The Preanalytic Phase

An Important Component of Laboratory Medicine

Sheshadri Narayanan, PhD*

Key Words: Preanalytic phase variables; Physiologic; Specimen collection and processing; Analyte stability; Endogenous interference factors

Abstract

The preanalytic phase is an important component of total laboratory quality. A wide range of variables that affect the result for a patient from whom a specimen of blood or body fluid has been collected, including the procedure for collection, handling, and processing before analysis, constitute the preanalytic phase. Physiologic variables, such as lifestyle, age, and sex, and conditions such as pregnancy and menstruation, are some of the preanalytic phase factors. Endogenous variables such as drugs or circulating antibodies might interact with a specific method to yield spurious analytic results. The preanalytic phase variables affect a wide range of laboratory disciplines.

In recent years, considerable attention has been given to the influence of preanalytic effects on laboratory results. Compendia of preanalytic effects and books have addressed the effect of the preanalytic phase on laboratory results. Preanalytic variables can be grouped broadly under 3 categories: physiologic, specimen collection, and influence or interference factors. In this review, I address these variables and provide examples of their effects across a broad range of laboratory disciplines. Examples of preanalytic variables affecting mainly clinical chemistry, hematology, and coagulation tests are discussed.

Admittedly, given the comprehensive range of subject matter that spans the realm of the preanalytic phase, this review must be selective. Hence, a mere passing mention is made in the concluding section, “Summary Perspectives,” of topics such as blood gases, molecular biology, tumor markers, and problem analytes, such as cytokines, homocysteine, and fibrinolytic activators and inhibitors, all of which could be the subject matter for more than 1 review article.

Even so, an attempt is made to provide a comprehensive overview of physiologic variables, specimen collection variables, including a discussion of traditional anticoagulants and newer stabilizing additives for newer problem analytes, and clinically relevant endogenous interferences.

Physiologic Variables

The effects of age, sex, time, season, altitude, conditions such as menstruation and pregnancy, and lifestyle are some of the physiologic variables that affect laboratory results.
Age

The effect of age on laboratory results has been well recognized to the point that separate reference intervals have been used to distinguish pediatric, adolescent, adult, and geriatric populations. Bone growth and development in a healthy growing child is characterized by increased activity of bone-forming cells, the osteoblasts, that secrete the enzyme, alkaline phosphatase. Hence, the activity of this enzyme is approximately 3 times higher in a growing child compared with the activity in a healthy adult.3,4

A substantial increase in the RBC count in the neonates compared with that in adults is the reason glucose is metabolized very rapidly in neonates.2 Increased arterial oxygen content within a few days of birth leads to an increase in hemoglobin levels owing to the destruction of the RBCs. Serum bilirubin levels then increase, because the immature liver lacks enzymes to convert bilirubin to the water-soluble bilirubin diglucuronide.2 The total number of neutrophils are at a maximum for 1 to 2 days after birth. In the neonate, the monocyte count is increased for up to 2 weeks after birth, while the eosinophil count stays elevated for up to 1 week after birth.5,6 The lymphocyte count is increased markedly at birth and remains elevated in children up to 4 years of age.6 In contrast, the basophil count is elevated only transiently for up to 1 day after birth. A substantial decrease in serum uric acid levels is noted between days 1 and 6 after birth.2

Age also affects renal function, as reflected by an effect on creatinine clearance, which decreases progressively with each decade.7 For example, the average value for creatinine clearance at age 30 years is 140 mL/min (2.33 mL/s) per 1.73 m² of body surface area. In contrast, at age 80 years, the creatinine clearance value decreases to 97 mL/min (1.62 mL/s) per 1.73 m² of body surface area.7 Beyond the age of 50 years, the decrease in creatinine clearance is also related to a decrease in muscle mass.7,8 However, when interpreting changes in renal function solely on the basis of aging, the overlap of disease processes, such as the presence of renal vascular disease in elderly patients, must be considered. Secondary risk factors, such as genetics and obesity, may have a role in the decrease in glucose tolerance with aging.9

Homeostatic control by the hypothalamic-pituitary-adrenal axis is compromised because of aging. Hence, there is an increase in plasma corticotropin and corticosteroid levels due to aging.10,11 Usually in young adults, the concentration of the cytokine, interleukin (IL)-6, in serum is low and is undetectable in absence of inflammation.11 However, IL-6 levels are detectable readily during middle age. Also, in healthy elderly adults, serum IL-6 levels are increased apparently due to the loss of the normal regulation of IL-6 levels. The decrease in dehydroepiandrosterone sulfate with age, which normally has a suppressive effect on IL-6, is related to the increase in IL-6 levels.12 Increased IL-6 levels, in turn, increase the percentage of cells bearing the receptor for the cytokine, transforming growth factor beta, a powerful immunosuppressive agent, thus providing an explanation for the age-related immunosuppressive phenomena.13

Increases in the level of plasma antidiuretic hormone (or arginine vasopressin) have been related to aging with levels significantly higher in 1 study of healthy subjects 53 to 87 years of age (4.7 – 0.6 pg/mL [4.4 – 0.6 pmol/L] in 68 healthy subjects) compared with subjects 21 to 51 years of age (2.1 – 0.2 pg/mL [1.9 – 0.19 pmol/L] in 45 healthy subjects).14

Thyroid homeostasis apparently is affected during aging. Thyrotropin levels in serum have been reported to be 38% higher in an elderly group (mean age, 79.6 years) compared with a younger group of subjects (mean age, 39.4 years).15 In the same study, serum triiodothyronine levels were 11% lower in the older age group compared with levels in the younger group of subjects.

<table>
<thead>
<tr>
<th>Age Group/Effects</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neonate</td>
<td>Guder et al², Monroe et al⁶, Weinberg et al⁶</td>
</tr>
<tr>
<td>Increased RBC count, neutrophil, monocyte, eosinophil, and lymphocyte counts</td>
<td>Guder et al²</td>
</tr>
<tr>
<td>Increased hemoglobin and unconjugated bilirubin values, decreased uric acid level</td>
<td>Weinberg et al⁶</td>
</tr>
<tr>
<td>Lymphocyte count elevated up to 4 years of age</td>
<td>Narayanan¹³; McComb et al⁴</td>
</tr>
<tr>
<td>Growing child</td>
<td>Narayanan and Appleton⁷, Narayanan¹¹</td>
</tr>
<tr>
<td>3-fold increase in alkaline phosphatase level due to bone growth</td>
<td>Pacini et al⁹</td>
</tr>
<tr>
<td>Progressive changes</td>
<td>De Kloet¹⁰; Narayanan¹¹</td>
</tr>
<tr>
<td>Creatinine clearance decreases with each decade; age-related change in renal function; may overlap with disease process in elderly persons</td>
<td>Daynes et al¹², Crawford et al¹⁴, Harman et al¹⁵</td>
</tr>
<tr>
<td>Decrease in glucose tolerance with age; overlaps with secondary risk factors, such as genetics and obesity</td>
<td></td>
</tr>
<tr>
<td>Homeostatic control by hypothalamic-pituitary-adrenal axis affected by aging; corticotropin and corticosteroid levels increase</td>
<td></td>
</tr>
<tr>
<td>Dehydroepiandrosterone sulfate levels decrease, interleukin-6 levels increase with age</td>
<td></td>
</tr>
<tr>
<td>Antidiuretic hormone and thyrotropin levels increase, triiodothyronine levels decrease with age</td>
<td></td>
</tr>
</tbody>
</table>
While changes in organ function occur due to aging, which in turn affects the circulating level of various analytes, one must also keep in perspective the overlapping effects due to disease and dietary deficiencies of trace elements, such as zinc and other nutrients.11

Sex Differences

Between 15 and 55 years of age, the levels of total and low-density lipoprotein cholesterol (LDL-C) increase progressively, with levels slightly higher in premenopausal females compared with the levels in males Table 2. Thus, in female subjects, a change in cholesterol value from 174 mg/dL (4.50 mmol/L) at age 25 years to 213 mg/dL (5.51 mmol/L) at 55 years has been reported.2 In the corresponding age span for men, cholesterol values have been reported to range from 166 mg/dL (4.29 mmol/L) at age 25 years to 232 mg/dL (6.00 mmol/L) at 55 years.2 In contrast, the high-density lipoprotein cholesterol (HDL-C) level does not fluctuate in either sex between the ages of 15 and 55 years; levels are slightly higher in females, presumably owing to the stimulating effect of estrogens.2

Analytes with levels that depend on muscle mass, such as creatinine and creatine kinase (CK), are generally at a higher concentration and activity, respectively, in males compared with females.2,7 However, when increased muscle mass is acquired as a consequence of strenuous athletic activities in female subjects, the sex difference in levels of creatinine and CK is abolished.2 Serum iron levels generally are lower in premenopausal female subjects compared with those for male subjects, with the difference disappearing in subjects older than 65 years.2

Sex differences are seen for several analytes as can be noted by reviewing a current list of reference laboratory values.16 The differences for some of the analytes based on sex can be explained as due to differences in muscle mass and to endocrine and organ-specific differences.

Time

There are time-related fluctuations in the level of some analytes (Table 2). Circadian rhythm is responsible for the diurnal changes seen in the circulating levels of some analytes. A classic example of an analyte subject to diurnal variation is cortisol, which generally peaks at around 6:00 AM, with levels becoming lower toward the evening and midnight.2 Because cortisol is a powerful immunosuppressant, its diurnal rhythm affects proinflammatory cytokine production. Indeed the level of proinflammatory cytokines, such as IL-1 alpha, IL-12, tumor necrosis factor alpha and interferon gamma have been shown to be inversely correlated with the level of plasma cortisol.17 The inverse relationship with plasma cortisol levels extends to the number of circulating T lymphocytes, especially the CD4+ T cells, and to the urinary secretion of neopterin, which is a marker of cellular immune activity.18,19 Even routinely measured analytes such as glucose, potassium, and iron exhibit diurnal variation.1,2

<table>
<thead>
<tr>
<th>Variable/Effects</th>
<th>References</th>
</tr>
</thead>
</table>
| Sex              | Guder et al⁷  
|                  | Guder et al²  
|                  | Kratz and Lewandrowski¹⁶  
| Cortisol peak, 6:00 AM; lower toward evening and midnight | Guder et al²  
| Proinflammatory cytokines (IL-1 alpha, IL-12, TNF-alpha, INF-gamma, CD4+ T cells) and urinary neopterin levels opposite the cortisol rhythm | Petrovsky et al¹⁷, Miyawaki et al¹⁸; Auzeby et al¹⁹  
| Glucose tolerance test values higher in afternoon | Guder et al²  
| LH, FSH, and testosterone released in short bursts | Narayanan²¹  
| Potassium, iron, thyrotropin, and growth hormone subject to diurnal effects | Young¹; Guder et al²; Keffer²⁰; Narayanan²¹  
| Vitamin D levels higher | Narayanan³  
| Triiodothyronine levels 20% lower | Guder et al²; Harrop et al²⁵  
| Slight (2.5%) increase in total cholesterol level | Narayanan²; Statland and Winkel²⁴  
| At 1,400 m, hematocrit and hemoglobin values 8% higher | Cooper et al²²; Narayanan²³  
| At 3,600 m, 65% increase in C-reactive protein level | Young¹; Guder et al²  
| As altitude increases, levels of renin, transferrin, and estriol and creatinine clearance decrease | Young¹; Guder et al²  

CK, creatine kinase; FSH, follicle stimulating hormone; HDL, high-density lipoprotein; IL, interleukin; INF, interferon; LDL, low-density lipoprotein; LH, luteinizing hormone; TNF, tumor necrosis factor.
Glucose values obtained during an oral glucose tolerance test tend to be higher when the test is performed in the afternoon than when the test is performed in the morning. The cortisol rhythm apparently may be responsible for the discrepancy noted in the results of an oral glucose tolerance test performed in the afternoon. ²

Thyrotropin levels peak during the late evening hours, with the lowest values observed around midday. ²,21 Growth hormone levels during waking hours are minimal, with increases noted during sleep. ¹

Reproductive hormones, such as luteinizing hormone, follicle-stimulating hormone, and testosterone, are released in bursts lasting barely 2 minutes, making accurate assessment of their concentration problematic. ⁸

Seasonal Changes

Vitamin D levels tend to be higher during the summer (Table 2), apparently due to prolonged exposure to sunlight. ³ A slight increase in the total cholesterol level (average, 2.5%) has been observed during the winter compared with values measured during the summer. ²²,²³ The decrease in the level of triglycerides in serum from summer to winter is more striking than the extent of decline noted between spring and autumn months when the temperature tends to be milder. ¹,²⁴

The levels of the thyroid hormone, triiodothyronine, are reported to be 20% lower during the summer compared with during the winter. ²,²⁵

Altitude

Changes in levels of some of the constituents in blood occur when measured at sea level as opposed to measurement at a higher altitude (Table 2). For example, hematocrit and hemoglobin levels can be up to 8% higher at an altitude of 1,400 m. ¹,² A 65% increase in C-reactive protein has been reported at 3,600 m. Concentrations of some analytes, such as plasma renin, serum transferrin, urinary creatinine, and estriol, and the creatinine clearance rate decrease with increasing altitude. ¹,²

Menstruation

At the onset of menstruation, a low estrogen level triggers the release of follicle stimulating hormone from the pituitary gland. The ovaries thus are stimulated to produce estrogen, and the level begins to increase noticeably from the sixth or seventh day after menstruation; the peak level is reached on approximately the 13th day. A day later, a burst of luteinizing hormone released from the pituitary gland signals ovulation. With the onset of ovulation, the progesterone level continues to rise until it decreases together with the estrogen level, just before the commencement of the next menstrual cycle. Thus, the reference intervals for estradiol, follicle stimulating hormone, luteinizing hormone, and progesterone are influenced by the stages of menstrual cycle (ie, follicular, midcycle, and luteal phases) (Table 3). ¹⁶

Coincident with ovulation, serum cholesterol levels are lower than at any other phase of the menstrual cycle. ² During midcycle or the luteal phase, the aldosterone concentration is approximately 2-fold higher compared with values during the follicular phase. ¹,² Renin activity may increase during the luteal phase of the cycle. ¹ In contrast, serum phosphate and iron levels decrease during menstruation. ¹,²

Pregnancy

A dilutional effect is observed due to an increase in the mean plasma volume, which in turn causes hemodilution (Table 3). ²,³ The effect is more pronounced when measuring trace constituents such as trace elements in serum. ³ During pregnancy, there is a considerable increase in the glomerular filtration rate. Hence, the creatinine clearance rate is increased by 50% or more over the normal rate. ⁷ During the third trimester, the urine volume may increase by approximately 25%.

During the second half of pregnancy, the placenta progressively begins to produce hormones antagonistic to insulin. As a result, the levels of hormones, such as estrogen, progesterone, and human placental lactogen (also called chorionic somatomammotropin), increase, leading to the onset of gestational diabetes. ²⁶

The increased metabolic demand during pregnancy causes an increased mobilization of lipids. As a result, especially during the second and third trimesters, the serum levels of apolipoproteins A I, A II, and B, triglycerides, and total cholesterol (especially LDL-C) are increased substantially. ²²,²³,²⁷ The levels of these lipids in serum return to normal within 10 weeks after delivery unless the mother breast-feeds her infant.²⁸

Human chorionic gonadotropin elaborated by the placenta has weak thyrotropic activity. Placental human chorionic gonadotropin levels are at the highest concentration toward the end of the first trimester of pregnancy, thus suppressing serum thyrotropin levels accompanied by a slight increase in the free thyroxine level. ²¹,²⁹ During the second and third trimesters of pregnancy, thyrotropin levels are more reliable. Hence, trimester-based reference intervals for thyrotropin are needed in pregnancy.

Associated with an increase in the glomerular filtration rate in pregnancy is an increase in the clearance of iodide and a transfer of iodide and iodothyronines to the fetus. As a result, the inorganic iodide level in serum decreases to such an extent that pregnant women with marginal iodine intakes of less than 50 µg/d develop iodine deficiency and, in turn, enlargement of the thyroid gland. ²¹,²⁹
The clearance by the liver during the first trimester of pregnancy of the sialylated thyroxine-binding globulin, which is induced by estrogens, is decreased. Hence, the level of thyroxine-binding globulin increases, leading to the well-known increases in serum thyroxine and triiodothyronine levels during pregnancy.

Other analytes with increased levels during pregnancy include the heat-stable alkaline phosphatase elaborated by the placenta, alpha-fetoprotein, copper binding proteins such as ceruloplasmin, and, in turn, copper, acute phase proteins, and coagulation factor VII. The increased level of acute phase proteins increases the erythrocyte sedimentation rate approximately 5-fold. Plasma concentrations of the fibrinolysis inhibitor, plasminogen activator inhibitor type 2, are increased up to 2 µmol/L during the third trimester of pregnancy. An increased metabolic requirement during pregnancy leads to a deficiency of iron, and the depletion of iron stores is reflected by a decrease in ferritin.

Lifestyle

Diet influences the results obtained for some analytes. Caffeine inhibits the enzyme phosphodiesterase, which normally regulates the levels of cellular cyclic adenosine monophosphate (c-AMP) by converting it to the inactive product 5′-c-AMP. The increase in c-AMP levels due to the caffeine-induced effect promotes glycolysis coupled with energy production, and the subject feels alert, awake, and energetic.
Narayanan / THE PREANALYTIC PHASE

Activation of triglyceride lipase by c-AMP results in a 3-fold increase in nonesterified or free fatty acid levels.2,3,24 The ability of free fatty acids to compete for binding sites on the albumin molecule can lead to the displacement of a drug or hormone bound to albumin, thus affecting the measurement of free drug or hormone levels.8 The increase in free fatty acid levels results in a decrease in pH, which, in turn, can strip calcium from the binding protein. Consequently, a spurious increase in ionic calcium concentration can occur.8 Plasma renin activity and catecholamine levels increase have been increased 3 hours after the consumption of 250 mg of caffeine.2,34

The effect of caffeine on lipid levels has been controversial, and the results obtained seem to be influenced by coffee-brewing methods.23 The finding in a double-blind study replacing caffeine-containing coffee with decaffeinated coffee for a period of 6 weeks of an insignificant change in serum triglyceride, total cholesterol, and HDL-C levels suggests that caffeine is not the substance in coffee that elevates the total cholesterol level.35 Consumption of ethanol can induce short- and long-term effects altering the results of several analytes (Table 4).2 Short-term effects reflected rapidly within the first 2 to 4 hours of consumption include a lowered blood glucose level and an increased plasma lactate level.2 Uric acid levels increase owing to the metabolism of ethanol to acetaldehyde and further to acetate. The net effect of lactate formation is to cause metabolic acidosis owing to the consumption of bicarbonate.2

Sustained consumption of ethanol can, by enzyme induction, lead to the increase in the activity of liver enzymes, such as gamma-glutamyltransferase. There are, however, marked intrindividual and interindividual variations in the level of serum gamma-glutamyltransferase, as demonstrated in a study in which subjects were challenged for 3 days with a 0.75-g of ethanol dose per kilogram of body weight.36 Other liver enzymes that become elevated owing to the direct cytotoxic effect of ethanol on liver are aspartate aminotransferase (AST) and alanine aminotransferase (ALT).2 Thus, consumption of 225 g of ethanol per day for 1 month caused skeletal muscle changes that led to the release of liver cell enzymes into blood.37 Depending on the extent of consumption, the effect of ethanol on serum lipid levels is variable.23 The effect of short-term alcohol intake, especially by subjects who usually do not consume alcohol, is to increase plasma triglyceride levels, especially the VLDL fraction.38 A moderate intake of ethanol (<40 g/d) is reported to increase the plasma levels of HDL-C, HDL₂-C, and the apolipoproteins AI and AII. However, if alcohol intake

Table 4

<table>
<thead>
<tr>
<th>Lifestyle/Effects</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet</td>
<td>High-protein and high-purine diet increase levels of uric acid, urea, and ammonia in blood compared with vegetarians</td>
</tr>
<tr>
<td>Differences in lipogenicity of saturated fatty acids: stearic acid, less effect; palmitic acid, substantial effect in raising LDL-C</td>
<td>Narayanan23; Cooper et al²⁷</td>
</tr>
<tr>
<td>Complex carbohydrates, monounsaturated and polyunsaturated fatty acids lower the levels of LDL-C</td>
<td>Narayanan23; Cooper et al²⁷</td>
</tr>
<tr>
<td>Fish oils lower the levels of triglycerides and VLDL</td>
<td>Narayanan23; Harris et al²²</td>
</tr>
<tr>
<td>Lipid profile (LDL-C, HDL-C) differences for lactovegetarians vs strict vegetarians</td>
<td>Narayanan³³; Sacks et al²³</td>
</tr>
<tr>
<td>Caffeine</td>
<td>Inhibits phosphodiesterase; raises cAMP; activation of triglyceride lipase causes 3-fold increase in free fatty acids</td>
</tr>
<tr>
<td>Decreased pH; increased ionic calcium level; free drug or free hormone stripped from albumin</td>
<td>Narayanan²⁸; Guder et al²²; Robertson et al³⁴</td>
</tr>
<tr>
<td>Plasma renin activity and catecholamine levels increase</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>Short- vs long-term effects</td>
</tr>
<tr>
<td>Increased levels of GGT, AST, and ALT subject to interindividual and intraindividual variation</td>
<td>Freer and Statland²⁶,²⁷</td>
</tr>
<tr>
<td>Moderate intake increases levels of HDL-C and apolipoproteins AI and AII</td>
<td>Narayanan²³; Freer and Statland²⁷; Taskinin et al³⁸</td>
</tr>
<tr>
<td>Smoking</td>
<td>Long-term smoking increases carboxyhemoglobin, hemoglobin, RBC, WBC and MCV values; WBC level related to number of packs smoked</td>
</tr>
<tr>
<td>Cadmium levels higher</td>
<td>Narayanan³,²³</td>
</tr>
<tr>
<td>Reduction in HDL-C dependent on extent of smoking</td>
<td>Narayanan²³; Cooper et al²⁷</td>
</tr>
<tr>
<td>Increased levels of plasma epinephrine, aldosterone, cortisol, free fatty acids, and free glycerol within 1 h of smoking</td>
<td>Young³¹; Guder et al²</td>
</tr>
<tr>
<td>ACE activity reduced owing to destruction of lung endothelial cells</td>
<td>Guder et al²; Haboubi et al³⁹</td>
</tr>
</tbody>
</table>

ACE, angiotensin-converting enzyme; ALT, alanine aminotransferase; AST, aspartate aminotransferase; cAMP, cyclic adenosine monophosphate; GGT, gamma-glutamyltransferase; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; MCV, mean corpuscular volume; VLDL, very-low-density lipoprotein.
exceeds 80 g/d, the synthesis of VLDL is stimulated, together with the activation of the enzyme lipoprotein lipase. Lipoprotein lipase hydrolyzes VLDL triglycerides, with the result that plasma VLDL levels will be apparently normal even when there is increased VLDL synthesis.23,27,38

Long-term smoking induces alteration in both biochemical and cellular profiles (Table 4).3,8 Carboxyhemoglobin levels in blood are higher in long-term smokers since cigarette smoke contains carbon monoxide, which has a much greater affinity for hemoglobin than does oxygen. As a consequence of long-term smoking, there is an increase in hemoglobin levels and the RBC and WBC counts. The RBCs become larger, thus increasing the mean corpuscular volume (MCV). Striking differences in the WBC count have been observed between healthy nonsmokers and healthy long-term smokers. Healthy smokers who smoked 1 pack of cigarettes per day for 1 year had a WBC count of approximately 240% more than the nominal value.3,24 With prolonged fasting, there is a decrease in the level of specific proteins, such as the C₃ component of complement, prealbumin, and albumin. Normal levels of these proteins are restored rapidly with protein supplementation.24

Cadmium levels in blood are higher in healthy long-term smokers compared with levels for healthy nonsmokers, thus requiring separate reference intervals to distinguish the 2 populations.3,8

Smoking can reduce HDL-C levels in plasma; the extent of reduction apparently is related to the number of cigarettes smoked per day.23,27 Short-term changes occurring within 1 hour of smoking 1 to 5 cigarettes include an increase in the plasma levels of epinephrine, aldosterone, cortisol, free fatty acids, and free glycerol.1,2

Angiotensin-converting enzyme (ACE) activity is decreased in smokers, presumably owing to the destruction of lung endothelial cells with the resultant decrease in the release of ACE into the pulmonary circulation or, alternatively, to inhibition of enzyme.2,39

The extent of smoking (moderate or heavy) parallels the blood levels of thiocyanate and cotinine. Compared with nicotine, which is the parent compound, cotinine has a longer half-life, approximating 20 to 28 hours, making it an ideal marker for the assessment of the extent of smoking.2

Specimen Collection Variables

The preparation of subjects for blood specimen collection, such as the duration of overnight fast, time of specimen collection, and posture during blood sampling Table 5; the effects of the duration of tourniquet application, infusion, and exercise (Table 5); the effects of anticoagulants Table 6; and stabilizing additives Table 7 used for blood collection; the anticoagulant/blood ratio; specimen handling and processing; and the relative merits of anticoagulants and other variables need to be delineated and standardized to minimize preanalytic error.

**Duration of Fasting**

Ideally, subjects should be instructed to fast overnight for at least 12 hours before specimen collection (Table 5).2,23 The reason for the 12-hour stipulation is based on the fact that an increase in the serum triglyceride level after a fatty meal can persist for up to 9 hours, but there is little effect on the levels of total cholesterol or apolipoproteins AI and AII.40

The effect of prolonged fasting, however, can affect some laboratory results. Thus, during a 48-hour fast, the hepatic clearance of bilirubin could be approximately 240% more than the nominal value.3,24 With prolonged fasting, there is a decrease in the level of specific proteins, such as the C₃ component of complement, prealbumin, and albumin. Normal levels of these proteins are restored rapidly with protein supplementation.24

The effect of fasting, however, varies depending on body mass.23 In a lean person, the use of fat as reflected by an increase in acetoacetic acid concentration in blood is minimal during a 1-day fast but increases rapidly with prolonged fasting.41 In an obese person, however, the acetoacetic acid level increases markedly during a 1-day fast. In contrast, while lipolysis, as reflected by a 3-fold increase in the serum glycerol concentration with 3 days of fasting is observed in a lean person, little change is noted in an obese person.23,41

**Time of Specimen Collection**

The section “Time” referred to diurnal changes seen in the circulating level of some analytes. It is, therefore, important that the time of specimen collection be kept constant from day to day to rule out variations introduced by diurnal effects (Table 5).

Specimens for therapeutic drug monitoring (TDM) should be obtained after a steady therapeutic concentration of drug or steady state is achieved in the blood. A blood sample obtained just before administering a drug dose after the steady-state level is reached will reflect the trough level or the lowest concentration to be obtained with the established drug dose. If only 1 blood sample is to be collected for TDM, it is preferred that it reflect the trough level.42

Blood samples obtained when the drug concentration is at its maximum, will, of course, reflect the peak level. Peak-level specimens need to be obtained within 1 to 2 hours after intramuscular administration of the drug, 15 to 30 minutes after intravenous administration, or 1 to 5 hours after oral administration.42 Timing of specimen collection for TDM, however, depends on the rate of distribution of the specific drug. If the drug is infused intra-
venously, approximately 1 to 2 hours after the completion of infusion are needed for the distribution phase to be completed, with exceptions such as digoxin and digitoxin, which need 6 to 8 hours for the completion of the distribution phase.\textsuperscript{2}

In general, since the rate of oral drug absorption differs from person to person, blood specimens obtained for TDM usually are timed to reflect trough levels. For the monitoring of certain drugs, such as theophylline, antibiotics, and antiarrhythmic drugs, it may be necessary and useful to measure peak levels after intravenous administration.\textsuperscript{2,43} The time for collection of urine specimens depends on the constituent being measured. For clearance measurements or for the measurement of analytes that have diurnal variation, a 24-hour urine sample is needed.\textsuperscript{7} A timed overnight urine sample or the so-called first-morning specimen, provides not only an assessment of the concentrating ability of the kidney, but also sufficient time for formed elements or sediment to be produced for easy enumeration.

While a random or spot sample may be suitable for routine screening, such a specimen may have its limitations because it may be too diluted to detect accurately slight increases in the concentration of analytes such as glucose, protein, cells, and casts. Thus, a spot or random sample may be of limited value in the initial screening for microalbuminuria.\textsuperscript{44} However, in a strategy designed to detect and

---

**Table 5**

**Effects of Specimen Collection Variables on Laboratory Results**

<table>
<thead>
<tr>
<th>Variable/Effects</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting</td>
<td>Guder et al\textsuperscript{2}; Narayanan\textsuperscript{23}</td>
</tr>
<tr>
<td>12-h overnight fast recommended, since increase in triglycerides persist up to 9 h after fatty meal</td>
<td>Narayanan\textsuperscript{2}; Statland and Winkel\textsuperscript{24}</td>
</tr>
<tr>
<td>After 48-h fast, 240% increase in bilirubin level; decreased levels of prealbumin, albumin, and C_3</td>
<td>Narayanan\textsuperscript{23}; Young\textsuperscript{81}</td>
</tr>
<tr>
<td>Effects dependent on body mass</td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td></td>
</tr>
<tr>
<td>Standardize time to rule out diurnal effect</td>
<td>Guder et al\textsuperscript{2}</td>
</tr>
<tr>
<td>Therapeutic drug monitoring trough vs peak levels; timing regimens</td>
<td>Guder et al\textsuperscript{2}; Narayanan\textsuperscript{62, 23}; Pippenger\textsuperscript{23}</td>
</tr>
<tr>
<td>Urine, timed overnight or first morning specimen concentrated, ideal for sediment enumeration</td>
<td>Narayanan\textsuperscript{44}; Narayanan and Appleton\textsuperscript{7}</td>
</tr>
<tr>
<td>24-hour sample for clearance measurements and for analytes with diurnal variation</td>
<td>Guder et al\textsuperscript{45}</td>
</tr>
<tr>
<td>Random or spot urine generally suitable</td>
<td></td>
</tr>
<tr>
<td>But, sensitivity to detect slight increases in numbers of cells and casts and glucose and protein levels affected by dilution</td>
<td>Narayanan\textsuperscript{44}</td>
</tr>
<tr>
<td>Posture</td>
<td></td>
</tr>
<tr>
<td>In general, 5%-15% increase for most cellular and large molecules (eg, albumin) in sitting posture</td>
<td>Guder et al\textsuperscript{2}; Narayanan\textsuperscript{6}; Statland and Winkel\textsuperscript{24}</td>
</tr>
<tr>
<td>Increase in upright supine has dilutional effect: 10% and 12% decreases in cholesterol and triglyceride levels, respectively</td>
<td>Narayanan\textsuperscript{23}; Young\textsuperscript{81}</td>
</tr>
<tr>
<td>Change in posture has no effect on free drug and small molecules</td>
<td></td>
</tr>
<tr>
<td>Postural effect substantial in patients with reduced plasma volume (eg, congestive heart failure, cirrhosis)</td>
<td>Narayanan\textsuperscript{2}</td>
</tr>
<tr>
<td>Increased aldosterone, norepinephrine, epinephrine, renin, and atrial natriuretic peptide levels</td>
<td>Guder et al\textsuperscript{2}; Tan et al\textsuperscript{46}</td>
</tr>
<tr>
<td>Tourniquet application time</td>
<td></td>
</tr>
<tr>
<td>&gt;1 minute increase in concentration of large molecules; for total cholesterol level, 5% increase at 2 minutes; up to 15% increase at 5 minutes</td>
<td>Guder et al\textsuperscript{2}; Narayanan\textsuperscript{8, 23}; Cooper et al\textsuperscript{22}; Statland and Winkel\textsuperscript{24}; Young\textsuperscript{81}</td>
</tr>
<tr>
<td>Decreased pH; increased potassium, lactate, ionized calcium, and magnesium levels with increased application time</td>
<td>Narayanan\textsuperscript{2}</td>
</tr>
<tr>
<td>Repeated fist clenching can increase potassium by 1.2 mmol/L, up to 2.7 mmol/L</td>
<td></td>
</tr>
<tr>
<td>Infusion</td>
<td></td>
</tr>
<tr>
<td>Spuriously increased blood glucose level if specimen obtained from arm receiving glucose infusion</td>
<td>Guder et al\textsuperscript{2}</td>
</tr>
<tr>
<td>Waiting times before blood specimen collection: subjects receiving fat emulsion, 8 h; carbohydrate, protein, or electrolytes, 1 h</td>
<td>Guder et al\textsuperscript{2}</td>
</tr>
<tr>
<td>Extent of hemolysis related to age of transfused blood</td>
<td>Guder et al\textsuperscript{2}</td>
</tr>
<tr>
<td>Exercise</td>
<td></td>
</tr>
<tr>
<td>Strenuous exercise increases total CK and CK-MB; CK-MB as percentage of total CK normal; decreased haptoglobin value due to intravascular hemolysis</td>
<td>Narayanan\textsuperscript{6}; Statland and Winkel\textsuperscript{24}</td>
</tr>
<tr>
<td>Individual variability in CK related to extent of exercise training</td>
<td>Guder et al\textsuperscript{2}</td>
</tr>
<tr>
<td>Mitochondrial size and number increase with vigorous training; mitochondrial CK increases to &gt;8% of total CK</td>
<td>Guder et al\textsuperscript{2}</td>
</tr>
<tr>
<td>Increased epinephrine, norepinephrine, glucagon, cortisol, corticotropin, and growth hormone levels; decreased insulin level</td>
<td>Guder et al\textsuperscript{2}</td>
</tr>
<tr>
<td>Increased serum and decreased urine uric acid levels due to increased lactate level</td>
<td>Guder et al\textsuperscript{2}; Narayanan\textsuperscript{23}; Cooper et al\textsuperscript{27}; Hardman et al\textsuperscript{20}</td>
</tr>
<tr>
<td>Increased apolipoprotein AI and HDL-C levels; decreased LDL-C, apolipoprotein B, and triglyceride levels with long-term strenuous exercise or brisk walking</td>
<td>Narayanan\textsuperscript{23}; Cooper et al\textsuperscript{27}; Hardman et al\textsuperscript{20}</td>
</tr>
</tbody>
</table>

CK, creatine kinase; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.
**Table 6**

**Anticoagulants for Blood Collection**

<table>
<thead>
<tr>
<th>Anticoagulant</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>Narayanan and Hamasaki(^{21}); Novotny et al(^{31}); Narayanan(^{52,54}); Guder et al(^{55}); Berg et al(^{56}); Doumas et al(^{56}); Narayanan(^{52}); Toffaletti et al(^{57}); Toffaletti(^{58}); Landt et al(^{59}); Narayanan(^{52}); Goosens et al(^{62}); Sacker(^{63}); Reardon et al(^{64}); Guder et al(^{65}); Narayanan and Hamasaki(^{31}); Narayanan(^{65}); Adcock et al(^{66})</td>
</tr>
<tr>
<td>For ionic calcium, heparin in blood gas syringe can be calcium-titrated, electrolyte-balanced, or zinc lithium–titrated</td>
<td></td>
</tr>
<tr>
<td>EDTA: salts of EDTA generally used for routine hematology testing (dipotassium or tripotassium EDTA, disodium EDTA); nominal amount, EDTA, 1.5 mg/mL of blood</td>
<td></td>
</tr>
<tr>
<td>Citrate: generally used for routine coagulation testing; trisodium citrate–dihydrate 3.2% (0.109 mol/L) or 3.8% (0.129 mol/L)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 7**

**Stabilizing Additives**

<table>
<thead>
<tr>
<th>Additive</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycolytic inhibitors</td>
<td>Guder et al(^{7}); Chan et al(^{67}); Sidebottom et al(^{68})</td>
</tr>
<tr>
<td>Sodium fluoride (2.5 mg/mL of blood with EDTA [1 mg/mL] or potassium oxalate [2 mg/mL])</td>
<td></td>
</tr>
<tr>
<td>Lithium iodoacetate (0.5 mg/mL of blood) alone or in combination with lithium heparin (14.3 U/mL of blood)</td>
<td>Guder et al(^{2}); Chan et al(^{67}); Sidebottom et al(^{68}); Guder et al(^{2})</td>
</tr>
<tr>
<td>Sodium fluoride and lithium iodoacetate require 3 h to fully become effective</td>
<td>Liss and Bechtel(^{71}); Nakashima et al(^{12})</td>
</tr>
<tr>
<td>Adding mannose (3 mg/mL of blood) to sodium fluoride achieves efficient glycolytic inhibition</td>
<td>Nakashima et al(^{12}); Ho et al(^{73})</td>
</tr>
<tr>
<td>Mannose, however, has concentration-dependent interference</td>
<td>Uchida et al(^{75})</td>
</tr>
<tr>
<td>Maintaining blood pH at 5.3-5.9 with mixture of citric acid, trisodium citrate, disodium EDTA, and 0.2 mg sodium fluoride stabilizes glucose</td>
<td>Guder et al(^{2})</td>
</tr>
<tr>
<td>Cell stabilizers</td>
<td>Narayanan and Hamasaki(^{31}); Contant et al(^{74}); Narayanan(^{75}); Van Den Besselaar et al(^{76}); Kuhne et al(^{77})</td>
</tr>
<tr>
<td>Acid-citrate dextrose A and B formulations; B formulation has greater amounts of dextrose available to metabolizing cells</td>
<td></td>
</tr>
<tr>
<td>Citrate, theophylline, adenosine, dipryridamole mixture minimizes in vitro platelet activation</td>
<td></td>
</tr>
<tr>
<td>Proteolytic enzyme inhibitors</td>
<td>Narayanan(^{52,79}); Narayanan(^{81}); Pandian et al(^{80}); Narayanan(^{81})</td>
</tr>
<tr>
<td>EDTA (1.5 mg/mL of blood) with aprotinin (500-2,000 KIU/mL) stabilizes several polypeptide hormones</td>
<td>Narayanan(^{52,79}); Narayanan(^{81})</td>
</tr>
<tr>
<td>Aprotinin also can be used with lithium heparin (14.3 U/mL)</td>
<td>Narayanan(^{81})</td>
</tr>
<tr>
<td>A mixture of EDTA, aprotinin, leupeptin, and pepstatin stabilizes parathyroid hormone–related protein</td>
<td>Pandian et al(^{80}); Narayanan(^{81})</td>
</tr>
<tr>
<td>Catecholamine stabilizers</td>
<td>Boomsma et al(^{82})</td>
</tr>
<tr>
<td>An antioxidant such as glutathione, sodium metabisulfite, or ascorbic acid at a concentration of 1.5 mg/mL is used with egtazic acid, EDTA, or heparin</td>
<td>Narayanan(^{52,79}); Narayanan(^{81}); Pandian et al(^{80}); Narayanan(^{81})</td>
</tr>
<tr>
<td>For urine, use 5 mL of a 6-mol/L concentration of hydrochloric acid per liter of urine or 250 mg each of EDTA and sodium metabisulfite per liter of urine</td>
<td>Narayanan(^{81})</td>
</tr>
<tr>
<td>Fibrinolytic inhibitors</td>
<td>Narayanan and Hamasaki(^{31}); Connaghan et al(^{83})</td>
</tr>
<tr>
<td>A mixture of thrombin and soybean trypsin or aprotinin</td>
<td>Narayanan and Hamasaki(^{31})</td>
</tr>
<tr>
<td>Reptilase and aprotinin</td>
<td>Narayanan and Hamasaki(^{31})</td>
</tr>
<tr>
<td>Thrombin inhibitor: 5-mmol/L concentration of a phenylalanine, proline, arginine chloromethyl ketone mixture with 10-mmol/L concentration of citrate or 4.2-mmol/L concentration of EDTA</td>
<td>Mohler et al(^{84})</td>
</tr>
</tbody>
</table>

KIU, kallikrein inhibitory unit.

Differentiate prerenal, glomerular, tubular, and postrenal causes of proteinuria by measurement of specific proteins, a morning spot urine specimen was adequate.\(^{45}\)

**Posture During Blood Sampling**

Changes in posture during blood sampling can affect the concentration of several analytes measured in serum or plasma (Table 5). Change in posture from a supine to the erect or sitting position can result in a shift in body water from the intravascular to the interstitial compartment. As a result, the concentration of large molecules that are not filterable is increased. Hence, albumin levels generally are higher among healthy subjects attending an outpatient clinic from whom blood specimens are obtained with the subjects in a sitting position compared with levels among healthy hospitalized subjects from whom blood specimens usually are obtained with the subjects in a supine position.\(^{8,24}\)

Small molecules, such as glucose, however, owing to their ability to move freely between the interstitial space and the circulation, are least affected by posture during
blood specimen collection. In general, molecules even as large as insulin can diffuse freely into and out of the interstitial space and, thus, are not subject to variation due to postural changes. While the free fraction of a metabolite, drug, hormone, or metal ion is not subject to postural variation, the fraction bound to protein, such as albumin, is affected by posture. Thus, bilirubin bound to albumin and calcium bound to albumin are affected by postural changes.

In blood specimens obtained with subjects in the sitting position, generally an increase in the range of 5% to 15% is seen for most cellular and macromolecular analytes compared with results when blood specimens were obtained with subjects in the supine position. The effect of postural change from supine to erect is accentuated in patients with a tendency to edema, such as patients with cardiovascular insufficiency and cirrhosis of the liver. In such patients, reduction in plasma volume in turn causes increased secretion of hormones, such as aldosterone, norepinephrine, and epinephrine, and the enzyme, renin. An increased level of atrial natriuretic peptide (ANP) is measurable in plasma as a compensatory mechanism to correct the decrease in blood pressure. Indeed, changing from supine to erect can dramatically influence the concentration of analytes such as renin and aldosterone, even in healthy persons. Conversely, moving from upright to supine can have a dilutional effect owing to an increase in plasma volume resulting from the transfer of fluid from the extravascular to the intravascular compartment. Thus, a change from upright supine can reduce (after 5 minutes in the supine position) the cholesterol level by 10% and triglyceride levels by 12%. The reduction in blood pH during prolonged tourniquet application can alter drug-protein or hormone-protein binding, leading to an increase in the free drug or free hormone concentration. Thus, at a low pH caused by anaerobic glycolysis during venous stasis, ionic calcium and ionic magnesium levels increase since they are released from albumin, the binding protein.

To minimize the preanalytic effects of tourniquet application time, the tourniquet should be released as soon as the needle enters the vein. Avoidance of excessive fist clenching during phlebotomy and maintaining tourniquet application time to no more than 1 minute can minimize preanalytic error.

Effect of Infusion

For persons receiving an infusion, blood should not be obtained proximal to the infusion site. Blood should be obtained from the opposite arm. Very often, exceptionally high glucose values are obtained by collecting blood from the arm in which an infusion of glucose is being administered (Table 5). Eight hours must elapse before blood is obtained from a subject who has received a fat emulsion. The waiting period for blood sampling from persons receiving a carbohydrate-rich solution or amino acid and protein hydrolysate or electrolytes is 1 hour after cessation of the infusion. For subjects receiving blood transfusions, the extent of hemolysis and, with it, increased values for potassium, lactate dehydrogenase, and free hemoglobin are related progressively to the age of the transfused blood.

Effect of Exercise

Exercise, such as running up and down several flights of stairs, or strenuous activity the night before specimen collection. The venous stasis that results from prolonged tourniquet application promotes anaerobic glycolysis with the accumulation of plasma lactate and a reduction in blood pH. The hypoxic effect drives potassium out of the cell causing a spurious increase in the serum potassium level. This effect can be accentuated if the phlebotomist requests the subject to repeatedly clench the fist to permit visualization of the vein.
collection, such as a workout in a gymnasium or marathon running, can affect the results obtained for several analytes (Table 5).

Strenuous exercise depletes the energy-yielding compound, adenosine triphosphate (ATP), from the muscle cells. As a result, there is a change in cell membrane permeability, and enzymes are released from the cells. Thus, CK levels can be very high after strenuous exercise (levels approaching 1,000 U/L) with a release of the CK-MB fraction, although CK-MB as a percentage of total CK would be normal.8

A high degree of individual variability is seen in a hypoxia-mediated increase in CK, which depends on the extent of exercise training.2 Mitochondrial size and number are increased in persons who maintain a vigorous training schedule, allowing their muscles to oxidatively metabolize glucose, fatty acids, and ketone bodies. As a result, even in the absence of myocardial damage, mitochondrial CK-MB levels increase to greater than 8% of total CK activity.2 Training increases muscle mass, thereby increasing the concentration of creatinine in plasma and the urinary excretion of uric acid, whereas decreasing the level of plasma lactate compared with untrained persons who indulge randomly in exercise.

During strenuous exercise, there is some amount of intravascular hemolysis. As a result, the level of plasma haptoglobin, an acute phase reactant, is reduced owing to its “complexation” with free hemoglobin released during hemolysis and the subsequent clearance of the complex from circulation.8,24

As a result of exercise, the concentrations of hormones such as epinephrine, norepinephrine, glucagon, cortisol, corticotropin, and growth hormone increase, while insulin levels decrease, thereby causing an increase in glucose levels because of the gluconeogenetic effect of hormones antagonistic to insulin.2

Volume shift from the intravascular to the interstitial compartment or volume depletion due to sweating during exercise can increase serum albumin levels.2 Increased lactate concentration due to anaerobic glycolysis during exercise can decrease the urinary excretion of uric acid, thereby increasing its serum concentration.2 There are, however, beneficial effects of long-term strenuous exercise or brisk walking in terms of reducing the levels of serum LDL-C, apolipoprotein B, and triglycerides and increasing the levels of apolipoprotein A1 and HDL-C.23,27,50

To minimize preanalytic variables introduced by exercise, subjects should be instructed to refrain from strenuous activity at least the night before testing and to not exert themselves by walking a long distance or running or climbing stairs before blood specimen collection.8

### Anticoagulants for Blood Collection

Various salts of heparin, EDTA, and sodium citrate are used widely in the clinical laboratory (Table 6).

#### Heparin

Heparin is the preferred anticoagulant for blood specimen collection intended for determination of electrolyte levels and other routine chemistry values. The standard commercial-grade heparin preparation, also called unfractionated heparin, with a molecular mass ranging from 3 to 30 kd (mean, 14 kd) exerts its anticoagulant effect by binding to antithrombin III. This interaction causes a conformational change in the antithrombin III molecule, thereby accelerating the inhibition of coagulation factors Xa, IXa, and thrombin.31 The standard commercial grade heparin also can inhibit thrombin with the aid of a plasma cofactor, called heparin cofactor II. In vivo, heparin can cause the release of a potent coagulation inhibitor, called tissue factor pathway inhibitor, from the endothelial surface.31,51

Various salts of heparin, such as lithium, sodium, and ammonium, have been used as anticoagulants for the collection of blood specimens intended for routine clinical chemistry determinations.52 The nominal amount of heparin used in blood specimen collection tubes is 14.3 U/mL of blood, although a range of 12 to 30 U/mL of blood has been deemed satisfactory.53

Although all of the 3 salts of heparin give comparable results for electrolyte determinations, lithium heparin has been favored over the others, more because of the perception that sodium heparin might overestimate sodium levels and ammonium heparin might spuriously increase serum urea nitrogen concentrations when measured by the urease procedure.52,54

Just how much sodium is present in a sodium heparin blood collection tube depends not only on the nominal amounts of heparin present (14.3 U/mL of blood), but also on other variables, such as the molecular weight of unfractionated heparin, the number of dissolvable milliequivalents of sodium per milligram of heparin, and the consistency of the heparin preparation from lot to lot. Indeed, in 1 study when blood was filled to half of the tube’s nominal volume, thereby increasing 2-fold the nominal amounts of sodium heparin present in the blood collection tube, plasma sodium values were not increased artificially.54

Obvious differences in results for certain analytes between serum and heparinized plasma are related to the consumption of fibrinogen and the lysis of cellular elements during the process of clotting. Thus, potassium values are higher in serum than in heparinized plasma, while in contrast, total protein values are higher in plasma than in serum. The effect of heparinized plasma on some of the enzyme measurements may be related, in part, to the bias introduced by the instrument-reagent combination.55,56
Since the commonly used anticoagulant, such as lithium or sodium heparin, in the syringe to obtain specimens for blood gas measurement binds to ionic calcium, with the effect dependent on the concentration of heparin, attempts have been made to eliminate this bias. One approach has been to use calcium-titrated heparin, in which heparin in an amount less than 50 U/mL of blood is titrated to an ionized calcium concentration of 1.25 mmol/L. The use of electrolyte-balanced heparin that involves the use of dry heparin in the amount of 40 U/mL of blood balanced with calcium, sodium, and potassium decreases bias to less than 0.02 mmol/L of calcium in the analytic range of 0.9 to 1.8 mmol/L. The use of dry heparin eliminates the dilution effect associated with liquid heparin.

Other approaches to minimize interference with ionized calcium measurements have involved saturating the high-affinity binding sites on heparin to which divalent cations, such as zinc, can bind. Zinc heparin, however, in the presence of excess zinc ions is reported to cause a positive interference in ionized calcium and total magnesium measurements. One successful approach is to saturate high- and low-affinity binding sites on heparin, the former with zinc and the latter with lithium. This calcium-neutralized lithium-zinc heparin has been satisfactory not only for ionized calcium measurements, but also for measurement of blood gases, total calcium, sodium, and potassium.

For TDM, the drug level in heparinized plasma would be expected to be higher than that in serum, especially if the drug was bound to fibrinogen. In reality, the serum value for drugs such as amiodarone, desethylamiodarone, chloroquine, and desethylchloroquine was higher than the plasma value, apparently due to the release of cell-bound drug during the clotting process. Thus, results obtained for amiodarone were 11.5% to 13.5% lower in heparinized plasma than in serum, while results for desethylamiodarone were 4.4% to 8.5% lower in plasma than in serum.

The concentration of chloroquine was approximately 2-fold higher in serum than in plasma, while the concentration of its metabolite, desethylchloroquine, was approximately 4 times higher in serum than in plasma. These 2 drugs were present mainly in platelets and granulocytes. The increased levels of these 2 drugs noted in serum apparently were related to the release of these drugs from platelets during the coagulation process. Thus, for some drugs, it is important to specify whether plasma or serum was used for measurement.

**EDTA**

EDTA is the commonly used anticoagulant for routine hematologic determinations. It functions as an anticoagulant by chelating calcium ions, which are required for the clotting process.

Potassium and sodium salts of EDTA have been used for blood specimen collection. The solubility of potassium salts is better than sodium salts of EDTA. The pH of EDTA varies depending on the salt. As more ions are added to the free acid, the acidity of EDTA decreases. Thus, while a saturated solution of EDTA as free acid has a pH of 2.5 – 1.0, the tripotassium salt (K₃EDTA) as 1% solution has a pH of 7.5 – 1.0. The disodium (Na₂EDTA) and dipotassium (K₂EDTA) salts of EDTA in a 1% solution have a lower pH. Na₂EDTA has a pH of 5.0 – 1.0, while K₂EDTA has a pH of 4.8 – 1.0. This difference in pH affects the size of the RBC. Although all salts of EDTA are hyperosmolar, causing the water to leave the cells and the cells to shrink, this shrinking effect is noticed only with K₃EDTA and not with K₂EDTA or Na₂EDTA. This is because although the cells shrink with K₃EDTA and Na₂EDTA, the lower pH of the anticoagulant causes the cells to swell as well, thus balancing for the osmotically dependent shrinkage, and the cell size is not altered. As a result, while the microhematocrit (centrifuged hematocrit) is lowered with K₃EDTA, it is not affected by K₂EDTA or Na₂EDTA. The effect of dilution of the sample in automated analysis restores cells to their native shape. At an optimum EDTA concentration of 1.5 mg/mL of blood, no differences in microhematocrit were noted between samples collected in K₂EDTA and K₃EDTA and analyzed between 1 and 4 hours after collection.

In the same study, the MCV measurement and the calculated hematocrit values were influenced not only by the instrument used, but also by the salt of EDTA (K₃ or K₂) used for anticoagulation of the blood specimen. Thus, MCV values were not affected with blood anticoagulated with K₃EDTA, even at concentrations up to 10 times the nominal value. In contrast, with 3 of the 4 instruments tested, increasing the concentration of K₂EDTA beyond the nominal value of 1.5 mg/mL of blood resulted in an increase in MCV. One explanation may lie in the ready dissolution of liquid K₂EDTA compared with powdered K₂EDTA, in which the effectiveness of mixing might become a variable, perhaps accounting for the discrepancy in results.

The neutrophils are subjected to morphologic changes upon collection of blood in EDTA. These changes depend on the time the peripheral blood smear is prepared and the concentration of EDTA used for blood specimen collection. Minor changes begin to appear in the morphologic features of neutrophils even at an optimal EDTA concentration of 1.5 mg/mL of blood. These changes include swelling of the neutrophils and loss of structure in the neutrophil lobes. Thereafter, there is a loss of granulation in the cytoplasm and the appearance of vacuoles in the nucleus or cytoplasm of the cells. Between 1 and 3 hours after blood collection in
1.5 mg/mL of EDTA, the nucleus swells further, with a crossover of the nuclear chromatin. Beyond 3 hours, the morphologic features of the neutrophils become unclear as the cell disintegrates. If a peripheral blood smear is made from blood anticoagulated with an EDTA concentration of 2.5 mg/mL of blood, approximating to a conventional blood collection tube filled to one half of its nominal volume, the morphologic features of the neutrophils are disintegrated totally.

Similar time-related and EDTA concentration–related changes also occur in the mononuclear cells, although to a lesser extent than in the neutrophils. Platelets also undergo changes, including swelling, giving rise to giant platelets that ultimately disintegrate, producing fragments in the same size range as the platelets that are mistakenly counted as platelets by electronic counters, thus spuriously increasing the platelet count.

Platelets rapidly change their shape from discoid to spherical when blood specimens are collected in EDTA, making determinations of mean platelet volume unreliable. The limited stability of the WBC differential count (up to 6 hours at room temperature storage) achieved in most of the 5-part differential analyzers is another drawback with EDTA.

**Citrate**

Traditionally, a 0.129-mol/L concentration or a 0.109-mol/L concentration of trisodium citrate has been used as the anticoagulant for collection of blood specimens intended for global coagulation tests, such as prothrombin time (PT) and activated partial thromboplastin time (aPTT). Citrate has virtually replaced sodium oxalate as an anticoagulant for performing global coagulation tests, since factor V is more stable in citrate than oxalate. Furthermore, citrate rapidly complexes with calcium, forming a soluble complex in contrast with the slow formation of the insoluble complex of calcium with oxalate. The effective molarity of citrate depends on whether the dihydrate or the anhydrous salt has been chosen in the preparation of the citrate solution. Nominally, a 3.2% solution of the dihydrate trisodium citrate salt corresponds to a 0.109-mol/L concentration, whereas a 3.8% solution of the same dihydrate salt corresponds to a 0.129-mol/L concentration. However, a 3.2% solution of the anhydrous trisodium citrate solution would correspond to a 0.124-mol/L concentration, whereas a 3.8% anhydrous sodium citrate solution would have a molarity of 0.147 mol/L. Hence, it is important that the dihydrate salt be used to prepare either the 3.2% or 3.8% solution of trisodium citrate.

A laboratory that has been using one of the concentrations (3.2% or 3.8%) to perform PT determinations for patients receiving oral anticoagulant therapy should not interchange the formulations. Doing so will affect the international normalized ratio (INR) units that are used to report the results of the PT. INR values generally are higher with a 0.129-mol/L concentration of sodium citrate than with a 0.109-mol/L concentration of sodium citrate, especially when a responsive PT reagent, such as a recombinant thromboplastin that has a sensitivity similar to the World Health Organization thromboplastin (with an international sensitivity index [ISI] equal to 1), is used. The differences in INR between the 2 concentrations of citrate can vary from 0.7 to 2.7 INR units.

The INR will be in error when a laboratory that routinely uses, for example, a 0.109-mol/L concentration of citrate, occasionally analyzes a specimen collected with a 0.129-mol/L concentration of citrate, but uses the mean of the normal range obtained with the 0.109-mol/L concentration to calculate the PT ratio (the patients PT divided by mean of normal range for PT), which together with the ISI are needed for the calculation of INR.

**Stabilizing Additives**

Sodium fluoride and lithium iodoacetate have been used alone or in combination with an anticoagulant such as potassium oxalate, EDTA, citrate, or lithium heparin for blood collection (Table 7). The concentration of sodium fluoride when used alone to inhibit glycolysis is approximately 4.3 mg/mL of blood. In combination with an anticoagulant, such as Na₂EDTA or K₂EDTA (1 mg/mL of blood) or potassium oxalate (2 mg/mL of blood), the nominal concentration of fluoride that is used to inhibit glycolysis is 2.5 mg/mL of blood (60 mmol/L).

Similarly, lithium iodoacetate has been used alone (0.5 mg/mL of blood) or at that concentration in combination with lithium heparin (14.3 U/mL of blood). Even with the use of either of these glycolytic inhibitors, at least 3 hours are needed for these inhibitors to become fully effective and stabilize glucose levels. During this 3-hour period in healthy subjects, an approximately 9 mg/dL loss of glucose occurs. However, in neonates or subjects with an increased RBC, WBC, or platelet count, the decrease in glucose during the 3-hour period, even in presence of glycolytic inhibitors, is greater than in healthy subjects. Once the glycolytic inhibitors become fully effective after the initial 3-hour period, glucose levels remain stable for at least 3 days.

In the absence of glycolytic inhibitors, a decrease in the glucose level of as much as 24% can occur in 1 hour after blood collection in neonates in contrast with a 5% decrease in healthy adults when specimens are stored at room temperature. By 5 hours after blood collection in specimens stored at room temperature, as much as 68% of glucose can be consumed in specimens collected without glycolytic inhibitors from neonates with a high hematocrit.
The reason that fluoride and iodoacetate take approximately 3 hours to stabilize glucose levels is that they do not act on the first enzyme in the glycolytic pathway, which is hexokinase. Instead, fluoride acts on enolase, which is the eighth enzyme in the glycolytic pathway, while iodoacetate acts on glyceraldehyde-3-phosphate dehydrogenase, which is the fifth enzyme in the pathway in which glucose is metabolized.2

Potassium oxalate, which is used in combination with fluoride, inhibits pyruvate kinase in the enzymatic step immediately after enolase in the glycolytic pathway and, thus, can effectively retard lactate formation 1 hour after blood collection.

The drawback with both fluoride and iodoacetate is that they promote hemolysis, which increases progressively as the concentration of the inhibitor used and the time elapsed after blood specimen collection increases. Apparently both inhibitors deplete the organic phosphate (ATP) content of the RBC, thus causing an efflux of potassium out of the cell.

A variety of approaches have been attempted to improve the efficacy of glycolytic inhibition. In one approach, a mixture of citric acid, trisodium citrate, Na2EDTA, and a very small concentration of sodium fluoride (0.2 mg) effectively inhibits the enzymes in the glycolytic pathway by maintaining blood pH in the range of 5.3 to 5.9.70 With this additive mixture, glycolysis was inhibited for 8 hours in specimens stored at room temperature, while at 24 hours, a mean – SD decrease of 1.3 mg – 1.1 mg/dL (0.07 – 0.06 mmol/L) of glucose was noted.70

Another strategy for achieving efficient glycolytic inhibition is to include mannose in addition to fluoride.71 The rationale is that mannose, which is an epimer of glucose (differs structurally from glucose in the configuration around just 1 carbon atom), will compete with glucose for hexokinase, the first enzyme in the glycolytic pathway. Since mannose is a competitive inhibitor, the inhibition is short-lived, lasting just 4 hours. However, by this time, fluoride, which is also in the additive mixture, would have become effective thus achieving a better glucose stabilization.2,71

At a mannose concentration of 3 mg/mL of blood, no interference was noted in hexokinase and glucose oxidase procedures for the measurement of glucose.72 However, when the mannose concentration was increased to 3 times the nominal concentration of 3 mg/mL of blood, an overestimation of glucose by 18 mg/dL (1.0 mmol/L) was observed.72 Some of the mannose preparations also may be contaminated with glucose. Negative interference has been reported in the hexokinase procedure when mannose concentrations exceed 3 times the nominal concentration.73

The extent of reduction in glucose levels depends on the concentration of hexokinase used in the glucose assay, with less interference noted at higher concentrations of hexokinase used in the assay.73

In practical terms, if plasma is separated from cells by centrifugation promptly after blood specimen collection, the glucose level in plasma is expected to be stable, since the enzymes that metabolize glucose are in the cellular fraction.

Stabilization of RBCs by including a nutrient such as glucose together with an anticoagulant mixture is the basis of 2 acid citrate dextrose (ACD) formulations.2 These formulations, labeled ACD A and B, are similar in pH; ACD A has a pH of 5.05, and ACD B has a pH of 5.1. The difference between the formulations is the additive/blood ratio; the A formulation has a ratio of 1:5.67. The B formulation, however, with a ratio of 1:3, has relatively greater amounts of dextrose available for the metabolizing RBCs and, hence, can preserve them better. While ACD formulations can preserve RBCs for 21 days when blood is stored between 1 C and 6 C, their stability can be extended further by including additives such as phosphate and adenine (citrate phosphate dextrose and citrate phosphate dextrose adenine, respectively).

The maintenance of increased levels of cyclic adenosine monophosphate (c-AMP) within the platelets is the basis of an additive mixture intended for blood specimen collection to minimize in vitro platelet activation.74,75 The additive is a mixture of citric acid, theophylline, adenosine, and dipyridamole with the final pH adjusted to 5.0.74 Adenosine activates the enzyme adenylyl cyclase, thus increasing c-AMP levels within the platelets. Theophylline and, to a certain extent, dipyridamole prevent the degradation of c-AMP by inhibiting the enzyme phosphodiesterase. Dipyridamole, in addition, prevents the uptake of adenosine by the RBCs, thus effectively increasing the amount of adenosine available to the platelets to activate adenylyl cyclase and, in turn, the inhibition of platelet aggregation and release of contents of platelet alpha granules.31,74 The citrate in the additive mixture functions as an anticoagulant by chelating calcium. The citric acid, theophylline, adenosine, and dipyridamole mixture is used for monitoring heparin therapy by aPTT or the chromogenic substrate assay and in measurement of platelet activation markers, such as P-selectin (CD62), by flow cytometry.76,77

Stabilizing additives to inhibit proteolytic enzymes are needed for some analytes. Although EDTA itself is a metalloprotease inhibitor, sometimes EDTA alone is insufficient to inhibit proteolytic enzymes. For example, when a synthetic protease inhibitor, such as nafamostat mesylate, is added to EDTA, the mixture can substantially improve the stability of complement components C3a, C4a, and C5a.52,78

A proteinase inhibitor, aprotinin (Trasylol), when used in combination with an anticoagulant such as EDTA or heparin, has been useful for the stabilization of labile
polypeptide hormones and enzymes.\textsuperscript{52} Aprotinin inhibits specific proteases of kinin and coagulation and fibrinolytic enzyme systems, in addition to inhibiting the enzyme systems of leukocytes and damaged tissue cells. Thus, it inhibits kallikrein, trypsin, chymotrypsin, coagulation factors in the preliminary phase of blood clotting, the fibrinolytic enzyme plasmin, and lysosomal proteinases. The fact that aprotinin inhibits kallikrein is the basis for the expression of its potency in terms of kallikrein inhibitory units (KIUs). Aprotinin has been used in combination with an anticoagulant such as EDTA or heparin. A mixture of EDTA (1.5 mg/mL of blood) and aprotinin in the activity range of 500 to 2,000 KIU/mL has been used to stabilize polypeptide hormones such as glucagon and corticotropin, the enzyme renin, and gastrointestinal hormones, such as beta-endorphin, secretin, neurotensin, gut glucagon, somatostatin, vasoactive intestinal peptide, and peptide yy.\textsuperscript{52,79}

In the absence of aprotinin, spuriously higher values may be obtained apparently because of the fragments of the analyte produced by proteolytic enzyme degradation are measured as intact molecules by the polyclonal antibody used in the assay. Thus, in a radioimmunoassay for glucagon, a 26\% spurious increase was noted in plasma obtained with blood specimens collected with EDTA alone compared with plasma obtained with blood specimens collected in a mixture of EDTA and aprotinin.\textsuperscript{52,79}

A mixture of lithium heparin (14.3 U/mL) and 1,000 KIU/mL of aprotinin also has been used to stabilize immunoreactive somatostatin, secretin, cholecystokinin (pancreozymin), glucagon, and C-peptide.\textsuperscript{52}

In addition to aprotinin and an anticoagulant such as EDTA, additional proteolytic enzyme inhibitors may be needed to stabilize a labile tumor marker, such as parathyroid hormone related protein (PTH-rP) that has been associated with the humoral hypercalcemia of malignant neoplasms.\textsuperscript{80,81} Thus, with an additive mixture of EDTA (1.5 mg/mL), aprotinin (500 KIU/mL), leupeptin (2.5 mg), and pepstatin (2.5 mg), PTH-rP levels were stable for 1 hour after blood specimen collection for specimens stored at room temperature and for 24 hours, if specimens were refrigerated at 4°C.\textsuperscript{80} Even when blood is collected with this additive mixture, if the sample is stored at room temperature for 24 hours, as much as 60\% of the PTH-rP activity can be lost.\textsuperscript{80} Without a stabilizing additive mixture, PTH-rP is so unstable that as much as 60\% of its activity can be lost within 1 hour of specimen collection and storage at room temperature.

Analytes that are susceptible to oxidative damage, such as catecholamines, require stabilization. Typically together with an anticoagulant such as heparin, EDTA, or egtaic acid, an antioxidant such as glutathione, sodium metabisulfite, or ascorbic acid at a concentration of 1.5 mg/mL should be included in the blood specimen collection additive mixture.\textsuperscript{81} The specimen collected with this additive mixture should be centrifuged within 1 hour of collection. It is preferable, however, if possible to separate plasma within 15 minutes of specimen collection and store it in plastic tubes or vials at ~20°C in case analysis is delayed. Prompt processing of plasma obtained with an antioxidant-anticoagulant mixture stabilizes catecholamines in plasma for 1 day at room temperature, for 2 days when stored at 4°C to 8°C, and for 1 month at ~20°C. Stability at ~20°C can be extended to 6 months with added glutathione.\textsuperscript{2,82}

Apparently, at the higher concentrations of catecholamines present in urine compared with plasma, the hormones and their metabolites are stable for 4 days, even when stored at 20°C to 25°C without preservatives.\textsuperscript{2,82} However, for long-term stabilization of catecholamines in urine specimens, collection is recommended in a container with 5 mL of a 6-mol/L concentration of hydrochloric acid per liter of urine or in a mixture of 250 mg each of EDTA and sodium metabisulfite per liter of urine, which will extend stability to 3 weeks at 20°C to 25°C, and 4 months to 1 year at 4°C to 8°C.\textsuperscript{2,82}

The laboratory assessment of fibrinolysis by the measurement of the nonspecific fibrinogen-fibrin degradation products requires inhibition of the in vitro generation of the fibrinolytic enzyme plasmin with an inhibitor, such as soybean trypsin or aprotinin, and the conversion of residual fibrinogen to fibrin with thrombin.\textsuperscript{31} However, in patients receiving heparin therapy, the conversion of residual fibrinogen to fibrin, even in presence of thrombin, will be slow, thus resulting in spuriously high fibrinogen degradation product values owing to the remaining unconverted fibrinogen. In such cases, the addition of snake venom, also known as reptilase, instead of thrombin to the additive mixture, which also contains a plasmin inhibitor such as aprotinin, can result in the conversion of residual fibrinogen to fibrin, even in the presence of heparin.\textsuperscript{83}

For monitoring fibrinolytic therapy, the additive used to inhibit plasminogen activation depends on the therapeutic agent administered. Thus, while aprotinin together with EDTA is effective for inhibiting plasminogen activation by the therapeutic agent urokinase, it is ineffective for inhibiting the recombinant tissue plasminogen activator (rt-PA) from activating plasminogen.\textsuperscript{75} Specific synthetic peptides of arginine chloromethyl ketone (PPACK) are needed to inhibit in vitro rt-PA activity. PPACK irreversibly inactivates rt-PA by alkylating the active center amino acid histidine, in addition to functioning as a potent thrombin inhibitor.\textsuperscript{84} A 5-mmol/L concentration of PPACK combined with a 10-mmol/L concentration of citrate or a 4.2-mmol/L concentration of EDTA effectively inhibited rt-PA upon blood collection.\textsuperscript{75} However, since PPACK is unstable at neutral pH, blood upon
collection should be maintained at 4 C, centrifuged promptly to obtain plasma, and processed without delay or kept frozen until analysis can be performed.31

As new diagnostic markers are proposed, the labile nature of some poses challenges. A case in point is the measurement of ANP, which is a marker of chronic congestive heart failure. An additive mixture of EDTA and aprotinin is needed to stabilize ANP; the blood specimen collected with this mixture must be centrifuged immediately at 4 C and plasma maintained frozen at –20 C or below until ready for analysis.85 Furthermore, hemolysis invalidates ANP measurements. Alternatively, the measurement of the N-terminal fragment of the prohormone proatrial natriuretic peptide (proANP) (which is formed when proANP is split into equimolar amounts of ANP and the N-terminal fragment) can be performed in blood specimens collected in EDTA alone with storage at room temperature for up to 6 hours. At 4 C, N-terminal proANP is stable for 3 days and at –20 C for 4 weeks, with hemolysis not markedly affecting the results.86

Anticoagulant/Blood Ratio

The anticoagulant/blood ratio is critical for some laboratory tests. Obviously, if too little blood is obtained, osmotic effects can be expected to result in cell shrinkage and, thus, to introduce dilutional changes in the concentration of extracellular analytes.

A well-known example of the critical effect of the anticoagulant/blood ratio is for the global coagulation test, aPTT. Traditionally, a 1:9 ratio of anticoagulant (sodium citrate, a 0.109-mol/L concentration or a 0.129-mol/L concentration) to blood is used. However, if less blood is collected, simulating a 1:7 anticoagulant/blood ratio, there is a significant increase in the aPTT result compared with that obtained using the nominal 1:9 ratio.31 In 1 study, a 2.4-second increase was observed at a 1:7 ratio compared with the aPTT result obtained at the nominal 1:9 ratio.65

However, for the global coagulation test, PT, the effect of the anticoagulant/blood ratio becomes meaningful only when the ratio reaches 1:4.5, which would occur when the blood collection tube is filled to just less than half of its nominal volume.65 The anticoagulant/blood ratio also is influenced by the sensitivity of the thromboplastin reagent that is used. In a study of the effect of the anticoagulant/blood ratio using 3.2% (0.109-mol/L concentration) buffered citrate, the results for the PT were valid even for specimen collection tubes that were filled to 65% of the nominal anticoagulant/blood ratio (1:9). However, filling the tube to at least 80% of the nominal ratio was needed to obtain reliable results in the therapeutic range using a moderately sensitive thromboplastin reagent with an ISI of 2.06. Using a highly sensitive thromboplastin reagent with an ISI of 1.01 required maintaining the anticoagulant/blood ratio to at least 90% of the nominal ratio.87 In the aforementioned study,87 the need to fill blood collection tubes to the nominal capacity (1:9) was reinforced for the aPTT, since tubes filled to less than 90% of the nominal capacity caused prolongation of the aPTT result.

In addition to maintaining the nominal anticoagulant/blood ratio (1:9), the amount of head space above the blood also has been reported to be a variable affecting the aPTT result. Thus, collecting less blood (2.7 mL as opposed to the nominal 4.5 mL) but maintaining the anticoagulant/blood ratio without decreasing the tube size effectively increases the head space above the blood. This, in turn, generally leads to a shortened aPTT in values outside the upper limit of the reference interval and in the therapeutic range for monitoring unfractionated heparin therapy.88 Thus, in the abnormal range, the mean value obtained with the partially filled tube was 11 seconds shorter than the mean value obtained with the full tube.88 One possible explanation for the head space effect is the increase in surface area relative to the volume of blood collected, favoring platelet activation and the subsequent release of platelet factor 4, which in turn neutralizes some of the heparin, resulting in a shortened aPTT.88

For polycythemic patients with hematocrit values above 60% (0.60), the PT and the aPTT can be prolonged substantially, even when the nominal 1:9 anticoagulant/blood ratio is maintained.89 This is because the plasma is overcitrated to the extent that it complexes much of the calcium chloride added to perform the PT and aPTT, effectively reducing the calcium ions needed to clot the plasma and thereby prolonging the clotting time. This effect can be minimized by adjusting the citrate concentration in accordance with the hematocrit value by using empiric formulas or by using a 1:19 anticoagulant/blood ratio.65,89 The latter ratio dilutes the citrate concentration to a point that results of PT and aPTT are not affected across a spectrum of hematocrit values ranging from anemia to polycythemia.89

In general, collecting blood specimens to less than the nominal volume increases the effective molarity of the anticoagulant and induces osmotic changes affecting cell morphologic features as noted for the morphologic features of neutrophils when the EDTA concentration changes from the nominal 1.5 mg/mL of blood to 2.5 mg/mL of blood. Furthermore, the binding of analytes, such as ionic calcium or ionic magnesium, to heparin can be enhanced when the effective concentration of unfractionated heparin increases beyond the nominal 14.3 U/mL of blood.52

Maintenance of the anticoagulant/blood ratio also is critical when the inhibitory effects of the anticoagulant are concentration-dependent. Thus, a 10% reduction in the gentamicin level was noted in an enzyme-multiplied immunoassay technique when the concentration of heparin used for specimen collection increased to 25 U/mL of
blood, presumably because of the inhibition of the enzymes used in the immunoassay procedure, as the concentration of heparin increased from the nominal value.90

At the other extreme is exceeding the anticoagulant/blood ratio to a point that the tube is overfilled with blood to such an extent that proper mixing of anticoagulant with blood becomes problematic. Thus, in 1 study, the hemoglobin values had doubled compared with a value obtained a week before, and the WBC and platelet counts were reduced drastically. Reanalyzing the specimen for the fourth time from the same tube yielded values similar to values obtained initially the week before. Apparently, proper mixing of the specimen on a rocking mixer was not achieved since there was no head space or bubble left in the tube to assist in the mixing of the specimen. After a sufficient amount of blood was withdrawn by repeating the analysis 4 times, there was sufficient space in the tube for the air bubble to move and effect thorough mixing on the rocking-type mixing device.91

For bacteriologic determinations, inhibition of the normal bactericidal properties of blood requires appropriate dilution of blood in broth. Incorporation of a 0.025% solution of sodium polyanethole sulfonate in blood culture media is intended to inhibit phagocytosis, complement, and lysozyme. Even when blood culture is supplemented with sodium polyanethole sulfonate, at least a 1:10 blood/broth ratio is considered optimal.92 However, a blood/broth ratio less than 1:10 has been acceptable when at least 2 separate blood cultures are obtained routinely or when a biphasic medium is used to culture blood (a broth bottle to which an agar slant with media is attached).93

Since spurious blood culture results can be obtained for patients receiving antibiotics, a 1:10 dilution of blood in broth dilutes many of the antibiotics in the blood to sub-inhibitory levels. The incorporation of sodium polyanethole sulfonate inactivates aminoglycosides. Alternatively, however, devices containing antibiotic-adsorbent resins can be used to neutralize antibiotics, and cell lysis followed by filtration or centrifugation neutralizes the antimicrobial properties or components of blood and provides a concentrate for culture on antibiotic-free media.93

Specimen Handling and Processing

The time and temperature for storage of the specimen and the processing steps in the preparation of serum or plasma or cell separation using density-gradient techniques can introduce a preanalytic variable. Analytes subject to cellular metabolism need prompt separation from cells.

Storage of anticoagulated whole blood or clotted blood in a refrigerator (4 C) inhibits the Na+,K+-ATPase pump, resulting in potassium leaking out of the cells and causing a spurious increase in levels measured in serum or plasma. This effect is seen even at 4 hours of storage of clotted blood at 4 C.2,8,94 Inhibition of the Na+,K+-ATPase pump also drives sodium into the cell. Since sodium is mainly extracellular, in contrast with potassium that is mainly intracellular, the decrease in sodium becomes noticeable only when whole or clotted blood is stored beyond 24 hours at 4 C. Ideally, when separation of plasma or serum from cells is needed, blood should be processed within 1 hour of collection to obtain plasma or serum.2

Storage of clotted blood beyond 8 hours at room temperature (23 C) can cause an increase in inorganic phosphate owing to the hydrolysis of cellular organic phosphate by the enzyme alkaline phosphatase; the effect is more pronounced when clotted blood is stored at 30 C.2 Thus, while a 10% increase was noted in the inorganic phosphate value at 24 hours compared with the 8-hour value during storage at 23 C, the increase was 120% at a storage temperature of 30 C.2

A time-dependent increase in ammonia is accentuated in specimens with increased gamma-glutamyltransferase activity.95 The time between collection of blood and its storage temperature before processing to yield plasma can introduce a variable. Thus, in the measurement of catecholamine levels in specimens collected with a stabilizing additive mixture and stored at 4 C, there is an increase in the level of catecholamines owing to a delay in the processing of plasma that exceeds 1 hour.81,82 A delay in the preparation of plasma for 2 hours after blood collection and storage at 4 C can increase the mean plasma norepinephrine level by 13% (N = 8).82 This effect is due to the lysis of some of the RBCs at 4 C, leading to a release of catecholamines into plasma and to the slow reuptake of these hormones by cells at 4 C. In addition, cryoprecipitation of plasma proteins at 4 C also can contribute to the increased level of catecholamines measured in plasma.81,82 In contrast, storage of specimens at 20 C and a delay beyond 1 hour in the processing of plasma after specimen collection can lower the catecholamine level measured in plasma owing to the uptake of catecholamines by cells such as RBCs or platelets.82 Thus, the mean plasma norepinephrine levels decreased by 14% (N = 8) from the initial value if blood specimens kept at 20 C were centrifuged 2 hours after collection, while mean plasma epinephrine values decreased by 20%.82

The storage temperature of a specimen influences the results of global coagulation tests such as PT and aPTT. Since factor VII, which is measured by PT, is activated during prolonged storage at 4 C, storage of plasma in contact with cells beyond 7 hours shortens the PT.65,96,97

Indeed, when a blood collection tube is kept well stoppered and stored at room temperature (25 C), the PT is stable for up to 48 hours.89,97 As is well known, the aPTT should be performed preferably within 2 to 4 hours of
specimen collection, and some investigators maintain plasma on ice (4 C). In 1 study, a 10% to 15% increase in aPTT was observed after 24 hours in specimens that were maintained well stoppered and at room temperature.

In contrast with refrigerated temperatures, freezing plasma at −20 C and −70 C did not activate factor VII. The PT and aPTT results were stable in plasma maintained frozen at −20 C for 10 days and at −70 C for 21 days.

Factors II, V, VII, and X are stable in plasma maintained under refrigeration for up to 6 hours. All except factor V had stability for up to 14 days when frozen at −20 C or −70 C. Typically, clotted blood or anticoagulated blood is centrifuged at 1,000 g to 1,200 g for 10 to 15 minutes. Insufficient centrifugation time and lower centrifugal forces can result in a gradient of platelets in plasma derived from heparinized blood, thus affecting some of the laboratory results, such as potassium levels.

While centrifugation at 2,000 g for 15 minutes is recommended for whole blood to obtain platelet-poor plasma for global coagulation tests, at slightly lower speeds (1,800 g), some residual platelet contamination was reported based on a shortened thrombin clotting time by 10% in plasma frozen for 48 hours and thawed to room temperature before testing.

Despite the use of robotics and improved pneumatic tube delivery systems, and despite the fact that erstwhile preanalytic problems such as hemolysis during specimen transportation may have been overcome by improved design of transport systems, the existence of preanalytic problems should not be discounted. A case in point is the likelihood of erroneous results for PO₂ if the blood has been contaminated with air before pneumatic tube transportation.

Relative Merits of Anticoagulants

Platelets rapidly change their shape from discoid to spherical when blood is collected in EDTA, thus making the determination of mean platelet volume unreliable. Approaches to minimize this artifact have been attempted by the use of an alternative anticoagulant, which is a mixture of trisodium citrate, pyridoxal phosphate and tris(hydroxymethyl)aminomethane. The rationale is that pyridoxal phosphate is an effective platelet antiaggregant and disaggregant. Mean platelet volume measurement using the aforementioned additive mixture was stable at room temperature for 24 hours.

ACD, heparin, and EDTA have been used for the separation of mononuclear cells. However, regardless of whether ACD, EDTA, or heparin was used as the anticoagulant, the lymphocyte fraction was contaminated with granulocytes if the blood specimen was more than 24 hours old. RBC contamination is a problem with aged EDTA blood specimens to the extent that 9 RBCs may be encountered for every lymphocyte separated from a 2-day-old blood specimen.

For flow cytometric analysis, ACD and heparin are superior to EDTA for maintaining viable WBCs overnight. However, if analysis is to be performed the same day, EDTA, ACD, and heparin give equivalent results. However, at 24 hours and beyond, a substantial decrease in granulocyte viability may occur in blood collected in EDTA. K₂EDTA also has been reported to be associated with a loss of function in lymphocyte-mitogen stimulation assays.

Heparin has been reported to be problematic for use in molecular biology procedures using restriction enzymes and DNA amplification by the polymerase chain reaction (PCR). While heparin inhibition can be overcome by a variety of approaches (eg, treatment with heparinase, or separation of leukocytes by centrifugation followed by a minimum of 2 washings in a saline buffer or use of an appropriate buffer), many investigators prefer not to use heparin for DNA amplification by PCR.

In vitro platelet, monocyte, and neutrophil activation studies are influenced by the anticoagulant used for blood collection. Thus, monocyte activation as measured by release of tissue factor and tumor necrosis factor activity was lowest with EDTA compared with citrate, heparin, and hirudin. Neutrophil activation as measured by lipopolysaccharide-induced release of lactoferrin also was the lowest with EDTA. EDTA seems to suppress platelet degranulation. Platelet factor 4 levels as an indicator of platelet activation was the lowest with EDTA–platelet-poor plasma (217 ng/mL) compared with platelet-poor plasma obtained with citrate (440 ng/mL), hirudin (469 ng/mL), and unfractionated heparin (1,180 ng/mL). A low-molecular-weight heparin preparation, however, gave platelet factor 4 values comparable to those obtained with hirudin.

Endogenous Interferences and Related Variables

The effect of drugs and their metabolites on laboratory tests is so extensive that compendia of interferences are available for consultation. Several are discussed in the following text.

Although the subject of endogenous interferences is broad, some mention should be made of interferences that can affect clinical interpretation of laboratory data. In that context, patients who are exposed to mouse immunoglobulins through imaging or therapeutic techniques are prone to develop
antibodies to mouse immunoglobulins. These antibodies, called human antihistone antibodies, or HAMA, can give rise to false-positive or false-negative results in 2-site immunometric assays using murine monoclonal antibodies as reagents.81,106

An increased level of a chemical analyte can affect some hematologic determinations. Thus, glucose levels more than 600 mg/dL (33.3 mmol/L) can lead to a transient increase in the MCV as water enters the RBC, as the RBC is placed in an isotonic diluent, owing to the osmotic effect of glucose.107 However, as the glucose level slowly equilibrates, water exits from the RBC along with glucose, thus restoring the original MCV. This transient hypoglycemic effect can be overcome by a microdilution of the blood sample and a 5-minute waiting period for the equilibration to be complete before analysis.107

Triglyceride levels exceeding 1,000 mg/dL (11.29 mmol/L) and hyperlipidemic specimens can cause a spuriously high hemoglobin value. This effect can be overcome by resuspending the RBCs in saline before analysis or by making corrections with a plasma hemoglobin blank.107

Turbidity resulting from the precipitation by lysis reagents of high concentrations of monoclonal proteins seen in patients with myeloma or macroglobulinemia may spuriously elevate the hemoglobin level and WBC count.107

Endogenous antibodies can affect both coagulation and hematologic results. Circulating antibodies directed to platelet membrane phosphatidylycerine and phosphatidylinositol, which are called anticardiolipin antibodies, also inhibit phospholipid-dependent global coagulation tests. An in vitro phenomenon of platelet agglutination seen in blood collected in EDTA is due to the presence of antibodies in blood that are reactive to platelets. These antibodies are directed to antigens such as the glycoprotein IIb/IIIa complex that is hidden within the platelet membrane. EDTA, upon chelating calcium, exposes these antigens. The exposed platelet antigen becomes modified at low and room temperature, permitting platelet antibodies to agglutinate platelets. This EDTA-induced pseudothrombocytopenia is, however, not seen when blood specimens are warmed to 37 °C, since the glycoprotein IIb/IIIa complex is dissociated at the higher temperature. EDTA-induced pseudothrombocytopenia at 4 °C or room temperature can cause a spuriously low platelet count and a spuriously increased WBC count if the platelet clumps are in the same size range as WBCs. Apparently an epitope on platelet membrane glycoprotein IIb is recognized by EDTA-dependent IgG antibodies, causing pseudothrombocytopenia.108

EDTA-dependent IgM antibody, most active at room temperature and of low titer, has been reported to cause leukocyte agglutination noticeable on a peripheral blood smear but to provide a spuriously low automated WBC count.109 This phenomenon was not seen when the same patient’s specimen was obtained in a tube with heparin or citrate.

Both pseudothrombocytopenia and pseudoleukopenia have been reported at room temperature owing to the EDTA antibody–induced platelet aggregates not being counted as platelets and to large neutrophil-platelet clumps falling outside the size range for WBCs.110 This phenomenon was abolished by warming the specimen to 37 °C.

An in vitro phenomenon that is referred to as platelet satel- litis is observed when the platelets surround the neutrophils owing to EDTA dependent IgG autoantibodies that are directed...
to the glycoprotein IIb/IIIa complex in the platelet membrane and neutrophil FC-gamma receptor III of neutrophils (CD16).\textsuperscript{111} This phenomenon, which is seen at room temperature, is not seen when blood is exposed to a temperature of 37 °C or peripheral blood smears are made immediately with EDTA-anticoagulated or capillary blood. Occasionally, the phenomenon also has been noticed with citrated blood, as well as with heparinized blood.\textsuperscript{112} Severe platelet satellitism can cause spurious pseudothrombocytopenia.

A discussion of endogenous interferences would not be complete without discussing the most common interferences, such as hemolysis and turbidity due to hyperlipidemia. Apart from a few analytes, there are conflicting data in scientific literature on the effect of hemolysis on a wide range of analytes. The discrepancy is related not only to differences in methods for the same analyte, but also to differences in instrumentation. While to some extent instruments that can subtract or blank the hemoglobin interference by taking spectrophotometric measurements at the wavelength of light where hemoglobin absorbs, in addition to the wavelength at which the analyte absorbs (bichromatic measurements), can minimize the interference, the hemoglobin interference is not eliminated totally, especially when the reagent system used is affected by hemoglobin.

In general, slight hemolysis would have a negligible effect on many of the routine clinical chemistry laboratory procedures. Severe hemolysis, while having a substantial effect on constituents that are at a much higher concentration within the RBC than in plasma, could induce a slightly dilutional effect on the constituents present at a lower concentration in the RBC than in plasma.

Typical of the enzymes present within the RBC, the concentration of lactate dehydrogenase (LDH) is 160-fold greater than that present in the plasma, acid phosphatase is 68-fold greater, and AST is 6.7-fold greater than the plasma levels.\textsuperscript{113} The concentration of potassium inside the RBC is fold greater, and AST is 6.7-fold greater than the plasma.

Naturally one would expect the aforementioned tests to be invalidated when the specimen is hemolyzed severely. The visual evidence of hemolysis is provided when the hemoglobin concentration exceeds 20 mg/dL. It has been reported that the appearance of serum when 0.1% of the RBCs are lysed was virtually identical to that of nonhemolyzed serum and, consequently, would go undetected by laboratory personnel looking for hemolyzed specimens.\textsuperscript{114} However, the appearance of serum when 1.0% of RBCs were lysed was clear and cherry red. Such a specimen would be characterized as having moderate hemolysis. Hemolysis of 1% of the RBCs affected the measurement of LDH, potassium, AST, and ALT.\textsuperscript{114}

Hemolysis also has been classified based on measurement of free hemoglobin levels.\textsuperscript{115} The nonhemolyzed samples had a mean – SD free hemoglobin value of 4.8 – 3.2 mg/dL, and moderately hemolyzed serum samples had a value of 43.5 – 13.9 mg/dL. Even slight hemolysis invalidated the results for LDH, total acid phosphatase, prostatic acid phosphatase, and potassium, and such specimens should be rejected.\textsuperscript{115} That the effect of hemolysis is method-dependent was demonstrated by the absence of statistically significant differences for the levels of AST, ALT, inorganic phosphate, glucose, bilirubin, total protein and albumin.\textsuperscript{115}

Adenylate kinase released from RBCs can spuriously increase CK activity measurements unless a sufficient amount of inhibitors of adenylate kinase are incorporated in the assay mixture.\textsuperscript{2}

The peroxidative effect of heme can cause a positive and negative interference with various diazotization methods used for the measurement of bilirubin, depending on the regents used in the assay system.\textsuperscript{116} In classic diazotization procedures for the measurement of bilirubin, hemoglobin in the hemolyzed sample competes with nitrite for the sulfanilic acid reagent, leading to spuriously lower results.\textsuperscript{115}

Depressed glucose values (values lower than the true value) have been reported with the glucose oxidase–peroxidase procedure, which apparently is related to the reagent composition. The negative interference has been explained as resulting from premature decomposition by hemoglobin of the hydrogen peroxide generated in the glucose oxidase reaction or from the dilution by cellular components.\textsuperscript{117}

The effect of hemolysis on glucose oxidase procedures depends not only on the composition of the glucose oxidase–peroxidase reagent system, but also on how the assay is performed. Thus, differences on the effect of hemolysis have been reported when using a kinetic assay in which the color development is monitored between 60 and 120 seconds after initiating the reaction as opposed to a bichromatic assay in which color is monitored at 2 different wavelengths; the additional wavelength is used to blank out the color resulting from substances other than glucose. For example, 2 grossly hemolyzed pediatric samples with a low true glucose value were diagnosed correctly by the kinetic procedure that yielded glucose values of 29 and 27 mg/dL (1.6 and 1.5 mmol/L; reference range, 70-105 mg/dL [3.9-5.8 mmol/L]). The bichromatic procedure, on the other hand, overestimated the glucose values on the 2 pediatric samples by reporting glucose values of 65 and 68 mg/dL (3.6 and 3.8 mmol/L).\textsuperscript{117}

Hemolysis also interferes with the hexokinase procedure for the measurement of glucose.\textsuperscript{118} However, owing to the multiplicity of instrumentation and variations in methods for the measurement of glucose, the magnitude of the effect of hemolysis on each glucose procedure is unpredictable. As such, for accurate glucose measurements, it is desirable to avoid hemolysis or, at the very least, not exceed levels that would make hemolysis visually apparent in serum.
While total acid phosphatase activity was, as expected, affected by hemolysis due to the release of RBC acid phosphatase, even the prostatic tartrate-sensitive fraction has been reported to be affected by hemolysis. Among the hormones very sensitive to hemolysis is insulin. Apparently the release of proteolytic enzymes during hemolysis degrades insulin. While some analytes clearly are affected by hemolysis, in general, method-dependent variations should be considered when assessing the influence of hemolysis.

Hyperlipidemia, by introducing turbidity, can be a source of interference that also is method-dependent. Interference could be due to a variety of mechanisms, such as inhomogeneity of the sample, displacement of water, and adsorption of lipophilic constituents. A visibly hyperlipidemic sample can be expected to interfere with the measurement of total protein and electrophoretic and chromatographic procedures.

Summary Perspectives

So much knowledge on the preanalytic phase has accumulated during recent years that this review can only highlight key issues affecting several contemporary topics.

On the subject of blood gases issues such as maintaining a steady state of ventilation with the patient in a supine position before and during arterial blood collection, the effect ofcontainer material (eg, glass vs plastic syringe), dry vs liquid heparin as the anticoagulant, leakage of gases owing to syringe material and conditions of storage, and time between collection and analysis need to be recognized and standardized.

Molecular biology is another area in which information on the preanalytic phase is accumulating constantly. The type of detergent used for cell lysis, its effect on DNA amplification by PCR, the effect of RBC contamination due to the effect of hematin on the DNA polymerase enzyme, taq polymerase, used in PCR, isolation of RNA including steps to eliminate RNase contamination, treatment of tissue, the effect of fixatives and duration of fixation, and protection of viral HIV RNA load in plasma from the inhibiting effect of neutrophils by prompt separation of plasma from cells after blood collection are just a few of a wide range of preanalytic variables that merit consideration.

As our knowledge of cell function advances and studies on stimulation of cells to secrete cytokines become diagnostically important, one must ensure that there is no artifactual effect induced by the anticoagulant used for blood collection. A case in point is the endotoxin- or pyrogen-contaminated heparin stimulating monocytes to secrete tumor necrosis factor, thereby invalidating the measurement of this cytokine.

Information related to the preanalytic phase of tumor marker measurements is voluminous. The preservation of labile tumor markers, including handling of tumor tissue for the processing of labile hormone receptors, half-life, circadian rhythm, distribution in cellular fractions, the effect of exercise on markers such as prostate-specific antigen, amplification by molecular methods, and sample quality for cytogenetics and flow cytometry assessment of DNA histograms are just some of the many issues that come under the preanalytic umbrella.

As new markers are proposed, preanalytic problems associated with their measurements also surface. For example, homocysteine has received considerable attention as a marker of cardiovascular risk. Yet the measurement of this amino acid presents challenges since it continues to be formed by cellular enzymes in vitro, thus making the prompt separation of cells from plasma or serum mandatory. Alternatively, measures should be taken to inhibit homocysteine generation and converting enzymes immediately after blood specimen collection.

With advances in coagulation and fibrinolysis, including increasing use of molecular methods to study mutations that predispose a person to hypercoagulability, preanalytic variables affecting these measurements need to be delineated and controlled. Some physiologic variables that affect the measurement of fibrinolytic activators and inhibitors, including the extent of diurnal variation during a 24-hour period, the effects of smoking and alcohol need to be recognized. Challenges in measurement of free t-PA in plasma by sample treatment to dissociate the t-PA-plasminogen activator inhibitor I complex should not be underestimated.

Finally, preanalytic problems that are unique to a method should be kept in perspective as we begin to better understand the complexities of the effect of new technology and therapy, inasmuch as they affect the preanalytic phase.

From the Department of Pathology, New York Medical College–Metropolitan Hospital Center, New York, NY.

Address reprint requests to Dr Narayanan: Dept of Pathology, New York Medical College–Metropolitan Hospital Center, 1901, First Ave, New York, NY 10029.

*Dr Narayanan is a Becton Dickinson Fellow at Becton Dickinson, Franklin Lakes, NJ.

Acknowledgments: I thank Carol Perello for secretarial support and preparation of the tables.

References


37. Freer DE, Statland BE. The effect of ethanol (0.5 g/kg body weight) on the activities of selected enzymes in sera of healthy young adults, I: intermediate term effects. Clin Chem. 1977;23:830-834.


