC device, the consistency of the cut-off among lot numbers of reagents should be determined. In a recent evaluation, a reference laboratory learned that a commonly used POC device detects drugs at a different cut-off than those claimed by the manufacturer. For some drugs, the device detected concentrations 20 times smaller than the claimed cut-off, while for other drugs, the amount of drug to be measured needed to be 5–10% higher than the cut-off to be detected [26]. The study performed as part of the evaluation of this device also showed that the detection of a positive specimen changed to negative if the reading time was extended beyond recommended time. For methadone, extending the reading time to 15 min could possible lead to false negative results. Other issues with POC include urine matrix effects, cross-reacting substances, and user misinterpretation of results. The vendors recommend the detection of a line no matter how faint to be a negative. The user should wait the prescribed amount of time for development before reading the device. Good lighting and, if necessary, a magnifying glass should be used to interpret the result. Some users will interpret a faint line as a "weak" positive. Thus, begins a "slippery slope" and issues of device reliability develop. Urine matrix can vary in color and consistency: medications, food, degree of hydration, blood, and crystals among other factors can affect the sample matrix. This matrix can interfere with the assay as well as with interpretation of the POC device. As with any immunoassay the risk of cross-reacting substances exist. The user should consult with the device manufacturer about possible cross-reacting substances.

While oral fluids have been used as alternative samples to urine, limitations apply on using oral fluid in drug-of-abuse testing when using POC devices. Oral fluid is usually collected on a device that contains a buffer as preservative. The ratio between the buffer and the oral fluid is not necessarily constant and may preclude accurate drug concentration measurements [27]. The distribution of a drug in the body will vary and cross-studies comparing different body fluids are needed to interpret oral fluid results accordingly [28]. Several factors may need to be taken into account and rigorous studies demonstrating that the presence of the drug is not affected by contaminants, stability, or method of collection should be performed. D. J. Crouch has described the use of oral fluid to replace urine or blood specimens and concluded that collecting a valid and representative specimen is intimately related to

the collection device [29]. A more recent systematic meta-analysis of previously published studies evaluating the reliability of detecting 5 commonly monitored drugs in oral fluid using POC devices also warns about the variability in specificity and sensitivity of POC devices. The authors also point out that a standardized study comparing devices, specimen types, and cut-offs may be needed in order to accurately appreciate the differences in devices, when oral fluid is used [30].

Cardiac markers

While myoglobin, CK-MB, and cardiac troponin POC assays have been used for the last two decades, cardiac care has evolved focusing on high sensitivity troponin (hsTnI or hsTnT) and BNP assays. With the need of rapid diagnosis of acute myocardial infarction in Emergency Centers, POC testing for cardiac troponins has grown. The POC tests for troponins are not as sensitive as CL troponin tests yet. Furthermore, the same interferants for CL troponin tests (heterophilic interference, biotin, prozone effect) also affect the POC troponin tests. Similarly, POC tests for the heart failure markers, BNP and NT-proBNP are now available. Thus, a newly available NT-proBNP assay was found to be interfered by biotin (above 10 mg/L), human anti-mouse antibodies (HAMA), and cardiac drugs (bispropolol and BNP) [31].

Diabetes markers – hemoglobin A1c (HbA1c)

There were three National Glycohemoglobin Standardization Program (NGSP)-certified POC HbA1c devices available in 2015 [32]: handheld A1CNow (PTS Diagnostics) and two bench-top models - Afinion (Axis-Shield) and DCA Vantage (Siemens). The Afinion assay uses borate affinity chromatography, and thus measure total glycated hemoglobin percent, not HbA1c, whereas other systems are immunoassays specific for HbA1c. Most of HbA1c POC assays are FDA approved to monitor known diabetics, not as screening tests. Many are interfered by fetal hemoglobin (HbF). The A1cNow suffers from interference from some of the hemoglobin variants (HbC and HbS), but not from HbD or HbE.

Pregnancy tests (hCG)

There are many POC tests for pregnancy tests (tests for hCG in urine) available. They are qualitative competition immunoassays. Noting how biotin is often used as over-the-counter dietary supplement, and how it interferes in many commercial CL immunoassays (Chapter 8), Williams et al. assessed biotin interference with seven POC pregnancy test kits. They found that one of the seven kits showed marked interference from biotin above 5 ng/L [33]. Of course, if the POC hCG assays are used for qualitative testing with serum or plasma samples, they will be subject to the same interferences that CL suffer from (for example, heterophilic antibody interference) [34]. False negative hCG results were reported in a POC in urine samples containing excess hCG beta core fragments when cartridge lots were changed [35]. One case study [36] reported false positive POC hCG (urine) pregnancy tests because of the presence of leukocytes in the specimen.

Multiplexed POC assays

Just like those for Drugs-of-Abuse, multiplexed POC immunoassays are available for various infectious, allergy, and autoimmune diseases. One advantage of multiplexing is the availability of a panel of biomarker results, which precludes erroneous diagnostics from a single marker result alone. For example, Chinnasamy et al. reported a paper-based vertical flow microarray with a multiplexing capacity of at least 1480 microspot binding sites, colorimetric readout, high sensitivity, and assay time of <10 min before imaging and data analysis [37]. Randox Biochip has a multiplexed single sample POC immunoassay system for Drugs of Abuse, Molecular, and custom application use. Such multiplexed assays, however, need a Reader to interpret the results, and are prone to interferences that have not been fully studied.

Infectious disease (ID)

Most of these POC tests are CLIA waived. Several commercial POC systems test for blood ID testing are available: for example, *Alere* HIV 1 testing, which could be performed on a finger stick. The *Murex* from Abbott is a 10-min POC that can use whole blood, serum, or plasma. The *Trinity Biotech* POC for HIV 1 uses recombinant antigens and gold nano-particles to enhance the signal. Some of them (for example, *OraQuick* from OraSure) can even use saliva as sample, but saliva is not the best specimen for HIV testing.

Other ID POC assays have been used for the testing of streptococci, methicillin-resistant *Staphylococcus aureus* (MRSA), Candida, influenza virus, sexually transmitted diseases, TB (*mycobacteria tuberculosis,* especially in third world countries), respiratory pathogens, and urinary tract infecting pathogens. The POC ID assays allow faster diagnosis and treatment of various pathogens. Recently, POC assays for procalcitonin (a sepsis marker) have been launched in the market.

Blood gas

The cost-effectiveness of POC over CL in blood gas testing and CO-oximetry has been questioned; the authors concluded that POC cost savings are dependent upon the existing laboratory structure and turnaround time of the central lab [38]. Lam et al. showed that hand disinfectant products, unless washed off well, may cause interference in electrolyte results. Therapeutic levels of hydroxycobalamin may interfere with Hb and some CO-Oxymetry fractions of POC blood gas instruments. While reviewing the many benefits of POC devices for blood gas and lactate measurements, Kapoor et al. noted the major concerns of POC use that must be addressed for successful therapy: analyzer inaccuracy, imprecision, performance (interfering substances), poorly trained non-laboratorians, high cost of tests, operator-dependent quality of testing, and difficulty in integrating test results with hospital information systems [39].

Coagulation meters

POC Coagulation meters are used to report prothrombin time and international normalized ratio (INR), activated partial thromboplastin time (aPTT), and activated coagulation time (ACT) to monitor coagulation therapy (warfarin or heparin). Poor correlation for high INR (for example in samples from lupus anticoagulant) with heparin interference has been reported. More interference in POC D-Dimer assays (used to assess thromboembolism) have been observed than in CL assays (though both are immunoassays). Shermock et al. analyzed their institution's POC INR values in comparison with parallel CL INR values to show that even if the POC unit's common statistical QC parameters were acceptable, the POC results made about 30% incorrect clinical decisions, because the bias was different in different INR ranges [40].

Other POC devices

The importance of monitoring parathyroid hormone (PTH) during parathyroidectomy for hyperparathyroidism, and the hormone's short stability have led to a growth in use of POC PTH assays. However, like all immunoassays, differences in antibodies used and reaction conditions often produce discordant results of biomarkers among the various POC assays, and between POC and CL assay results [41].

Tests for some cancer markers have been commercially available. Ludecke [42] compared two POC tests for NMP-22 antigen (using urine samples, for diagnosing bladder cancer), and found one of them consistently gave false positive tests with macro- and micro-hematuria. The stool occult blood test, used to detect colon cancer, can be considered a POC test.

McGann et al. evaluated a POC test for sickle cell disease diagnosis using both whole blood and dried blood spots and an assay to identify HbS, HbC, and HbA. They tested and found no interference from several hemoglobin variants (HbA_2, Hb Bart's, HbD, HbE, HbF, Hb Lepore, Hb Hope, Hb I-Texas, and HbG-Philadelphia); the results were not quantitative [43].

GUIDELINES FOR USING POCT DEVICES

The Executive Summary of the National Academy of Clinical Biochemistry: Evidence Based Practice for Point of Care, published in 2004 [19], strongly recommends that POC device training include quality monitoring and limitations. Users must be familiar with the devices analytical specifications, and the common interferences or other limitations to prevent misinterpretation errors. If a result from a POC device is going to trigger a penal or legal decision, confirmation of the results must be mandatory.

The Clinical and Laboratory Standard Institute (CLSI) created several guidelines related to POC [44–47]. These guidelines describe connectivity for engineers and users (POCT01 and POCT02 respectively), approaches and quality practices to reducing errors at the POC (POCT07-A, POCT08-A), and selection criteria for POC devices (POCT09-A). Guidelines POCT01 and POCT02 were created to address connectivity standardization, and follow the Connectivity Industry Consortium vision to "develop, pilot, and transfer the foundation for a set of seamless 'plug-and-play' POC communication standard" … These guidelines have helped the development of better instrument connectivity, and therefore decreased errors due to manual data entry. However, when POC devices are used outside a data framework of a university or hospital system, the compliance with these guidelines may not take place. Immediate care and primary care clinics where manual entry is often performed may have data entry issues. This type of errors will continue to exist unless incorporation of results in the patient medical record is completely automated. Some devices created for monitoring glucose concentration are capable of connecting to a software application via wireless communication. In similar fashion, all POC testing should incorporate the same capability to avoid manual data entry errors.

Guidelines POCT07-A, POCT08-A, and POCT-09-A are all related to error reduction but cover the topic from a different perspective. POCT07-A provides information on quality indicators for analytical processes and emphasizes the importance of creating indicators to identify common causes of errors. It also provides

guidance for creating error tracking processes. POCT08-A translates good laboratory practices into terms useable by testing personnel that is not trained in the laboratory. Sometimes personnel using POC devices consist of medical personnel that are required to attend to patients at the same time they perform testing. Under these circumstances, device common errors need to be more evident in all testing phases. Finally, POCT09-A describes important criteria for the selection of POC devices based on the clinical setting and clinical needs. It also provides information on regulatory and accreditation requirements referring to POC devices and their use.

In addition to the CLSI guidelines described above, the Center for Medicare Services (CMS) has recently approved a new plan for laboratories aiming at assessing risk of failure and allowing laboratories to customize a quality control plan according with the method and its use, the environment, and personnel competency. This plan is called "Individualized Quality Control Plan" or IQCP.

One of the reasons why the use of POC devices has grown steadily in the last few years is that they appear to provide fast and accurate results without the hassle of sending the specimens to a central laboratory. The perception is that laboratory results correlate really well with POC device results. As described above, there are serious caveats with this perception. Laboratory personnel can be of valuable assistance in implementing, training, and monitoring POC devices. Laboratory personnel expertise as part of an integrated healthcare team has value added to the use of POC devices. POC device complexity as defined by CLIA can be waived on non-waived. In general, the non-waived devices are moderately complex. A waived or non-waived device may become of high complexity if the manufacturer's instructions are not followed.

Waived devices are believed to be more reliable and less error prone than high complexity laboratory assays. However, that is hardly the case. Evaluating and monitoring the performance of a waived device is important to understand the limitations of the device and the concomitant implications of those limitations [48]. While many point-of-care tests are designed to be relatively simple and low risk to use, they are not error-proof. Individuals using point-of-care tests, even healthcare practitioners, must carefully follow test directions and be familiar with the test system. Some point of care tests such as those used to adjust doses of medications, have the potential to lead to serious health consequences if not performed properly. Many large hospitals have point-of-care coordination teams to ensure that testing procedures are properly followed.

It's important that the convenience of point-of-care testing does not tempt users to apply them beyond their intended purpose or to misinterpret results. For example, glucose meters and point-of-care hemoglobin A1c tests are designed only for monitoring diabetes and should not be used for diagnosis or screening.

Lastly, preanalytical errors are to be guarded against both in CL and POC testing. These were identified by Plebani [49]:

(1) Patient preparation errors
- Incorrect sampling time

(2) Blood collection errors
- Patient identification errors
- Sampling errors
- Tubes under filled

(3) Sample handling and transport errors
- Inadequate sample mixing (clotted sample, micro clots, incomplete clotting − fibrin interference)

(4) Interferences
- Hemolyzed sample

Unfortunately, most of those errors are not detectable within the testing process, but luckily, many are preventable by standardization of the process and proper staff education.

CONCLUSIONS

Laboratory personnel should be involved in the early adoption of a POC testing. Rigorous training of all personnel who may be involved in POC testing is of utmost importance. The routine user of the device should aware of workflows, cognitive errors, or unintended misuse of the device in use [26]. They should be trained in Quality Control activities. The laboratory personnel should be involved in the initial selection and evaluation of the POC device; implementing policies, procedures and training manuals to minimize misinterpretation and operator errors; training and competency assessments to meet regulatory requirements; monitoring device and personnel performance using quality control materials; identifying errors, and creating process improvements; and creating an individualized quality control plan to assess risk and prevent errors. POC testing has many benefits; only with strict training of correct usage and limitations of the devices and quality control, can POC benefits be fully realized.

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CHAPTER

31

Special concern: sources of inaccuracy in breath alcohol analysis

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INTRODUCTION

Alcohol levels are measured in various body fluids including blood, breath, urine and saliva. In addition, transdermal alcohol sensors are used in the criminal justice system. Blood alcohol as well as breath alcohol are measured both in medical and legal situations. Although breath alcohol measurement is commonly conducted in the roadside for screening drivers suspected of driving under the influence of alcohol, breath alcohol analysis is also conducted in the hospital emergency room in intoxicated patients. Breath alcohol analysis may also be used in workplace drug testing. Breath alcohol analyzers can be classified broadly under two categories; screening devices and evidential breath alcohol analyzers. Result obtained by using an approved evidential breath analyzer is usually accepted in the court of law as evidence. However, for medical breath alcohol testing, evidentiary breath alcohol analyzers may also be used due to better precision and accuracy of such analyzers compared to screening devices.

Although DWI (driving while intoxicated) and DUI (driving under the influence) are very similar in nature, in some states DUI may indicate a lesser degree of impairment. Therefore, conviction under a DUI charge may carry a lesser penalty than a DWI charge. Alcohol measurement in blood is discussed in Chapter 17. In this chapter limitations of measuring alcohol in breath are discussed.

ALCOHOL ANALYSIS USING BREATH ANALYZERS: LEGAL ISSUES

The legal limit for driving in all states in the US is 0.08% blood alcohol (80 mg/dL). However, personnel involved in safety sensitive positions are subjected to more stringent requirements. Usually commercial drivers should have blood alcohol lower than 0.02% which is virtually no alcohol in the body. Breath analysis is often used to ensure that commercial drivers are virtually alcohol free before joining their duties. Commercial drivers with blood alcohol level of 0.04% (40 mg/dL) or higher should be suspended immediately. Those who register a blood alcohol between 0.02 and 0.03% should be removed from their duties for 24 h. In the case of an accident all involved drivers are required to submit to alcohol testing within 2 h [1]. The employee's refusal to be tested for breath alcohol or not cooperating with such testing is considered as a violation of the rule and appropriate action can be taken against that person.

The Federal Aviation Administration (FAA) also has strict guidelines for alcohol consumption for security and safety sensitive personnel involved in the aviation industry. Airline pilots must abide by the current regulations governing alcohol. Moreover, FAA has the authority to take emergency revocation action against a pilot's airman certificate when a pilot is in violation of the agency's alcohol and drug policy. The FAA has a longstanding policy of 8 h of prohibition against pre-duty alcohol consumption. Different airlines may have policies where pilots must refrain from consuming alcohol longer than 8 h. Because of extremely low acceptable limits for blood alcohol in pilots it is possible that a pilot may not able to fly the airplane despite not consuming alcohol for 8 h. The current regulation prohibits pilots from performing safety sensitive duties with a blood alcohol level of 0.02% or higher. In addition, alcohol levels between 0.02 and 0.039% may result in grounding of the pilot for an additional 8 h, unless the alcohol level is reduced below the 0.02% limit. Reporting for duty or

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being on duty with a blood alcohol level of 0.04% or higher as measured by a breath analyzer or other method is a serious violation of FAA regulations and the pilot may subject to stiff penalties.

The United States Department of Defense also enforces a strict policy to ensure military personnel are not affected by alcohol and drug abuse. Active duty members are not allowed to consume any alcohol if below the age of 21. In addition, alcohol cannot be consumed 12 h prior to joining active duty, while in uniform, within 12 h of operating a motor vehicle, or any other time as restricted by the commander. Evidential breath alcohol analyzers are widely used in military. In one study the authors used breath alcohol analysis to evaluate the effect of a alcohol misconduct prevention program on 10,087 Air Force technical trainees at Lackland Air Force Base in San Antonio, Texas and observed significant reductions in the number of alcohol related incidents after such training. The average rate of alcohol related incidents reduced from 7.30 per 1000 trainees per quarter to 4.06 per 1000 trainees per quarter after implementing the alcohol misconduct prevention program [2].

ALCOHOL MEASUREMENT IN BREATH

Breath alcohol is measured using various devices as a reliable estimate of blood alcohol concentrations over the last 50 years. Police may often use any breath alcohol analyzer for screening purpose at the roadside but an initial result is not admissible in the court of law as evidence. Nevertheless initial screening results indicating intoxication may be sufficient grounds for arrest and after bringing that person to the police station breath alcohol is measured again with an evidential breath alcohol analyzer approved by National Highway Traffic Safety Administration. Results obtained by using evidential breath measurement devices are reliable and values are admissible to court as evidence. Though interlock devices in the car also work on the principle of breathalyzers, such devices are not designed for legal alcohol testing.

Partition of alcohol between blood and alveolar air

The principle of breath alcohol measurement is partition of alcohol between blood and alveolar air (breath) following Henry's Law, which states that when equilibrium is reached at a fixed temperature the liquid to air solubility coefficient of a gas is expressed by the following equation:

$$\text{Solubility coefficient} = \text{concentration in the liquid phase/concentration in the vapor phase}$$

Although original Henry's Law is applicable to gas, such approach can be adopted for alcohol, which is a volatile substance. The passage of alcohol from blood to breath follows the same process as other volatile gases in the exhaled air such as carbon dioxide and acetone. Alcohol diffuses from the pulmonary blood into the alveolar air at the body temperature of 37 °C until equilibrium is reached. The ratio of concentration of alcohol in the blood and in the alveolar air at equilibrium is known as blood/breath partition ratio or Ostwald solubility coefficient. The value selected for the blood/breath ratio is an essential element of breath alcohol analysis because this ratio determines the value of blood alcohol level when measured breath alcohol level is converted into blood alcohol concentration. In the United States, the accepted ratio is 2100:1. Therefore, one liter of blood contains the same amount of alcohol as 2100 litters of breath at 34 °C. Although the normal body temperature is 37 °C, the temperature of exhaled air is 34 °C because the temperature of exhaled air drops from the physiological temperature during passage through the respiratory tract [3].

The concentration of breath alcohol measured as mg/L should be multiplied by 2100 to calculate blood alcohol concentration in mg/L. In the United States, the driving legal limit is 0.08% whole blood alcohol (80 mg/dL). Therefore, the breath alcohol level measured as mg/L should be multiplied by 210 in order to calculate blood alcohol concentration in mg/dL. As a result, the legal limit of breath alcohol concentration is 0.38 mg/L or 38 μg/dL. However, in the US if breath alcohol concentration is reported as mg/210 L by the device, then that value is equivalent to mg/dL blood alcohol level. For example 80 mg/210 L breath alcohol is equivalent to 80 mg/dL blood alcohol.

Although in the United States, Canada, Norway and Sweden, the accepted ratio between blood alcohol and breath alcohol is 2100:1, in Great Britain and Netherland, the ratio is 2300:1. In Austria, the ratio is 2000:1. The legal limit of driving in Great Britain is also 80 mg/dL but due to higher blood/breath partition ratio, the limit of driving is 35 μg/dL of breath alcohol level which is slightly lower than legal limit of breath alcohol in the US [4]. Therefore, if the blood/breath alcohol ratio is lower than 2100:1 in an individual, breath alcohol analysis may overestimate true blood alcohol in the US. In contrast, if the ratio is higher than 2100:1, then breath alcohol analysis may underestimate the true blood alcohol concentration.

Technical aspect of breath alcohol measurement

Analyzers used for breath alcohol measurement utilize one of the following technologies:

- Color change due to a chemical reaction of a cocktail of chemicals with alcohol is used to determine alcohol level

- Alcohol in breath is measured using principles of infrared spectroscopy
- Electrochemical oxidation of alcohol (fuel cell technology)
- Analyzer based on mixed technology (infrared and fuel cell)

The earliest developed breath alcohol analyzers utilized chemical oxidation of ethanol with potassium permanganate (Drunkometer) or iodine pentoxide (Alcometer). Than another colorimetric method of analysis of alcohol concentration in breath was developed where exhaled air was allowed to pass through a cocktail of chemicals containing sulfuric acid, potassium dichromate, silver nitrate and water. Silver nitrate catalyzes the reaction where alcohol in the presence of sulfuric acid turns orange potassium dichromate solution into green due to conversion of potassium dichromate into chromium sulfate. The intensity of the green color can be used to estimate the amount of alcohol in the exhaled air. Captain Robert Borkenstein of the Indiana State Police used this chemical principle to develop breath analyzers in 1954 and some breath alcohol analyzers still use this principle today. Breathalyzer is the oldest technology of breath alcohol analyzer based on the principle of color change of potassium dichromate solution in the presence of alcohol and then analysis by using spectroscopy after a specified time to ensure complete reaction. The analyzer contains two vials of chemical cocktail. After a subject exhales into the device, the air is passed through one vial and if alcohol is present in the exhale, a color change occurs. A system of photocells connected to a meter measure the color change associated with the chemical reaction by comparing the response from the second vial (where no air is passed through) thus producing an electrical signal proportional to the color change in the reaction vial. This electrical signal can move the meter (more alcohol, more signal and higher reading) and the alcohol level in the subject can be determined. Breathalyzer was the brand name originally developed and marketed by Smith and Wesson and the company then sold that brand to a German Company called Draeger. The old Breathalyzer 900 model was replaced by newer versions such as model 1100 but this technology is subjected to interferences from a variety of substances and because of that other companies focused on developing more robust technologies for breath alcohol analysis [5].

In 1970s, breath alcohol analyzers based on infrared spectrometry, and electrochemical oxidation (fuel cell technology) appeared in the commercial market offering more sensitivity and specificity compared to the original colorimetry based instruments. The infrared spectrum of alcohol shows a distinct absorption band close to 3.4 μm due to C−H bind stretching and also at 9.5 μm due to C−O bond stretching frequency. There are many evidential breath alcohol analyzers that are based on the principle of infrared spectroscopy (IR spectroscopy) for quantitative determination of alcohol in exhaled air. The Intoxilyzer was originally developed by Omicron in Palo Alto, CA, and later sold to CMI, Inc., in Owensboro, KY. The earlier models were 4011 A, 4011 S and Intoxilyzer 5000 and more recently the Intoxilyzer 8000 model is available as an evidential breath analyzer. In addition to Intoxilyzer, Data Master cdm (National Patent Analytical System, Mansfield, OH), which is also used in many states as the evidentiary breath alcohol analyzer, is based on IR spectroscopy technology. The newer version of Intoxilyzer (Intoxilyzer 5000) uses a five wavelength filter at 3.36, 3.4, 3.47, 3.52, and 3.8 μm and thus can differentiate between ethanol and common interferences in exhaled air such as acetone, acetaldehyde and toluene. The 3.4 μm wavelength is used to detect alcohol while 3.47 identify interfering substances and 3.8 μm is used as the reference wavelength. The latest model Intoxilyzer 8000 uses a pulsed IR source instead of moving wavelength filter and uses dual wavelengths for measuring alcohol (3.4 and 9.36 μm) in the breath. It also has more advanced computer technology to accurately provide alcohol level results. The Data Master cdm, an evidentiary breath analyzer widely used by police officers in many states is also based on the principle of infra-red (IR) spectroscopy where alcohol is detected using two different wavelengths (3.37 and 3.44 μm). In Sweden, Evidenzer, an evidential breath analyzer, utilizes five wavelengths (3.37, 3.41, 3.47, 3.52 and 3.80 μm). In this instrument wavelengths at 3.37, 3.41, 3.47 and 3.52 are used for measuring alcohol and other volatiles such as acetone in breath while 3.80 μm wavelength is used as a reference wavelength [6].

There are several different brands of evidential breath alcohol analyzers which are based on the principle of fuel cell technology, such as Alcotest Models 6510, 6810, 7410, etc., (National Drager, Durango, Colorado), and Alco-Sensor III and IV (Intoximeters Inc, St. Louis, MO). The fuel cell is a porous disk coated with platinum oxide (also called platinum black) on both sides. The porous layer is impregnated with acidic solution containing various electrolytes so that charged particles such as hydrogen ions can travel through that medium. In addition, both sides of the disk containing platinum oxide are connected through a platinum wire. The manufacturer mounts this fuel cell in a case along with the entire assembly so that when a person blows to the disposable mouthpiece the air can travel through the fuel cell. If any alcohol is present in the exhaled air, then the alcohol is converted into acetic acid, hydrogen ion and electrons on the top surface

by the platinum oxide. Then hydrogen ions travel to the bottom surface (also containing platinum oxide) and are converted into water by combining with oxygen present in the air. In this process, electrons are removed from the platinum oxide. Because there is an electron excess on the top surface and electron deficit on the bottom surface, electrons flow from one surface to another generating an electric current which flows through the platinum wire and the intensity of the current is proportional to the amount of alcohol present in the exhaled air. The microprocessor of the instrument then converts that current to equivalent breath and or blood alcohol.

Some evidentiary breath analyzers are based on both fuel cell and infrared spectroscopy technology giving them good sensitivity and specificity. Various models of Intox EC/IR l desktop evidentiary alcohol breath analyzers, also manufactured by Intoximeters, Inc., combine reliable fuel cell analysis with real time analytical advantages of infrared technology. Semiconductor alcohol sensors are utilized in inexpensive breath analyzers marketed to the general public. However, the sensor response is non-specific to alcohol and also non-linear. For example, semiconductor sensors will respond to particles and gases present in cigarette smoke. Gas chromatography technique applied for analysis of blood alcohol may also be used for breath alcohol analysis [7,8]. Various technologies applied in breath alcohol analysis are summarized in Table 31.1.

ISSUES WITH PARTITION RATIO

Although in the US blood/breath partition ratio is 2100:1, wide variation of this partition ratio (1300:1 to 2700:1) in different individuals have been reported. Such fluctuations are due to difference between arterial blood and venous blood during the early stage of alcohol absorption by the body. After drinking, alcohol is found in higher concentration in arterial blood transporting newly absorbed alcohol to tissues and as a result the venous alcohol concentration is lower than arterial blood alcohol but after reaching equilibrium, both arterial and venous blood alcohol levels are comparable. Jones et al. compared time course of arterial and venous blood alcohol concentration differences using nine healthy male volunteers and observed that the mean peak arterial blood concentration was 98 mg/dL while the mean peak venous blood alcohol concentration was 84 mg/dL whereas the median time to reach the maximum blood alcohol concentration was the same (35 min). The arterial blood alcohol level was significantly higher than venous blood alcohol level 10 min after the end of drinking (mean difference 20 mg/dL; range: 9–40 mg/dL). At a median time of 90 min after drinking (range: 45–105 min), no difference between arterial and venous blood alcohol was observed [9].

Because alcohol is measured using venous blood, after drinking the blood to breath alcohol ratio is lower during the absorption phase. Nevertheless, in most individual the blood to breath ratio is close to 2100:1

TABLE 31.1 Examples of breathalyzers based on different technologies.

Technology	Comments	Examples of breathalyzers
Colorimetry	Cocktail of chemicals containing sulfuric acid, potassium dichromate, silver nitrate and water is used. If alcohol is present, orange color of potassium dichromate is changed to green.	Breathalyzer 900, Breathalyzer 1100 etc.
Infrared based technology	The infrared spectrum of alcohol shows distinct absorption bands close to 3.4 μm due to C–H bind stretching and 9.5 μm due to C–O bond stretching frequency. The newer version of Intoxilyzer (Intoxilyzer 5000) uses a five wavelength filter at 3.36, 3.4, 3.47, 3.52, and 3.8 μm and thus can differentiate between ethanol and common interferences in exhaled air such as acetone, acetaldehyde and toluene. Some analyzer uses dual wavelengths for measuring alcohol (3.4 and 9.36 μm) levels in the breath	Intoxilyzer 5000, Intoxilyzer 8000, Intoxilyzer 9000, etc. Data Master cdm etc.
Fuel cell based technology	Fuel cell technology is based on electrochemical oxidation of ethanol into acetic acid, hydrogen ion and electrons which generates an electric current proportional to amount of ethanol present in breath.	Alcotest various models, Alco-Sensor III and IV etc.
Mixed technology	Incorporates both infrared and fuel cell technology in one analyzer.	Intox EC/IR, Intox DMT, dual sensors etc.
Semiconductor	Breath alcohol analysis based on semiconductor technology utilizes increase in conductance of a tin oxide layer if alcohol is present in breath. Usually less precise than fuel cell or infrared technology.	AlcAlert etc., semiconductor

and as a result values provided by a breath analyzer such as Drager 7110 MKIII IL breathalyzer correlate with blood alcohol analysis with high sensitivity (97%) and specificity (93%). Moreover, mean blood alcohol and breath alcohol 29 min post-consumption were 70.9 mg/dL and 32.57 μg/dL respectively indicating a mean blood/breath alcohol ratio of 2164.1:1 (n = 60) while blood and breath alcohol 58 min post consumption were 82.6 mg/dL and 35.15 μg/dL respectively indicating that the blood/breath alcohol ratio was 2346.9:1 (n = 61) [10]. Breathalyzers can also measure very low alcohol concentrations in breath accurately. In one study involving 62 Intoxilyzer 500-D analyzers (evidential breath analyzer) used for screening suspected drunk drivers, the authors reported that breathalyzers accurately measured low breath alcohol levels of 0–0.059 g/210 L [11]. These values correspond to 0–0.059% blood alcohol (0–59 mg/dL).

Sometimes non-evidential breath analyzers (pre-arrest breath analyzers) are used for roadside screening of suspected drunk drivers and then at the police station evidential breath analyzers are used to verify initial screening results. Polissar et al. evaluated the validity of results obtained by using pre-arrest breath analyzers by comparing results obtained by an evidential breath analyzer using data from 1,779 DUI arrestees and observed excellent correlation between values obtained by using pre-arrest breath analyzers and evidential breath analyzer although values were slightly lower (120 mg/210 L breath) using evidential breath analyzer compared to results obtained by using pre-arrest breath analyzer (127 mg/210 L breath). The values obtained by the evidential breath analyzer were slightly lower because of time elapsed between both measurements (median time: 48 min) where alcohol continued to be eliminated from the body [12].

However, there are some problems in calculating blood alcohol levels from breath alcohol levels. Breath alcohol analyzers may underestimate blood alcohol concentration by an average of 15% which could be beneficial to the person being tested [13]. Nevertheless, over estimation of blood alcohol based on breath alcohol analysis is also possible because blood/breath partition ratio vary widely between different individuals as well as in the same individual during the different phases of alcohol absorption, metabolism and elimination. Dubowski after data analysis of 393 subjects using Gaussian distribution reported that the blood/breath partition ratio varied from 1555:1 to 3005:1 in 99.7% of population studied but the mean blood/breath partition ratio was 2280:1 [14]. Although the mean partition ratio in this study was comparable to 2100:1, in a person with a partition ration of 1555:1, breath alcohol should produce falsely elevated blood alcohol concentration. In contrast in a person with a partition ratio of 3005:1, breath alcohol analysis should produce falsely lower blood alcohol levels. For example, in a person with partition ratio of 1555:1, true blood alcohol of 0.07% (70 mg/dL) will be overestimated to 0.95% (95 mg/dL) if breath alcohol analyze is used for estimating blood alcohol. Similarly for a person with 3005:1 partition ratio true blood alcohol of 0.07% will be underestimated as 0.049% (49 mg/dL) using breath alcohol analysis.

Hartung et al. performed a controlled drinking experiment involving 78 volunteers and reported that the blood/breath partition ratio varied from 1571.1:1 to 2394.1:1 and increased with increasing blood alcohol levels. The authors used Drager Alcotest 9510 DE breathalyzers for measuring breath alcohol level. The difference between breath and blood alcohol measurements was not more than 10 min. The authors concluded that a blood alcohol measurement is superior to a breath alcohol measurement in legal cases [15].

ALCOHOL MEASUREMENT IN BREATH: COOPERATIVE VERSUS NONCOOPERATIVE PERSON

Performance of a breath alcohol analyzer depends on level of cooperation from the person being tested. Gibb et al. measured breath alcohol (Alco-Sensor III breath alcohol analyzer) and blood alcohol in 55 patients and observed that the mean blood alcohol level was 217 ng/dL but when blood alcohol was calculated based on breath alcohol analysis, the mean estimated blood alcohol was 187 mg/dL, which was substantially lower than the measured blood alcohol level. Interestingly, the mean blood alcohol level of uncooperative patients was 243 mg/dL compared to the calculated mean blood alcohol level of 183 mg/dL from breath alcohol analysis. In contrast, in cooperative patients, the mean blood alcohol was 194 mg/dL and the mean blood alcohol calculated from breath alcohol analysis was also 194 mg/dL. The authors concluded that in uncooperative individuals breath alcohol analysis may significantly underestimate true blood alcohol concentration [16].

Currier et al. investigated the relative accuracy of breath and serum alcohol readings in the psychiatric emergency service. Breath alcohol and serum alcohol were measured in 55 patients. However, the time of measurements was documented only in 32 patients and the authors analyzed these data. The authors reported that the mean breath alcohol concentration in these patients was 0.15 g per 210 L which was equivalent to 150 mg/dL of blood alcohol concentration. In contrast, the mean blood alcohol level in these patients was 230 mg/dL. The authors concluded that underestimation of blood alcohol based on breath alcohol analysis

was due to lack of cooperation from patients during breath alcohol analysis [17].

LUNG FUNCTION AND BREATH ALCOHOL ANALYSIS

The breath alcohol test involves a single exhalation maneuver where a subject is asked to inhale air (preferably a full inhalation to total lung capacity) and then exhale (preferable a full exhalation to residual volume) into the breathalyzer instrument. The assumption is that the alcohol concentration in exhaled breath is equal to that in alveolar air. In general, the alcohol level in end-exhaled air is always lower than in alveolar air. When performing a breath alcohol test, the subject is asked to inhale ambient air and exhale into the analyzer (usually 1.1−1.5 L of exhaled air is needed for the test). Therefore, a smaller subject with smaller lung capacity must exhale a greater fraction of air in their lung to fulfill the minimum volume requirement of the analyzer and as a result, the alcohol breath test may overestimate the blood alcohol level in smaller subjects compared to larger subjects with larger lung capacity [18].

Chronic obstructive pulmonary disease (COPD) may affect breath alcohol analysis. In one study based on 10 normal volunteers and 10 volunteers with COPD, the authors observed that the mean blood/breath partition ratio was significantly higher in subjects with COPD compared to normal subjects. Therefore, the blood alcohol calculated from breath analysis was significantly lower than true blood alcohol concentration. In addition, the authors further observed that breath analyzers also significantly underestimated the blood alcohol level as a function of age. For example in a 52 year old female subject, the blood/breath partition ratio was 2753:1 while in a 66 year old subject, the blood/breath ratio was 3504:1. Therefore, in older subjects breath analyzers may significantly underestimate true blood alcohol [19]. Although most patients with lung disease are able to supply an evidential breath sample (75% subjects with interstitial lung disease and 92% patients with COPD), some subjects with very severe lung disease (vial capacity below 1.5 L) may not be able to provide a specimen for breath alcohol analysis [20].

EFFECT OF HEMATOCRIT AND BODY TEMPERATURE ON BREATH ALCOHOL ANALYSIS

The average hematocrit is 46.2% in males and 40.6% in females. Because alcohol is water soluble, for two individuals with the same concentration of alcohol in whole blood, a person with a higher hematocrit will have a slightly higher alcohol concentration in serum as well as in breath. In addition, body temperature also has an effect on breath alcohol concentration. Usually the temperature of human breath varies between 33.3 to 34.4 °C and in the US it is assumed that the breath temperature is 34 °C whereas the blood/breath ratio is 2100:1. An in vivo study showed that the blood/breath alcohol ratio increased on average by 5.7% per 1 °C increase in body temperature. Lowering body temperature has the opposite effect. Therefore a drop in core temperature works as an advantage for the subjects with hypothermia because breath alcohol analysis should provide falsely lower values [4]. Rinsing the mouth with water reduces breath alcohol concentration determined using Lion AE-D2 alcolmeter but the magnitude of reduction was greater after rinsing mouth with cold water [21].

Fox and Hayward studied the effect of hyperthermia on breath alcohol analysis by submerging alcohol intoxicated subjects in hot water bath thus inducing mild hyperthermia to the extent of 2.5 °C increase above normal body temperature. Hyperthermia did not influence the blood alcohol decay curve in these subjects but hyperthermia did cause a significant distortion of the breath alcohol decay curve, up to as much as a 23% increase above the blood alcohol concentration. The authors calculated that for each 1 °C increase in body temperature, the breath alcohol concentration may increase by up to 8.62% over the blood alcohol concentration. The author suggested that mouth temperatures must be recorded prior to breath alcohol measurements to allow for potential use of a temperature correction factor [22].

SOURCES OF ERRORS IN BREATH ALCOHOL MEASUREMENT

There are several sources of errors in breath alcohol analysis. Sometimes a driver stopped by the police may use mouthwash to hide any alcoholic breath. Because some mouthwashes contain alcohol, use of a mouthwash prior to taking a breath alcohol analysis may cause falsely elevated breath alcohol result. However, residual alcohol evaporates from the mouth rapidly and this is the reason for waiting for 15 min in a police station under supervision so that the suspect cannot take anything by his or her mouth during the waiting period. Fessler et al. studied the effect of alcohol based substances such as mouthwash, cough mixture and breath spray just prior to breath alcohol measurement using Drager Evidential portable breath alcohol analyzer and twenty five volunteers. The authors concluded that 15 min waiting period was necessary to

ensure that there was no residual alcohol in the mouth after using mouthwash and other alcohol containing products. Otherwise alcohol from mouthwash may interfere in breath alcohol analysis causing falsely elevated values [23]. Harding et al. studied the effect of dentures and denture adhesives on mouth alcohol retention using Intoxilyzer 5000 and concluded that dentures had no significant effect on breath alcohol test results as long as a waiting period of 20 min was observed prior to testing [24].

Case report

A 37 year old man who was breath tested after following a traffic accident showed a breath alcohol concentration of 70 μg/dL using Lion Intoximeter which was twice as high as legal limit of driving in UK (limit breath alcohol: 35 μg/dL; blood alcohol 80 mg/dL; blood/breath ratio in UK is 2300:1). The defendant protested vigorously that he did not consume that much alcohol but no malfunction was found in the breath analyzer. Alcohol loading test was conducted in the laboratory in two different occasions and the results indicated that breath alcohol analysis significantly overestimated blood alcohol concentration. The observed breath alcohol concentration was 140 μg/dL where calculated breath alcohol concentration should be 35 μg/dL based on the amount of alcohol consumed. A blood sample was taken at a point when breath alcohol concentration was 70 μg/dL (calculated blood alcohol: 80 mg/dL) which was significantly higher than the actual blood alcohol concentration of 54 mg/dL. Dental examination of the defendant showed that he had extensive dental work carried out including three bridges. The author concluded that possible explanation of significantly elevated breath alcohol level might be due to retention of alcohol in the bridges and periodontal spaces [25].

Logan et al. evaluated the effect of asthma inhalers and nasal decongestant sprays on breath alcohol test and observed that the only product which had any effect on the breath alcohol test was Primatene Mist containing 34% ethyl alcohol but alcohol was eliminated from the breath within 5 min. The authors concluded that inclusion of a 15 min deprivation period when no food or drink could be consumed prior to an evidential breath test was adequate safeguard against interference in the test caused by alcohol containing inhalers [26]. Some mouthwashes contain alcohol. Based on a study using 40 young adult volunteers, the authors in one study showed that immediately after rinse using an alcohol containing mouthwash, a significant amount of alcohol was detected in breath due to the presence of residual alcohol in the mouth cavity but within 10 min after rinsing values were significantly reduced. In addition, 20 min after rinsing no alcohol was detected in the breath. For example, the mean blood alcohol value calculated from breath alcohol analysis was 375 mg/dL immediately after rinsing mouth with a mouthwash containing 21.6% alcohol but 10 min after the value was reduced to 7 mg/dL and finally after 20 min no alcohol was detected in the breath. As expected blood alcohol analysis did not detect any alcohol in these subjects [27]. In another study, the authors demonstrated that rinsing mouth with a mouthwash containing 18% alcohol, the mean time required for breath alcohol level to go back to zero in healthy volunteers was 11.32 min [28].

Drinking an energy drink while driving a car is legal but some energy drinks contain very low levels of alcohol. When volunteers drank various energy drinks, 11 out of 27 drinks gave positive results using evidentiary breath analyzers when testing was done just after drinking. However, after a 15 min waiting period all breath alcohol analysis reports were negative. The authors concluded that a 15 min waiting period eliminates the possibility of testing false positive after consuming an energy drink with low alcohol content [29].

In a hospital setting medical staff may wash hands using alcohol hand gel following World Health Organizations (WHOs) "five moments of hand hygiene" recommendation. Such alcohol hand gel contains 70% alcohol. In one study, the authors observed positive breath alcohol test results in some subjects when breath alcohol analysis was performed within 2 min of using hand gel (highest value: 0.064% blood alcohol calculated from breath alcohol analysis but blood alcohol measurement was negative). However, 62.5% of positive breath analyzer test results returned to zero in less than 7 min. The authors speculated that positive test results may be due to inhalation of alcohol vapor into the respiratory dead space following gel application. The authors concluded that for workplace breath alcohol analysis, a 15 min waiting period after hand ashing with alcohol containing gel is needed to avoid false positive breath test. Moreover, positive results must be confirmed using a direct blood alcohol measurement [30].

Mother tinctures used in homeopathic medicine often contain alcohol. In some countries where homeopathic medicines are commonly used and the legal limit of driving is 0.05% blood alcohol (50 mg/dL), defendants often claim that positive breath alcohol test results are due to use of homeopathic medicine. Boatto et al. based on a study of 30 subjects demonstrated that within 1 min of drinking homeopathic mother tincture, breath alcohol test may be positive (9 out of 30 subjects showed measurable breath alcohol levels) but after 15 min no alcohol was detected in breath. The authors concluded that mandatory 15 min waiting period prior to blood alcohol analysis eliminates possibility of false positive results due to use of homeopathic medicine [31].

Breath alcohol analysis and GERD

Studies have shown that breath alcohol analysis provides reliable results in patients suffering from gastroesophageal reflux disease (GERD). Kechagias et al. compared blood alcohol values with values obtained by breath alcohol analyzer (Data Master) in patients suffering from GERD and concluded that breath alcohol analyzers overestimating the true blood alcohol value due to eruption of alcohol from the stomach to the mouth due to gastric reflux is highly improbable [32]. In another study, the authors concluded that people with GERD can provide bias free end-expiratory breath alcohol result provided 15 min waiting period is followed [33].

Interferences of volatiles in breath alcohol analysis

Volatile organic compounds are produced in the body as a result of various metabolic processes. These endogenous volatiles are transported in the blood and can be exchanged across the alveolar-blood capillary membrane into exhaled air. Over 1,000 volatile compounds have been identified in exhaled air of healthy humans and also in various diseases. Some of these compounds which are present in extremely low amounts can also be used as biomarkers of various diseases such as lung cancer [34]. In general high concentration of carbon dioxide (approximately 4%) and water vapor are present in exhaled air but other organic compounds such as hydrocarbons, alcohols, ketones, and aldehydes are present in human breath as parts per million to parts per billion concentration. The major volatiles in human breath include isoprene (12−580 ppb), acetone (1.2−1,880 ppb), ethanol (13−1,000 ppb), methanol (160−2,000 ppb) and other alcohols [35]. In another study, the authors also identified many volatile organ compounds in human breath. These compounds include hydrocarbons (isoprene, 2-pentene, 2-methyl-1-pentene, benzene, toluene, p-cymene, limonene, 2,4-dimethylheptane, n-butane), ethers (dimethyl ether, 1, 3-dioxolane), esters (ethyl acetate), aldehydes and various alcohols including ethanol [36].

Laakso et al. studied the potential interference of volatile solvents with breath alcohol analysis using Drager 7110 evidential breath analyzer using a procedure stimulating a human breathing. The authors concluded that most of the compounds studied had either a negligible effect on breath alcohol analysis (acetone, methyl ethyl ketone, and methyl isobutyl ketone) or were detected in very low concentration (methanol, ethyl acetate and diethyl ether) with little effect on breath alcohol analysis. However, n-propyl alcohol and isopropyl alcohol showed significant interference [37]. Logan et al. also presented a case report where a person consumed both ethanol and isopropyl alcohol. The authors observed interference of isopropyl alcohol in breath alcohol analysis using Datamaster infrared breath alcohol instruments. Blood analysis using gas chromatography/mass spectrometry showed the presence of ethanol (76 mg/dL), isopropyl alcohol (23 mg/dL) and acetone (metabolite of isopropyl alcohol; 57 mg/dL). The breathalyzer recorded alcohol levels of 90−170 mg/210 L over a period of 3 h [38].

Jones and Rossner described a case where a 59-year-old man undergoing a weight loss program using a ketogenic diet attempted to drive a car which was fitted with an alcohol ignition interlock device, but the vehicle did not start. Because he completely stopped drinking, he was surprised and upset. Ketogenic diet used for treating obesity and controlling seizure in some epileptic children is high in fat, very low in carbohydrate and also has adequate protein. The goal is to burn fat to get energy rather than getting it from glucose which is formed by carbohydrate metabolism. However, consuming the ketogenic diet lead to a stage called ketonemia where concentrations of acetone, acetoacetic acid, and beta hydroxybutyric acid are high. This high amount of acetone may be found in the exhaled air. The interlock device in the car determines alcohol by electrochemical oxidation method and acetone does not interfere with the process. However, acetone is known to be converted into isopropyl alcohol by the action of liver alcohol dehydrogenase and isopropyl alcohol can be falsely identified as ethanol by the ignition interlock device. In addition, methanol and propanol can also be falsely identified as alcohol. The authors concluded that side effects of ketogenic diets need further evaluation by authorities especially for people involved in safety sensitive positions such as airline pilots and bus drivers who are subjected to much tougher alcohol tolerance policies [39].

Glue sniffing may cause false positive breath alcohol test result because glue contains aliphatic hydrocarbons, ethyl acetate and toluene [40]. Methanol poisoning is dangerous because it may cause death or blindness. Methanol poisoning may cause false positive test results with breath analyzers. In one report the authors observed that toluene, xylene, methanol and isopropyl alcohol in exhaled air can be mistakenly identified as breath alcohol by the Intoxilyzer 5000 evidentiary breath alcohol analyzer [41]. Diethyl ether is a common organic solvent used in laboratories. Prolonged exposure to this solvent (1 h) may cause false positive test results with breath analyzers [42]. Common interferences in breath alcohol analyzers are summarized in Table 31.2.

TABLE 31.2 Interferences in breath alcohol test.

Interfering substance	Comments
Propyl alcohol	Propyl alcohol interferes with breath alcohol analysis.
Isopropyl alcohol	Endogenously produced isopropyl alcohol has no effect on breath alcohol analysis due to very low concentration in breath but isopropyl alcohol if consumed interferes with breath alcohol measurement.
Methanol	Endogenously produced methanol has no effect on breath alcohol analysis due to very low concentration in breath but methanol if consumed interferes with breath alcohol measurement.
Toluene	Endogenously produced toluene has no effect on breath alcohol analysis due to very low concentration in breath but toluene if consumed interferes with breath alcohol measurement.
Xylene	Xylene is an industrial solvent and prolonged exposure to this compound may cause false positive results in breath alcohol analysis.
Diethyl ether	Only prolonged exposure to this common organic solvent commonly used in laboratories may cause false positive breath alcohol test results.
Ketogenic diet	Ketogenic diet lead to a stage called ketonemia where concentrations of acetone, acetoacetic acid, and beta hydroxybutyric acid are high. Acetone is converted into isopropyl alcohol by the action of liver alcohol dehydrogenase and isopropyl alcohol can be falsely identified as ethanol during breath alcohol analysis.
Glue sniffing	Glue aliphatic hydrocarbons, ethyl acetate and toluene and some of these compounds interfere with breath alcohol analysis.

Case report

A 47-year-old man who was found at a public park and acting intoxicated was given a breath analyzer test in the police station using Intoxilyzer 5000 EN which showed a concentration corresponding to 288 mg/dL blood alcohol. The subject admitted that he was suicidal and was transported to a hospital. In the emergency room, the patient admitted drinking gas line antifreeze which contains 99% methanol. The serum drug screen for alcohol showed a negative result indicating that the positive ethanol level determined by the breath analyzer was false positive due to interference of methanol. As expected, the patient's serum methanol level was 589 mg/dL. The patient was initially treated with fomepizole and then with ethanol infusion to reduce metabolism of methanol to toxic formaldehyde metabolite. Hemodialysis was initiated 9 h after ingestion and after 44 h of ingestion his serum methanol level was reduced to 22 mg/dL. The visual activity of the patient was not affected by methanol intoxication and finally he was transferred to a psychiatric facility [43].

CAN ALCOHOL BE PRODUCED ENDOGENOUSLY?

Endogenous production of alcohol (auto-brewery syndrome) is a common defense strategy adopted by some individuals charged with driving under the influence of alcohol. A small amount of alcohol is produced endogenously but it should not alter blood alcohol level. Jones et al. reported that endogenous alcohol level varied from none detected to 1.6 μg/mL (0.16 mg/dL) which is negligible [44]. Madrid et al. studied eight patients with liver cirrhosis and observed that during fasting no patient showed any endogenous ethanol level but after a meal two patients showed serum alcohol level of 11.3 and 8.2 mg/dL while another four patients showed negligible values [45].

In general healthy individuals as well as patients with metabolic disease such as diabetes, hepatitis or cirrhosis showed blood alcohol levels ranged from 0 to 0.08 mg/dL due to endogenous production of alcohol which is negligible. Hafez et al. investigated endogenous production of alcohol in healthy volunteers, patients with diabetes mellitus, liver cirrhosis as well as patients suffering from both diabetes mellitus and liver cirrhosis. Blood alcohol concentrations were measured using gas chromatography/mass spectrometry. In control group, the blood alcohol concentrations varied from 0.01 to 0.3 mg/dL, in patients with diabetes mellitus, the mean blood alcohol concentration was 4.85 mg/dL (maximum value: 12.90 mg/dL), in patients with liver cirrhosis, the mean value was 3.45 mg/dL (maximum value: 9.7 mg/dL) and in patients suffering from both diabetes mellitus and liver cirrhosis, the mean value was 10.88 mg/dL (maximum value: 22.3 mg/dL) [46]. The dermal absorption of alcohol is usually negligible after washing hands with alcohol containing hand gel.

CONCLUSIONS

Breath alcohol analysis is useful for screening drivers suspected of driving under the influence of alcohol but initial screening result must be confirmed in a police station using evidential breath analyzer. Although the result obtained by using an evidential analyzer is accepted in legal situation, there are many limitations

of breath alcohol analysis. The gold standard is blood alcohol measurement by using head space gas chromatography or gas chromatography/mass spectrometry. Breath analyzers are also used as a point of care device in the emergency room in evaluating intoxicated patients but a blood alcohol level determined in a clinical laboratory is recommended if the breath alcohol level is inconsistent with the clinical picture.

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