Section 7

CLINICAL BIOCHEMISTRY

Chapter

29

Biochemical Processes of the Body Under Normal and Pathogenic Conditions

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Chapter Outline

- Normal and Abnormal Biochemical Processes of the Body
- Basic Physiology and Biochemistry of the Body
- Interrelated Metabolic Processes of the Body
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NORMAL AND ABNORMAL BIOCHEMICAL PROCESSES OF THE BODY

The clinical biochemistry laboratory analyses the chemical constituents of various body fluids, notably serum (or plasma), urine and spinal fluid. Most of these analyses reflect the biochemical malfunction of various key organs of the body such as the liver, heart, kidney, brain and pancreas and the endocrine system. Thus it becomes imperative for any beginner to understand the basic biochemical setup of the body and the role of various organs to maintain homeostasis (chemical balance) in the body. This understanding helps in the diagnosis of a diseased state on the basis of specific biochemical changes.

These specific biochemical changes are called *biomarkers,* and they are often used as an efficient and standard means to access a certain adverse condition and compare it to other similar cases. For example, high levels of Troponin I, a muscle protein, in blood is strongly indicative of a myocardial infarction (MI), also known as heart attack. From a clinical perspective, the use of these biomarkers can allow for a faster diagnosis. Instead of running expensive, and sometimes invasive, diagnostic procedures, physicians can instead look for specific triggers that preclude a certain condition.

The practice of medicine popular in media today tends to fixate on acute cases on a specific organ/tissue; however, most people suffer from more chronic conditions. These chronic malfunctions are more difficult to identify and comprehend. However, an understanding of normal and abnormal biochemical processes of the body allows a diagnostician and physician to provide more holistic care and better medicine as a result.

BASIC PHYSIOLOGY AND BIOCHEMISTRY OF THE BODY

The human body is an incredible machine that utilizes 'fuel' to run a broad range of activities, from movement of extremities to reproduction. It delivers this function through the coordination of biochemical processes. The food that the body consumes (carbohydrates, fats and proteins) acts as the source that supplies energy and in addition, participates in building the structure of the body (anabolism).

The materials that the body consumes are classified in chemical terms as **organic** and **inorganic.** Inorganic compounds can be salts, water, acids, and bases. These compounds (e.g., sodium chloride, NaCl or common salt) do not contain carbon and do not originate from living matter. Organic compounds, however, are related to living material and have carbon atoms as their essential constituent. The intake of organic food, water, inorganic salts and vitamins leads to the synthesis of a variety of organic substances inside the body that play a vital role in its sustenance. This process of creating supportive compounds within the body is called *anabolism,* and is contrasted with *catabolism* of carbohydrates and other fuel sources. Organic compounds are used to build body structure, supply and store energy (e.g., glycogen, fats, etc.), regulate biochemical processes at the cellular level (e.g., enzymes), and regulate interrelated activities of various organs (e.g., hormones). The **inorganic salts** control many physical processes of the body such as osmotic pressure. They typically enter into structures of various organic compounds and are closely related to different physiological functions of the body (Figure 29.1). **Water** metabolism and **pH** (a measurement of acidity and alkalinity) also play a significant role in numerous biochemical processes. These will be discussed in subsequent sections.

The basic physiological functions of the body are carried out by a few organs (Figure 29.2). These can be broadly divided into the digestive system, circulatory system, respiratory system, excretory system and reproductive system. The **digestive system** breaks down complex organic molecules into simpler molecules, which are then absorbed into the blood. The **excretory system** helps to eliminate the materials that the body cannot absorb. The heart is a mechanical pump that maintains the constant movement of blood throughout the body. The lungs are utilized for gas exchange, whereby carbon dioxide is released as oxygen is inhaled. The kidneys and liver helps in filtering the blood. Unwanted metabolites and toxins are rejected and the essential ones are taken back into circulation. The pancreas is used to maintain blood sugar levels via the secretion of insulin. Figure 29.2 identifies the location of these organs in the human body.

The endocrine glands modulate many physiological processes over the long-term by secreting chemical messengers called **hormones.** These chemicals can be tissue-specific and typically act to alter systemic functions. **Enzymes,** on the other hand, regulate intracellular biochemical activities. The characteristic functions of various vital organs of the body (liver, heart, kidney, pancreas, etc.) are closely related to their enzymatic composition. Enzymes catalyse a specific chemical reaction by reducing the activation energy required to instigate the reaction. For example, carbonic anhydrase found in red blood cells catalyses the rapid conversion of carbon dioxide and water to bicarbonate. It is one of the fastest enzymes in the body and its function is critical to the transport of carbon dioxide from peripheral tissue to the lungs. Intracellular enzymes can also be secreted into the blood stream in cases of trauma or distress. The search for elevated levels of specific enzymes in blood sera forms the basis of **diagnostic enzymology.**

The chemical structures of some of the basic organic compounds connected with the physiology of the body are shown in Figure 29.2. These and many other organic compounds enter into the structure of the body and govern its complex physiological functions.

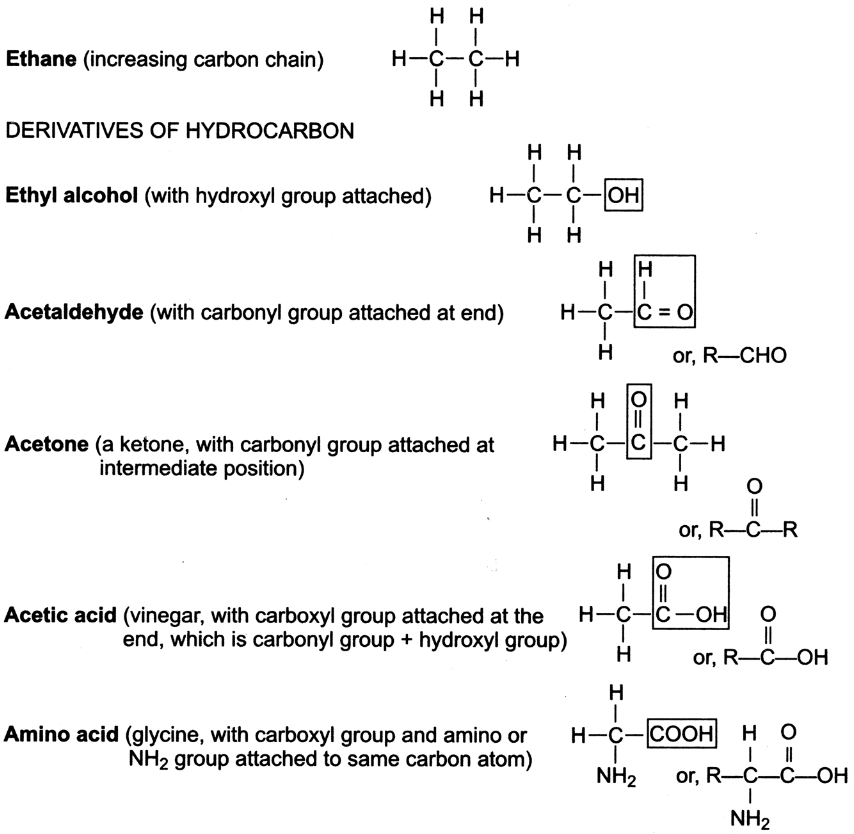

FIGURE 29.1 *Some important chemical structures that form the basis of various organic compounds of the body (R, alkyl group with varying numbers of carbon atoms).*

Carbohydrates are a class of organic compounds containing the elements carbon (C), hydrogen (H) and oxygen (O). They form the principal source of energy for the body. Sugars are carbohydrates, which are classified as **monosaccharides** or simple sugars, such as glucose, fructose and galactose, **disaccharides,** such as maltose and sucrose, and **polysaccharides,** such as starch and glycogen, which are complex carbohydrates formed by the union of monosaccharides. Hydrolysis is a chemical reaction by which complex organic compounds such as starch are broken down to simpler components (Figure 29.3). As the name implies, water molecules are utilized in the cleavage process. Polysaccharides are hydrolysed to sugars, proteins to amino acids, and fats to fatty acids and glycerol. Digestion of food in the digestive tract is largely a process of hydrolysis accomplished by various digestive enzymes that break down carbohydrates, fats and proteins.

Proteins are organic nitrogenous compounds of the body made of amino acids (principally), which are linked by

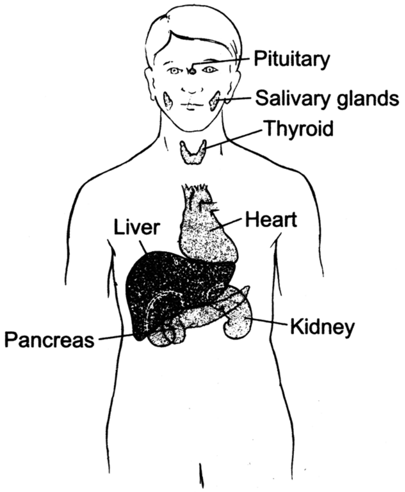

FIGURE 29.2 *Location of some important organs of the body*

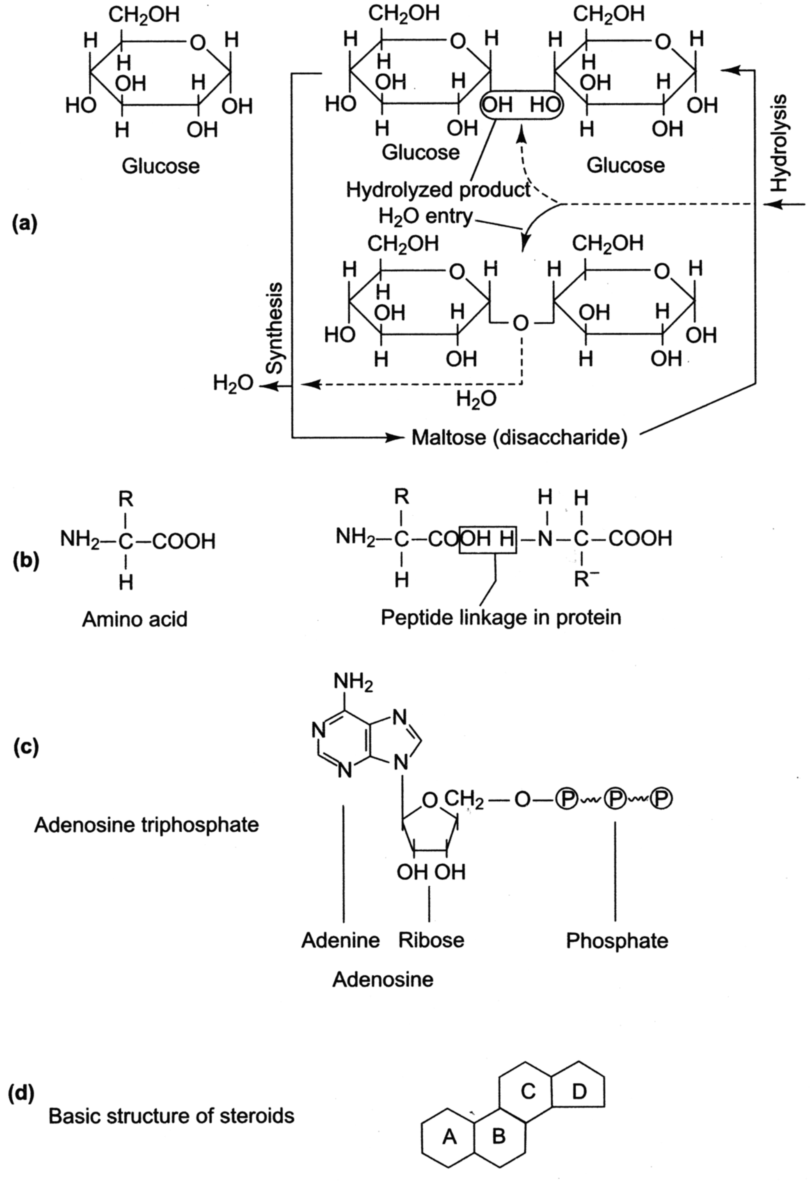

Figure 29.3 *Chemical structures of some of the important biochemical constituents of the body: (a) Carbohydrate, (b) Protein, (c) Nucleotide, and (d) Steroids.*

peptide bonds (Figure 29.3). The elemental composition of proteins includes carbon, hydrogen, oxygen and nitrogen, and occasionally sulphur, phosphorous, iron and other metals. Proteins form the basic structural component of the body. They may act as a secondary source of energy from the oxidation of their hydrolysed product, the amino acids. Serum proteins include albumin and four components of globulin—alpha$_1$, alpha$_2$, beta and gamma. Proteins are classified as simple or conjugated. Upon hydrolysis, the simple proteins (for example, albumin and globulin) are reduced to amino acids. Conjugated proteins, on the other hand, produce other organic compounds along with amino acids. Some examples of conjugated proteins are haemoglobin, nucleoprotein, phosphor-protein and lipoprotein.

Amino acids, the building blocks of protein, are formed by the amination of carboxylic acids. The basic formula for the amino acids is NH_2–CH(R)–COOH where R stands for various organic compounds, either **aliphatic** or **aromatic** (Figure 29.3). When amino acids are used as a source of energy by the body, the amino group (NH_2) 's first removed through the process of **deamination**. The ammonia generated by the deamination process is converted to urea ($CO[NH_2]_2$) in the liver through the ornithine cycle (Figure 29.4). The remaining organic acid then enters into the Krebs cycle and is oxidized (Figure 29.9). There are about 22 amino acids generally found in the living body, of which 10 are essential. Numerous combinations in the amino acid sequence lead to the existence of an unlimited number of plant and animal proteins. Amino acids can well be considered the alphabet that spells life.

Non-protein nitrogenous compounds are also important constituents of the body. These include urea, uric acid, creatinine, bilirubin, urobilinogen and nucleic acids.

Urea is synthesized in the liver by combining ammonia with carbon dioxide (Figure 29.4). It is eliminated through urine, which protects the body from ammonia toxicity. Uric acid is the product of purine metabolism and is a result of nucleoprotein breakdown. Uric acid cannot be metabolized by the body and is eliminated through the urine. Accumulation of uric acid in the blood results in renal failure.

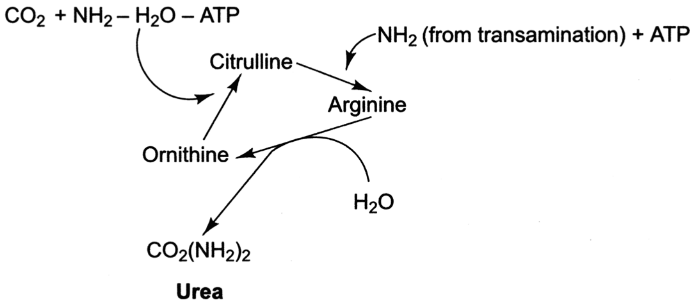

FIGURE 29.4 *Process of urea synthesis (ornithine cycle)*

Dehydration of creatine generates **Creatinine.** Creatine is associated with the anaerobic phase of muscle contraction and energy transfer. Creatinine is maintained at a constant level in the body and excess is eliminated through the urine.

Bilirubin is a pigmented non-protein nitrogenous compound of the body that originates from the breakdown of haemoglobin (Figure 29.5). Haemoglobin consists of a complex organic compound haeme (iron + protoporphyrin, a tetrapyrrole) and globin (a protein). The degradation of haemoglobin in the reticuloendothelial cells of the spleen produces free, or unconjugated, bilirubin (Figure 29.5). Unconjugated bilirubin is insoluble in water, and thus cannot be transported in the blood without first binding the serum protein albumin. Bilirubin-albumin complexes are transported to the liver, where they are conjugated with glucoronic acid. The conjugated bilirubin is now water-soluble, and is excreted from the liver into the duodenum via bile. In the intestine, bacterial enzymes convert bilirubin into urobilinogen. Most of the urobilinogen is eliminated through faeces, and a portion of it is reabsorbed from the intestine into blood circulation. The reabsorbed urobilinogen is excreted through urine or re-excreted in bile along with the conjugated bilirubin.

Nucleic acids are another group of complex organic nitrogenous compounds. They are primarily found in the cytoplasm and nucleus and form the physical manifestation of genetic information. The most important nucleic acids are **ribonucleic acid (RNA)** and

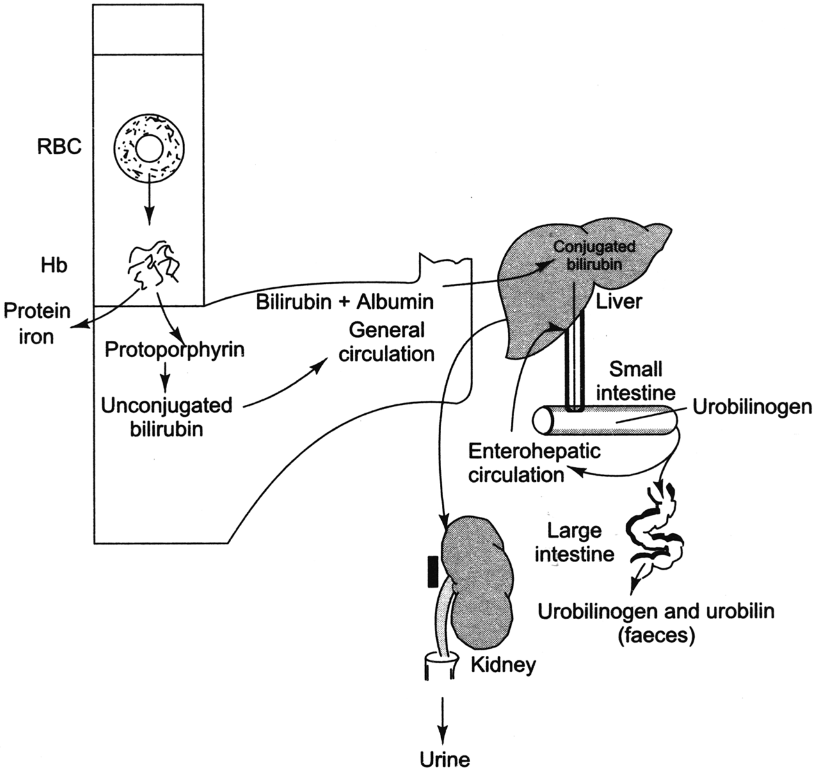

FIGURE 29.5 *Haemoglobin degradation and bilirubin metabolism*

deoxyribonucleic acid (DNA). In higher animals, DNA is the carrier of **genetic information** from the parent to offspring. The nitrogenous bases found in various nucleic acids include **purines** and **pyrimidines.** Pyrimidines are aromatic heterocyclic, while purines are a pyrimidine ring fused to an imidazole ring. Adenine and guanine are purines, whereas uracil, cytosine and thymine are pyrimidines. Nucleotides are composed of phosphoric acid, a sugar and a nitrogenous base (purine or pyrimidine). These come together to form nucleic acids. Adenosine is the most important nucleotide and is composed of an adenine base and a ribose—a pentose sugar molecule. Adenosine diphosphate (ADP) and adenosine triphosphate (ATP) are examples of nucleotides involved in energy transfer throughout the body (Figure 29.6). The formation, of ATP is mediated by the breakdown of various organic compounds. Bond energy within carbon-carbon bonds are utilized to bind phosphates to adenosine, thus creating ATP. ATP is utilized as mobile energy units and is actively transported throughout the body based on energy demand. The release of energy when the 3′ phosphate is cleaved from ATP is significant and it is used to drive millions of reactions every second. The resultant product ADP, is then recycled into ATP by binding an extra inorganic phosphate via catabolism.

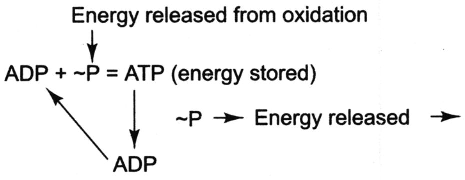

FIGURE 29.6 *Process of energy transfer in the body*

Lipids make up the fourth most important group of organic compounds after carbohydrates, proteins and non-protein nitrogenous compounds. They are insoluble in water but soluble in organic solvents such as chloroform, ether and alcohol. In chemical terms, lipids are esters of fatty acids with the basic elemental composition of C, H and O. Although the same elements form the basic structure of carbohydrates as well, lipids are in a more reduced state. Simply put, they have more hydrogen and thus can store more energy (calories) within a smaller unit area. Complex lipids may include phosphorus and nitrogen to form phospholipids and lipoproteins. Esters are formed by the chemical reaction of fatty acids with an alcohol (contains OH group). This is similar to the formation of salt by the reaction of an acid with a base or alkali (this also contains an OH group).

$$\text{HC1} + \text{NaOH} = \text{NaCl} + H_2O$$

$$\text{Fatty acid} + \text{Glycerol} = \text{Ester} + H_2O$$

Triglyceride is a simple lipid that is formed by the union of glycerol and three fatty acids—stearic, oleic and palmitic (Figure 29.7). Complex lipids are also esters but they include other chemical groups (phosphate to form phospholipids, protein to form lipoproteins and glucose to form glycolipids). In sphingolipids, glycerol is replaced by an amino alcohol called **sphingosine.** Vitamins A, D, E and K are fat soluble and bilirubin of bile, waxes, carotene and fatty acids are soluble in organic solvents; hence, they are also classified as lipids. Most dietary **fats** are first digested by the fat-digestive enzyme lipase, which hydrolyses the fat into fatty acids and glycerol. These enzyme products are water-soluble and absorbed directly into the body from the digestive tract, whereas fatty acids, which are not soluble in water, are absorbed by the body from micelles (colloidal suspension) produced in the digestive tract with the help of bile salts (sodium glycocholate and sodium taurocholate). **Glycerol,** other than from the dietary origin, is also a metabolic product of glucose breakdown. Thus the body can re-synthesize its own fat from the basic ingredients—fatty acids and glycerol. The liver plays an important role in the synthesis of body fats. Fat is transported in the body by the lipoproteins, which are synthesized in the liver and small intestine. In the body, the lipids can serve as structural and functional elements (e.g., plasma membrane), as precursors of many essential substances as a secondary energy source and as an insulator.

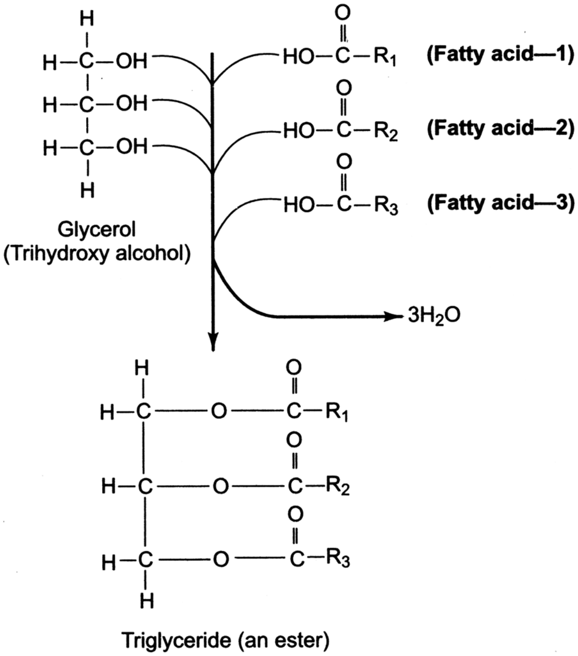

Figure 29.7 *Formation of triglyceride from glycerol and fatty acids through dehydration synthesis (opposite of hydrolysis)*

Sterols have fat like properties (insoluble in water but soluble in organic solvents). Steroids are chemically related to sterols (e.g., steroid hormones, vitamin D and bile acids). Steroids have a characteristic chemical structure (Figure 29.8) composed of four rings. Cholesterol is one of the important sterols of the body and is a precursor to a number of steroid hormones. Body cholesterol is found as free cholesterol or esterified cholesterol. Esterification of cholesterol occurs in plasma with the aid of lecithin-cholesterol acyltransferase (LCAT), an enzyme produced in the liver. Cholesterol is present in all lipoproteins and two-thirds of the plasma total cholesterol is esterified with long-chain fatty acids. Linoleic acid, specifically, is the predominant fatty acid in humans. The cholesterol esters in the plasma are in a state of constant turnover because of their continual hydrolysis and re-synthesis. It is suggested that esterified cholesterol is easy to transport through the plasma, from the tissues to the liver—the seat of body-cholesterol synthesis.

Ketone bodies include three organic compounds—acetone, acetoacetic acid and beta-hydroxybutyric acid. These are products of fat degradation through incomplete oxidation. Interestingly, these compounds are the only products of fat that can pass the blood-brain barrier and provide energy to the cerebrum.

INTERRELATED METABOLIC PROCESSES OF THE BODY

The body performs a number of anabolic and catabolic processes that are closely interconnected. Anabolism, the synthesis of complex organic compounds from simpler compounds, requires energy. Important anabolic processes include the storage of glucose as glycogen, creation of proteins from amino acids, creation of complex fats from fatty acids and glycerol, and the production of urea from ammonia and carbon dioxide.

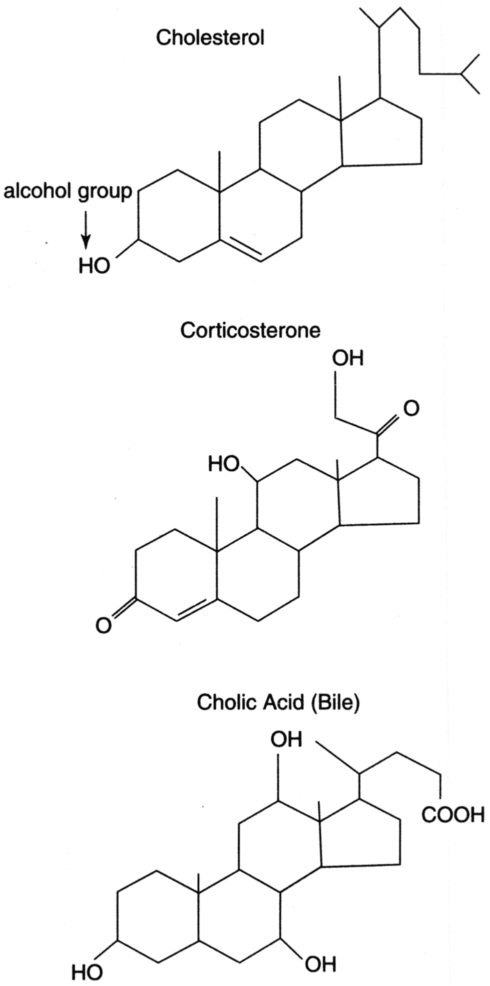

FIGURE 29.8 *Chemical structures of some of the important organic compounds in the body. The shared steroid structure (four shades rings) is seen in cholesterol, corticosterone and cholic acid (bile).*

Catabolism is the opposite of anabolism and results in the release of energy. The degradation processes yield simpler products from complex organic compounds. Digestion and oxidation are the two most common degradation processes. Often times, products of digestion are oxidized to water and carbon dioxide. Energy released by the breakdown of chemical bonds is available for the growth and development of the body. Mitochondria, which are specific organelles present in cells, leverage the energy generated by catabolism to generate ATP.

The interrelated metabolic processes, involving carbohydrates, fats and proteins, are shown in Figure 29.9. Dietary glucose is stored in the liver as polysaccharide glycogen (animal starch). When energy is required, glycogen is first converted to glucose, which is then anaerobically

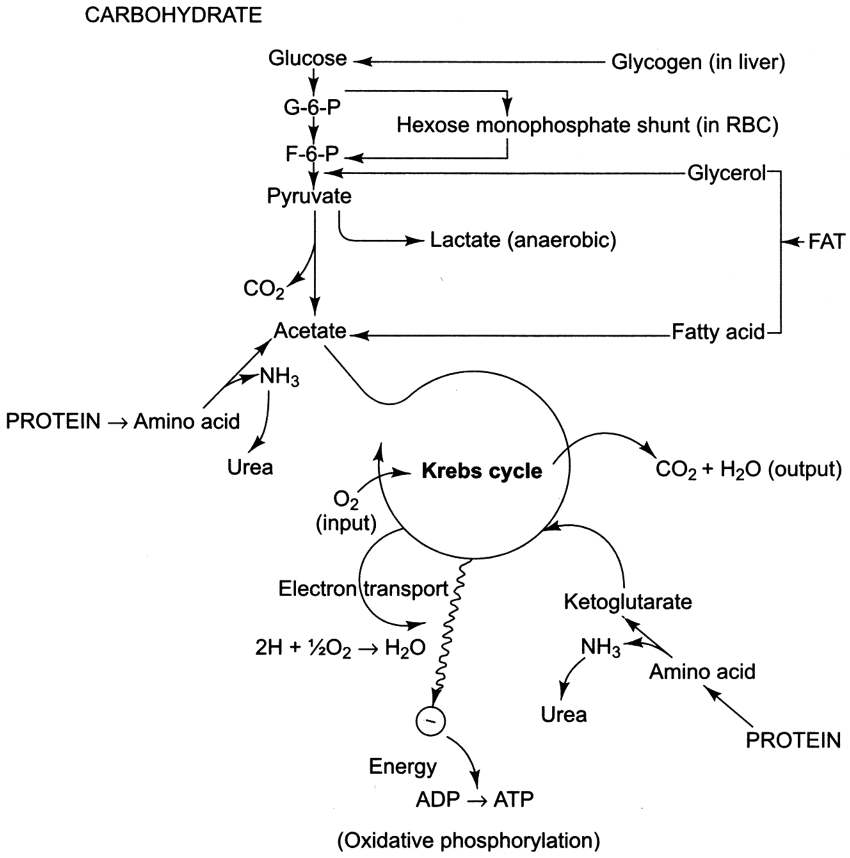

FIGURE 29.9 *Interconnected biochemical processes of the body leading to aerobic respiration*

degraded to pyruvic acid (a three-carbon compound) via a process called glycolysis. Pyruvic acid is the end-product of glycolysis that may either lead to the formation of lactic acid under anaerobic conditions, or to the formation of acetate (a 2-C compound, acetylcoenzyme A, acetyl-CoA) prior to entry into the Krebs cycle. Acetate also acts as a 'junction box' for the metabolic products of carbohydrates, fats and proteins in order to supply energy for the growth and development of the body. Simply put, the degradation of carbohydrates, fats, and proteins, all lead to the formation of acetyl-coA, which in turn enters the Krebs cycle and becomes fully oxidized.

FUNCTIONS OF VARIOUS ORGANS

The function of the human body is regulated by its various organs. These organs do not function independently, and coordinated by a network of chemical communication. In the following sections we will elaborate on some of the most important organs of the body, their normal functions, and signs of abnormal functions.

Liver

The liver plays a vital role in the metabolic processes of the body. These processes can be broadly classified as synthesis, storage and excretion. The liver **synthesizes** a variety of organic compounds—albumin, fibrinogen, urea, uric acid, prothrombin, lipoprotein, transferrin, glycoprotein, hippuric acid, cholesterol and other lipids. Glucose is **stored** in the liver as glycogen and returned to blood circulation when body glucose levels decrease. Besides glycogen, the liver stores fat-soluble vitamins (A, D, E and K) and vitamin B. The **excretory** and detoxifying functions of the liver are evident in conversation of unconjugated bilirubin to conjugated bilirubin. In the degradation of haemoglobin, the end-product (unconjugated bilirubin) is delivered to the liver via binding to albumin. In the liver, bilirubin is conjugated and later excreted as bile pigment into the intestine. Many other water insoluble, toxic substances are similarly removed from blood circulation through the bile duct, as they cannot be properly excreted through the kidneys.

Jaundice, hepatitis, cirrhosis (degeneration), fatty liver, infection (abscess) and amyloids are some of the many pathologic conditions of the liver. Diagnostic test results for the assessment of liver function include increased serum transaminase activity, decreased blood urea nitrogen (BUN), decreased serum albumin, increased prothrombin time (PT) in coagulation, increased ammonia concentration in the blood, decreased levels of conjugated bilirubin, and esterified cholesterol in serum (Table 29.1).

Table 29.1 *Routine clinical chemistry profiles*

Ordering physician:	**Name of patient:**			
Date:	**Date of birth:**			
	Outpatient		**Ward (Location) Nurse**	
General Profile	Glucose	BUN	Bilirubin	Date of specimen collection:
	Protein	Albumin	Calcium	Time of specimen collection:
	Phosphorus	Uric acid	Alkaline Phos.	
	AST	LDH	Cholesterol	Technician collecting specimen:
	A/G ratio	Globulin	T4	Date of reporting result:
Kidney (renal) Profile	Sodium	Potassium	CO_2	
	Chloride	Glucose	BUN	Time of reporting result:
	Creatinine			Technician reporting:
Liver (hepatic) Profile	Bilirubin total	Bilirubin direct	Protein	Other comments:
	Albumin	Alk. Phos.	AST	
	Serum LDH	A/G ratio	Globulin	
	ALT	Gamma GT	Liver LDH	
Cardiac Profile	AST	LDH	CK (CPK)	
	CK isoenzymes: Only done if CK is elevated			
Lipid Profile	Cholesterol	Triglyceride	HDL	
Thyroid Profile	T3	T4	TSH	Laboratory supervisor:
Iron Profile	Total iron Total iron binding capacity Unsaturated iron binding capacity			

A common test for liver disorders involves the injection of fructose and galactose into the blood stream. Normally, these sugars are converted to glucose by the liver when present in serum. If these sugar levels are maintained over time, this is indicative of a liver disorder. The detoxification action of the liver is assessed by administering bromosulphophthalein (BSP), 95% of which is removed from the blood within 15 min in normal liver function.

Kidney

The primary function of the **kidney** is to filter blood, however, it also reabsorbs essential substances from the filtrate. Kidney function is also closely related to the homeostasis of the body through the maintenance of the water-electrolyte balance and production of certain hormones. The chemistry panel in a kidney function test is provided in Table 29.1. This includes glucose, BUN, creatinine, uric acid and electrolytes. Renal failure is diagnosed by the increased level of serum creatinine, and urea. The glomerular filtration rate (GMR) for creatinine clearance is a reliable index to assess kidney diseases.

Heart

The heart is another important organ of the body and is the centre of the circulatory system. Heart diseases are caused by a number of agents. The clinical laboratory assesses heart condition through enzyme assays of creatine kinase (CK), AST and lactic dehydrogenase isoenzymes (Table 29.1).

Pancreas

The pancreas externally secretes digestive enzymes (e.g., amylase, lipase) into the digestive system. It also internally secretes insulin, an endocrine hormone, from the islets of Langerhans, which are tiny isolated masses of ductless glands. Pancreatic pathology is diagnosed in the laboratory by the assay of digestive enzymes in serum and in urine.

Endocrine glands

Endocrine glands are ductless glands that produce internal secretion of hormones. The brain, hypothalamus and pituitary act in concert to control the body functions through the mediation of several target organs. Hormonal regulation is typically used to modulate autonomic function that takes place over long periods of time (such as breathing and heart rate).

Hormones are either steroids or proteins. They regulate the interdependent metabolic processes of the body and its overall development. The latter involves the reproductive system, formation of a personality, ability to combat stress, as well as pathogen resistance. Insulin plays an important role in the carbohydrate metabolism and its deficiency leads to hyperglycaemia (increased glucose concentration of blood) and glucosuria (glucose excretion in urine). Thyroid hormones—triiodothyronine or T_3 and thyroxine or T_4—are also intimately connected with carbohydrate metabolism of the body and regulate several other metabolic processes.

There are two components of the **pituitary gland**—anterior and posterior. The **posterior** pituitary secretes only one hormone: vasopressin or anti-diuretic hormone (ADH). ADH controls water absorption from the intestines and the kidneys. The **anterior pituitary,** on the other hand, releases a number of hormones that trigger several target organs. The target organs respond by releasing appropriate hormones to meet the physiological needs of the body. Abnormal hormone levels may cause a number of pathophysiological problems which will be discussed in detail in the subsequent pages.

The following are some examples of anterior pituitary-mediated hormones which are of clinical significance.

1. **Thyroid-stimulating hormone (TSH):** Controls the release of thyroid hormones T_3 and T_4.
2. **Adrenocorticotropic hormone (ACTH):** Stimulates the adrenal gland cortex, which secretes corticosteroids (e.g., aldosterone, 17-hydroxycorticosteroids and 17-ketosteroids) and androgens.

3. **Gonadotropins:** Stimulates the release of testosterone and 17-ketosteroids in the testes (oestrogen and progesterone in the ovaries).

The following are the examples of **pituitary-independent hormones:**

- **Adrenal medullary hormones:** Epinephrine and norepinephrine (catecholamines); hypersecretion of catecholamines is seen in pheochromocytoma, a vascular tumour of chromaffin tissue of the adrenal medulla.
- **Insulin** (pancreatic secretion)
- **Parathormone** (parathyroid gland secretion)
- **Serotonin (5-hydroxytryptamine):** 5-hydroxyindoleacetic acid (5-HIAA) is produced during serotonin metabolism. Increased discharge of 5-HIAA in urine is diagnostic of certain intestinal tumours.
- **Human chorionic gonadotrophin or HCG:** Released from the placenta in pregnant women.

The chemistry panel for the thyroid function includes T_3, T_4 and TSH (Table 29.1).

Lungs

The lungs govern gas exchange in the body. Within alveoli, oxygen is provided to blood within pulmonary capillaries and carbon dioxide is removed. Haemoglobin acts as a carrier of these gases (CO_2 and O_2) between the lungs and the cells. The pathologic states of respiratory acidosis and alkalosis originate from fluctuations of the partial pressure of carbon dioxide in blood. Metabolic acidosis and alkalosis, on the other hand, are governed by serum bicarbonate level which is regulated by the kidney. Metabolic disorders like ketosis may also lead to metabolic acidosis.

Brain

The brain is the primary centre for regulating and coordinating various neural activities of the body. Pathologic states of the brain (infection, tumour, haemorrhage) can be assessed from the laboratory study of cerebral spinal fluid (CSF). CSF surrounds the brain and spinal cord; physical and chemical changes of the spinal fluid signal meningeal dysfunction.

BIOCHEMICAL CHANGES IN THE BODY UNDER PATHOLOGIC CONDITIONS

A delicate biochemical balance (homeostasis) is maintained by the body under normal conditions. When this is disturbed, a pathologic state is suspected. As the chemical composition of the body is greatly dependent on the functions of various organs and endocrine glands, a study of the chemical changes can be helpful in speculating on the source of trouble.

Here, we will consider the **abnormal carbohydrate metabolism** of a patient with **diabetes mellitus,** an insulin deficiency, in order to illustrate the interrelated nature of biochemical processes of the body (Figure 29.10).

Note *Diabetes insipidus* is caused by ADH deficiency. Diabetes that develops over time is referred to as type II diabetes or *diabetes mellitus.*

Four main abnormalities in diabetic patients are listed as follows:

1. Glucose is not readily removed from the circulation for storage in the liver as glycogen.
2. The Krebs cycle does not function efficiently and thus glucose not adequately utilized in generating energy.
3. Decreased availability of glucose, thus leading to a decreased ability match energy demand. This results in anaerobic degradation of fats, as the Krebs cycle is inhibited by lack of insulin.

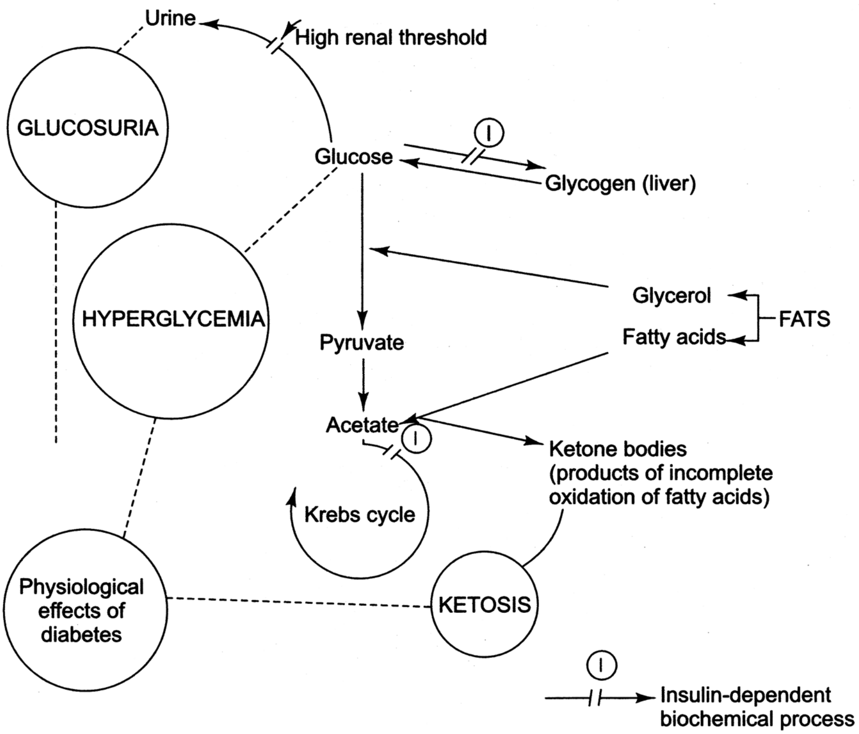

FIGURE 29.10 *Metabolic disorder in diabetes mellitus*

4. As a result, formation of ketone bodies (ketosis), metabolic acidosis and increased anion gap accompany untreated diabetes.

The abnormal chemical composition of blood sera also reflects organ or systemic dysfunction. Biochemical test profiles for various disorders of organs are presented in Chapter 34 of this volume. Decreased protein concentration (particularly albumin), decreased PT, increased bilirubin concentration and increased transaminase activity are some of many indications of liver disorder. On the other hand, increased protein levels could be a sign of infection and/ or lymphoproliferative disorders. The increase in protein in the blood is inferred to result from the increased synthesis and secretion of antibodies (globulin) from lymphocytes and plasma cells. Similarly, elevated urea and creatinine in blood sera is indicative of kidney abnormalities. The kidney typically clears waste materials, inorganic ions, and other by-products of degradation from the blood; however, these substances will build up in the blood during kidney dysfunction. Other laboratory information, such as the presence of casts in urine (urine analysis) and decreased GFR (glomerular filtration rate) will support the diagnosis of renal failure.

In recent years, accurate analysis of **serum enzymes** has greatly assisted in pinpointing organs with abnormal function. Most organs of the body have the predominance of some specific enzymes in their cells in order to carry out their biochemical functions. For example, the liver contains a high concentration of transaminases to carry out transamination. If the organ is in distress, its cellular enzymes are released into the blood stream. Thus, the elevation of specific enzyme levels in blood may indicate a pathologic organ. Another example of this is the increase in amylase and lipase activities in serum subsequent to pancreatic disorder. These will be further discussed in the following sections.

BASIC CLINICAL BIOCHEMISTRY

Chemistry Profiles

Blood chemistry tests are classified as 'routine' or 'special'. **Routine tests** are those that frequently ordered. Chemistry profiles, or metabolic profiles, are a group of tests performed simultaneously on a patient specimen to provide a general assessment of the patient's condition. The physician can use the results of the chemistry profile, in conjunction with a physical examination and patient history to assess the overall health of the patient (Table 29.1). Many automated chemistry analysers are capable of performing various chemistry profiles in a group.

Tests that are ordered less frequently, such as those that access hormone or certain drug levels, may be performed occasionally and are sometimes referred to as 'special' tests. These are typically carried out in special laboratories.

Types of Specimens for Chemical Analyses

Specimens arriving at the desk of a clinical laboratory commonly include blood, urine, etc., however, samples can also include CSF and synovial, pleural, or pericardial fluid. Specimens other than blood and urine are collected by the physician. Some collections of patient samples require prior preparations on the patient's side, such as samples for glucose. When measuring fasting sugar levels, a patient should not have consumed food for a number of hours. The technician collecting the specimen must also be aware of the specific contingencies involved in various tests so as to ensure accurate collection and processing of the sample itself. Many specimens (e.g., urine) change their chemical composition over time (compounds within the sample degrade quickly), and such specimens should be handled quickly.

Units of Measure in Clinical Chemistry

Clinical chemistry test results are usually reported in metric units or SI units (Table 29.2). Commonly used units are milligrams (mg) or micrograms (µg) per decilitre (dL), millimoles per litre (mmol/L) or in the case of enzymes, enzyme activity units per litre (U/L). In developing countries, however, older units may still be in use and, hence, a conversion factor has been provided in Table 29.2 for reporting these results in modern units (SI).

Reference (Normal) Ranges

The reference (or normal) range of a substance is determined by measuring the level of the substance in a portion of the general population and applying statistical methods to the data. The reference range is not universal. Hence each hospital should maintain its own reference range. The process of establishing reference ranges has been discussed in Chapter 7 of Vol. 1. Table 29.2 gives the reference ranges of commonly tested analytes in a clinical laboratory. If a patient's results are beyond normal ranges, it must be highlighted or flagged in order to draw the attention of the physician. If the results are excessively high compared to the norm, the physician should be informed immediately.

DIAGNOSTIC BIOCHEMICAL PROFILES

Biochemical profiles are groups of tests performed in the biochemistry laboratories that are used to assess the general function of the body or for the diagnosis of specific organ disorders

Table 29.2 *Clinical chemistry reference values in conventional and SI units*

Test (Substances measured)	Conventional Unit	Conversion Factor	SI Unit
Alanine aminotransferase (ALT)	3–30 U/L	1	3–30 U/L
Albumin	3.8–5.0 g/dL	10	38–50 g/L
Alkaline phosphatase (AP)	20–130 U/L	1	20–130 U/L
Aspartate aminotransferase (AST)	10–37 U/L	1	10–37 U/L
Bicarbonate (HCO_3^-)	22–28 mEq/L	1	mmol/L
Bilirubin (Total)	0.1–1.2 mg/dL	17.1	2–21 µmol/L
Bilirubin (Direct)	0–0.3 mg/dL	17.1	0–6 µmol/L
BUN	8–18 mg/dL	0.357	2.9–6.4 mmol/L
Calcium	8.7–10.5 mg/dL	0.25	2.18–2.63 mmol/L
Chloride	98–108 mEq/L	1	98–108 mmol/L
Cholesterol (Total)	140–250 mg/dL	0.026	3.6–6.5 mmol/L
Creatinine	0.7–1.4 U/L	88.5	62–125 umol/L
Creatinine kinase	30–170 U/L	1	30–170U/L
Gamma glutamyltransferase (GGT)	3–40U/L	1	3–40 U/L
Glucose	70–110	0.05	3.9–6.2 mmol/L
Iron	65–165	0.18	11.6–29.5 µmol/L
Lactate dehydrogenase (LD)	110–230 U/L	1	110–230U/L
Phosphorus	3.0–4.5 mg/dL	0.32	0.96–1.44 mmol/L
Potassium	3.5–5.4 mEq/L	1	3.5–5.4 mmol/L
Sodium	135–148 mEq/L	1	135–148 mmol/L
Thyroid stimulating hormone (TSH)	0.35–5.0 µIU/mL	1	0.35–5.0 mIU/L
Total protein	6.0–8.0 g/dL	10	60–80 g/L
Triglycerides	10–190 mg/dL	0.011	0.11–2.15 mmol/L
Uric acid	3.5–7.5 mg/dL	0.06	0.21–0.44 mmol/L

(Table 29.2). Measurements of the protein level of serum and electrolyte concentrations are included in most routine chemistry profiles, and hence are discussed separately.

Protein

Two major groups of serum proteins are **albumins** (60%) and **globulins** (40%). Albumin is made in the liver while globulins, particularly immunoglobulin, are made by lymphocytes and plasma cells.

Total protein is commonly measured in sera, but it can also be measured in urine and the CSF, where the concentration is normally low. In the laboratory, total serum protein and albumin are directly measured by colorimetric procedures. The difference is used to calculate the concentration of globulin (total protein – albumin = globulin). The reference value of serum proteins is 6.0–8.0 g/dL (60–80 g/L). This represents the sum of a variety of different proteins. Decreased levels of **albumin** can occur in liver disease, starvation, impaired amino acid absorption, increased protein catabolism, or protein loss through the kidney or gastrointestinal tract. The ratio of albumin and globulin (A/G) has diagnostic significance and can be computed and reported.

Electrolytes

In clinical chemistry, the term 'electrolyte' refers to major **cations** [sodium (Na^+), potassium (K^+), etc.] and major anions [chloride (Cl^-), bicarbonate (HCO_3^-), etc.]. These four ions have a great effect on hydration and acid–base balance (pH) in the body. This directly correlates with proper heart and muscle function. Electrolyte measurement is included in most routine chemistry profiles and renal profiles.

Of the two cations present in serum, sodium is typically found in higher concentrations [135–148 mmol/L (mEq/L)] than potassium [3.5–5.4 mmol/L (mEq/L)]. In regards to anion concentrations, chloride is found in higher concentration [98–108 mmol/L (mEq/L)] and bicarbonate is found at a lower range [22–28 mmol/L (mEq/L)].

Kidney function panel

Proper kidney function is necessary in order to maintain water and electrolyte balance of the body (homeostasis, Table 29.1). Creatinine is excreted by the kidney. It is the waste product of creatine phosphate, a substance stored in muscle and used for energy. The reference range for serum **creatinine** is 0.7–1.4 mg/dL (62–125 μmol/L). When renal function is impaired, blood creatinine level rises. This rapid rise typically happens after more than 50% of kidney function has been lost. Creatinine level is not affected by diet or hormone levels. Kidney panel tests include serum concentrations of creatinine, BUN and uric acid (Table 29.2). All aforementioned concentrations increase when there is an impairment of urine formation or excretion. This typically occurs in renal disease, shock and water imbalance or ureter blockage.

In mammals, surplus amino acids are converted to **urea** and excreted by the kidneys. This surplus is measured as blood urea nitrogen, or **BUN.** The reference range of **BUN** is 8–18 mg/dL (2.9–6.4 mmol/L). BUN concentrations, unlike creatinine, are influenced by diet, hormones and kidney function. Therefore, BUN level is not as good of an indicator of kidney disease as creatinine levels. BUN levels can be low during starvation, pregnancy, or it could be a sign of low-protein diet. An increase in BUN can occur during a high-protein diet, after administration of steroids and in kidney disease.

Uric acid is formed from the breakdown of nucleic acids and is excreted by the kidneys. It has low solubility and tends to precipitate as uric acid crystals or urates. Uric acid measurements are principally used to diagnose and treat gout, a disease where uric acid precipitates in tissues and joints, causing pain. Uric acid levels can also increase after tremendous exposure radiation or chemotherapy due to increased cell destruction. Many laboratories do not include uric acid as a part of a kidney function test. The reference range of serum uric acid is 3.5–7.5 mg/dL (0.21–0.44 mmol/L).

Liver function panel

The liver is both a secretory and excretory organ and has numerous metabolic functions. The liver functions in carbohydrate metabolism (synthesizes glycogen from glucose). Most plasma proteins are made in the liver, including albumins, lipoproteins and transport proteins, and blood coagulation proteins such as fibrinogen. The liver is also important in lipid metabolism and is also a prominent source of cholesterol. It is also a storage site for glycogen, vitamins, and many other substances. Numerous tests are used to estimate liver function. Most clinical laboratories include bilirubin and a number of liver enzymes in their liver function panel. However, these tests are not considered specific for a particular disease. They only reflect liver tissue damage or liver dysfunction, which can be manifested in numerous different systemic pathologies. In addition, significant liver function must be lost or impaired before laboratory tests show abnormal results.

Most laboratories include total protein, albumin, globulin, A/G ratio, bilirubin (total and direct), and a number of liver enzymes in their **liver function test panel** (Table 29.1). Clinical significance of the levels of total **protein** and albumin levels in serum has been discussed earlier.

Bilirubin is a waste product from the breakdown of haemoglobin. It is conjugated in the liver and excreted in bile. In liver, most bilirubin is bound to glucuronide prior to secretion into the bile duct. This binding to glucuronide is referred to as 'conjugation', and it results in conjugated or **direct bilirubin.** Bilirubin that is not conjugated is called indirect bilirubin.

The terms **'direct'** and **'indirect'** come from the way bilirubin is determined in the laboratory. The bilirubin assay usually measures both **total bilirubin** and direct bilirubin while **indirect bilirubin** is calculated from their difference. The reference range of total serum bilirubin (Table 29.2), or bilirubin in short, is 0.1–1.2 mg/dL (2.0–21.0 μmol/L) and that of direct bilirubin is 0–0.3 mg/dL (0–6 μmol/L). Serum bilirubin is measured to assess liver function and gall bladder dysfunction. An increase in the bilirubin level may indicate excessive destruction of haemoglobin, such as in haemolytic anaemias, biliary obstruction (an increase in direct bilirubin due to impaired excretion by the liver), gall bladder disease, and hepatitis (impaired bilirubin processing).

Various metabolic activities of the liver are accomplished by its intracellular **enzymes.** When liver cells are damaged, these enzymes are released in circulation and can be recognized by a serum assay. Some enzymes, however, are widely distributed in many body tissues, whereas others are found in only a few tissues. Hence, the measurement of enzyme levels is not always specific for damage to a particular organ; they can be most helpful when used alongside other tests, clinical symptoms and patient history. The **diagnostic enzyme assays** for monitoring liver function include alkaline phosphatase (ALP), lactate dehydrogenase (LDH), gamma-glutamyl transferase (GGT) and alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Generally, only one enzyme needs to be measured, as the levels tend to mirror each other.

The reference range of ALP, also called AP, is 20–130 U/L (Table 29.2). Its sharp rise in the serum level may be related to liver tumours and lesions, and a moderate increase is often associated with hepatitis.

Serum levels of aminotransferases (ALT and AST) increase with damage to liver cells and decrease during recovery. ALT was formerly called serum glutamic-pyruvic transaminase (SGPT) or GPT, in short. ALT levels are typically low in cardiac tissue and high in liver tissue. This enzyme usually rises higher than AST in liver disease, with moderate increases (up to 10 times normal) in cirrhosis, infections, or tumours, and increases up to 100 times normal in viral or toxic hepatitis. The reference range of serum ALT is 3–30 U/L.

AST was formerly known as serum glutamic oxaloacetic transaminase (SGOT) or GOT. This enzyme is not confined to the liver, and is also present in many other tissues, particularly cardiac, muscle. It can be elevated after myocardial infarction, as well as in liver disease. The reference ranges of these enzymes are given in Table 29.2.

GGT is found in kidney, pancreas, liver and prostate tissue. GGT can be more helpful than AP in determining liver damage because GGT levels remain normal in bone diseases while AP may rise with bone disease as well. It is also more helpful than AST because it remains normal in muscle disorders. GGT measurements are a standard practice to monitor recovery from hepatitis. The reference range for serum GGT is 3–40 U/L.

LDH, also called LD, is widely distributed in tissues. Serum LDH increases with liver disease as well as in cases of myocardial infarction (heart attack). Haemolysis also causes a rise in serum LDH levels as this enzyme is also present in red cells. The reference range for serum LDH is 110–239 U/L.

Cardiac function panel

Although cardiac function test panels include AST (formerly called SGOT) and LDH, CK, also called creatine phosphokinase (CPK), is more helpful in the diagnosis of myocardial infarction (Table 29.1). Although CK is present in large amounts in muscle and the brain, and in small amounts in organs such as liver and kidneys, its pattern of rise and fall in case of heart attack is very typical. An increase in serum CK level (normal range, 30–170 U/L) may be related to skeletal muscle damage and brain injury. In case of heart attack, CK is released from damaged heart muscle and rises five to eight times the upper limit of normal levels. It, however, falls rapidly back to normal levels within 3–4 days. When the CK is high, its isoenzyme assay should be performed to confirm myocardial infarction.

Lipid profile

Lipids are closely associated to cardiovascular diseases (CVD) associated with abnormal blood pressure and blood vessel problems. Lipids are synthesized in the body from dietary fats. The most commonly measured lipids are cholesterol, triglycerides and high-density lipoprotein (HDL).

Cholesterol is present in all body tissues, and serum concentrations tend to increase with age. Elevated cholesterol levels can increase the risk of coronary artery disease. It is recommended that total serum cholesterol levels be maintained below 200 mg/dL (normal range, 140–250 mg/dL). Cholesterol fractions such as low-density lipoprotein (LDL), high-density lipoprotein (HDL), and very low density lipoprotein (VLDL) are also measured for various diagnostic reasons. Triglycerides are the main forms of neutral lipids.

Thyroid function profile

The thyroid gland synthesizes a number of hormones that stimulates metabolism. Tyrosine, an amino acid, and iodine are necessary ingredients for the synthesis of these hormones. Excessive secretion of thyroid hormone (hyperthyroidism), as it happens in Grave's disease, or insufficient secretion of thyroid hormone (hypothyroidism), as it happens in myxedema, are both pathological states that disturbs the normal function of the body. Two major thyroid hormones are thyroxine, also known as $T_{4,}$ and triiodothyronine or T_3. Thyroid profiles or endocrine panels will include measurement of free or total T_4, free or total T_3 along with TSH, thyroid stimulating hormone. TSH is an anterior pituitary hormone that regulates thyroid gland activity. The reference value of TSH is 0.35–5.0 µIU/mL (Table 29.2). Measurement of thyroid hormones is usually not included as part of a routine chemistry profile.

Mineral metabolism

Minerals are necessary for good health, the more important of which include sodium, potassium, calcium, phosphorus (phosphate) and iron. These mineral concentrations are frequently measured as part of the chemistry profile. **Sodium** is the chief cation (positively charged ion) of extracellular body fluids that stays outside the cell. **Potassium,** on the other hand, is the chief cation of intracellular fluid (within cell). **Calcium** and **phosphorus** are necessary for proper bone and tooth development. Calcium is also needed for coagulation. Most phosphorus in the body is found as inorganic phosphate and hence the terms, phosphorus and phosphate are used interchangeably in this text. Pure phosphorus *per se* is highly unstable.

The reference value of serum **calcium** is 8.7–10.5 mg/dL (2.18–2.63 mmol/L). Compared to other minerals, it is found at the highest concentration. The value of body calcium is tremendously large; however, approximately 99% of this is bound in calcium complexes within the bone and is not metabolically active. The calcium assay measures only the unbound, metabolically active ions of calcium. Calcium is required for blood coagulation and normal neuromuscular excitation. The calcium balance is influenced by vitamin D, and several hormones (calcitonin, parathyroid hormone, among many others). These hormones control dietary absorption of calcium, calcium excretion by the kidneys, and calcium movements in and out of bone. Increased blood calcium concentration (hypercalcaemia) may be related hormone disorders, bone malignancies, excessive vitamin D and acidosis. This may lead to kidney stone formation in some cases. Decreased level of calcium (hypocalcaemia) can be life-threatening and could be a sign of osteoporosis or other kinds of bone degradation.

Reference ranges for serum **phosphorus** is 3.0–4.5 mg/dL (0.96–1.44 mmol/L). Approximately 80% of the phosphorus is in the bone (hydroxyapatite) and the rest is mostly in high-energy compounds such as ATP. Phosphorus levels are influenced by calcium and certain hormones.

Iron is needed for haemoglobin synthesis and is an integral component of many enzymes. Normally, the serum iron is in the range of 65–165 ug/dL (11.6–29.5 μmol/L). Iron is absorbed from dietary sources and is highly conserved by the body. In blood, iron is transported by a serum protein, called **transferrin.** Iron deficiency leads to anaemia which indicates poor haemoglobin concentration. The deficiency can be due to insufficient iron in the diet, poor iron absorption, impaired release of stored iron, or increased iron loss due to bleeding. Serum iron levels can be elevated with haemolytic anaemia, increased iron intake, or blocked synthesis of iron-containing compounds, such as occurs in lead poisoning.

REVIEW QUESTIONS

1. Give the characteristic features of the following organic compounds found in the body and how they originate: carbohydrate, protein, lipids (fats), urea, bilirubin and creatinine.
2. Discuss the following metabolic cycles.
   (a) Krebs cycle, and (b) Ornithine cycle
3. What are purines and pyrimidines? How do they form in the body?
4. What are the various breakdown products of haemoglobin? How are they discarded by the body?
5. With the help of a diagram show the metabolic processes involved in the formation and elimination of bilirubin by the body.
6. What is a simple lipid? How is this different from compound lipid?
7. What are the functions of liver, brain, kidney, pancreas and heart?
8. Which are the two major types of serum proteins and what are their major functions?
9. What three enzymes are useful in diagnosing liver disease?
10. What is the significance of bilirubin assay? What is the difference between direct and indirect bilirubin? What is conjugated bilirubin?
11. What are endocrine glands? How do they control and coordinate the functions of the body?
12. What is insulin? What is its clinical significance?
13. Discuss the abnormal carbohydrate metabolism of diabetic patients.
14. What patient preparation is needed before drawing blood for lipid profile?
15. A doctor ordered the following chemistry tests for a patient: Creatine kinase (CK), AST and cholesterol fractions. Which of the following the doctor is trying to diagnose?
    (a) Heart disease (b) Renal disease (c) Liver disease (d) Thyroid disease

Chapter

30

Specimen Collection and Processing for Biochemical Analyses

Chhotelaal Pande and Anant Kumar Pande

Chapter Outline

- Specimens of Biochemistry and their Handling
- Types of Specimens
  - Blood
  - Urine
  - Stool
  - Cerebrospinal Fluid (CSF)
- Review Questions

SPECIMENS OF BIOCHEMISTRY AND THEIR HANDLING

Clinical biochemistry tests comprise over one-third of all hospital laboratory investigations. Clinical biochemistry is that branch of laboratory medicine in which chemical and biochemical methods are applied to diagnose a disease. The specimens submitted for biochemical analyses are mostly confined to studies on blood and urine. This is because of the relative ease in obtaining such specimens although analyses are made on other body fluids such as gastric aspirate and cerebrospinal fluid.

Biochemical data are as good as the specimen which is analysed. Collection, preservation, handling, storage, identification, recording and other steps are necessary for the accuracy of test procedure. In this chapter, our primary focus is on the handling of specimens submitted for biochemical analysis in order to provide reliable and meaningful information to the physician. The technician should also be aware of the conditions that make the specimens unsuitable for analysis. We will concentrate on three types of specimens—blood, urine and cerebrospinal fluid (CSF), which are submitted for routine biochemical analysis.

Blood and urine are collected by the technician or phlebotomist but other body fluids are collected by the physician. They are highly precious and hence marked as ‘STAT’ for immediate attention.

General comments about the collection, care and disposal of specimens after analysis, have been discussed earlier (Chapter 5 of Vol. 1). In this chapter, we will concentrate on the specific requirements of a biochemistry laboratory for the specimen whose analytes become the diagnostic tool.

TYPES OF SPECIMENS

As mentioned earlier, urine, serum and plasma are the three most common specimens submitted to a clinical biochemistry laboratory. Many other fluid specimens, including cerebrospinal fluid, synovial fluid taken from the joint of the body, pleural fluid that accumulates in the pleural cavity (space that surrounds the lungs), semen, amniotic fluid, and peritoneal fluid from the abdominal cavity, are analysed for quantitative biochemistry. Only blood, urine and stool are collected by medical technician, medical assistant or nurse. Other body fluids are collected by the physician or his/her assistant.

Blood

Blood is the most commonly received specimen in clinical biochemistry laboratory. Blood for biochemical analysis can be capillary, venous or sometimes arterial (for blood gas measurement). Special attention must be paid to the type of specimen required for each test and to the handling and processing of the blood specimen. Laboratory analyses can only produce useful results if they are performed on a specimen that has been properly collected and maintained in an appropriate environment until the test is complete.

With the advent of machines now used as **"point of care (POC)"**, many physician offices collect capillary blood and analyse at the spot. The results, however, may not be as accurate as in a clinical biochemistry laboratory. The advantage is the use of "whole blood" and the methods are sensitive enough to overcome the interference of liquid protein present in blood. But the number of tests done by POC equipment is limited. In larger clinical biochemistry laboratories, most clinical biochemistry testing is performed on the plasma or serum specimens separated from the sample after centrifugation. Serum is obtained from clotted blood and plasma comes from anticoagulated whole blood.

Patient preparation and timing of specimen collection

In certain tests, **patient preparation** is as important as the specimen in order to get reliable and meaningful results. Some body fluid constituents are affected by meals, medications or the time of the day. Each test procedure contains specimen collection and handling instruction that must be strictly followed. Most blood constituents, however, do not change significantly after eating, so blood used for these can be collected at any time. However, concentrations of constituents such as glucose, inorganic phosphate, triglycerides and cholesterol will change after eating, and specimens for these tests are usually collected when the patient is fasting, generally in the morning before breakfast. Water intake may not be restricted during this period.

Specimens collected from patients with lipid metabolism disorder (lipaemia) or shortly after a patient has eaten may appear **lipaemic**. Since lipaemic serum or plasma is milky or cloudy, it can interfere with certain tests, particularly those that use photometry. Hence, a fasting specimen is recommended. If the laboratory receives a lipaemic specimen, the lipid can be removed from the top, after centrifugation, with the help of a Pasteur pipette. Sometimes, placing the specimen in the refrigerator (at 4°C) overnight also helps.

In some diseases, certain blood constituents follow patterns of increase or decrease that make the collection time very important. For example, **Creatine kinase (CK)** which is also known as **Creatine phosphokinase (CPK)**, an enzyme measured to detect heart attacks (myocardial infarctions), rises rapidly after a **heart attack** and falls back to normal levels in the 3–4 days following the attack. If this enzyme is not measured during this critical period, a heart attack can go undiagnosed. Similarly, iron and corticosteroids show a tendency to change with the time of day (diurnal variation). Hence, it is important, therefore, to note the collection time of specimens for these tests and to consider this when interpreting test results.

During therapy, the drug level increases after the administration of the medicine. Hence for therapeutic drug monitoring, specimens must be drawn at set time intervals before or after administering medications.

Components of blood

Plasma

Plasma is the liquid portion of the blood in our body in which the blood cells are suspended. This liquid makes up more than 50% of the total blood volume. But blood clots immediately after it is taken out of the body. Hence an anticoagulant is added to the blood specimen to prevent clotting. The liquid portion of this anticoagulated blood is the **plasma**. When centrifuged, the cells settle at the bottom and the semi-clear liquid at the top of the cell-sediment is the plasma.

The plasma is made up of 90% water, in which there are many dissolved substances and gases. Following are **too many chemical compounds present in plasma** to list them all separately, but general categories include the following:

- ***Plasma proteins***: These are large molecules that are not excreted as waste products and are designed to remain in the blood. These include antibodies (immunoglobulins), as well as blood-clotting proteins (fibrinogen and prothrombin) and albumin.
- ***Electrolytes***: Electrolytes are compounds that bear electrical charge (+ or –) when dissolved in water and are capable of transmitting an electrical impulse within the body.
- ***Hormones***: Endocrine glands present throughout the body secrete hormones (chemical messengers) directly into the bloodstream. Hormone tests are commonly ordered to verify the function of the various endocrine glands, such as the pituitary gland and thyroid gland.
- ***Lipids***: These are fat molecules (cholesterol and triglycerides) which are transported within the body through the bloodstream.
- ***Enzymes***: Protein-based molecules that catalyse (speed up) chemical reactions are known as enzymes. Numerous enzymes present in the body travel through the circulating blood.
- ***Foods***: When digestion is complete, the food we eat has been broken down into its components that can be used by our body for energy or building blocks for other molecules. These breakdown products are absorbed into our bloodstream and dissolve in the plasma for transportation. Fatty acids, proteins (as amino acids), and simple sugars (glucose) are carried as food in the plasma. In addition, essential trace elements such as minerals and vitamins also form important constituents of our food.
- ***Dissolved gases***: Carbon dioxide (CO_2) is present in plasma as a by-product of metabolism. Other dissolved gases include oxygen and nitrogen. Part of the oxygen is also attached to the red blood cells.
- ***Waste***: Cell metabolism creates waste products that are processed in liver but removed from the body through the urinary system (kidney). The process of transportation occurs through the bloodstream.
- ***Medications***: If an individual is taking medication, it is transported throughout the body in the plasma.

Serum

Serum is the liquid portion of blood without any anticoagulant. Plasma and serum are very similar. However, when the blood is drawn into a tube without any anticoagulant, it instantly clots. During the clot formation, the proteins and other blood clotting factors are utilized or removed from the liquid system as the clot forms (Figure 30.1). Serum provides better specimen for biochemical analyses as it creates least interference in measurement.

Specimen transport, storage and preservation

Specimen transport is one of the greatest challenges in developing countries. They not only have limited means of transportation but the weather can be hostile and the infrastructure of business may be far from satisfactory. The method of transportation is determined by the distance. Specimens for point-of-care testing (in physician's office or on the bedside of the hospital) require no transport. This technology is fast developing.

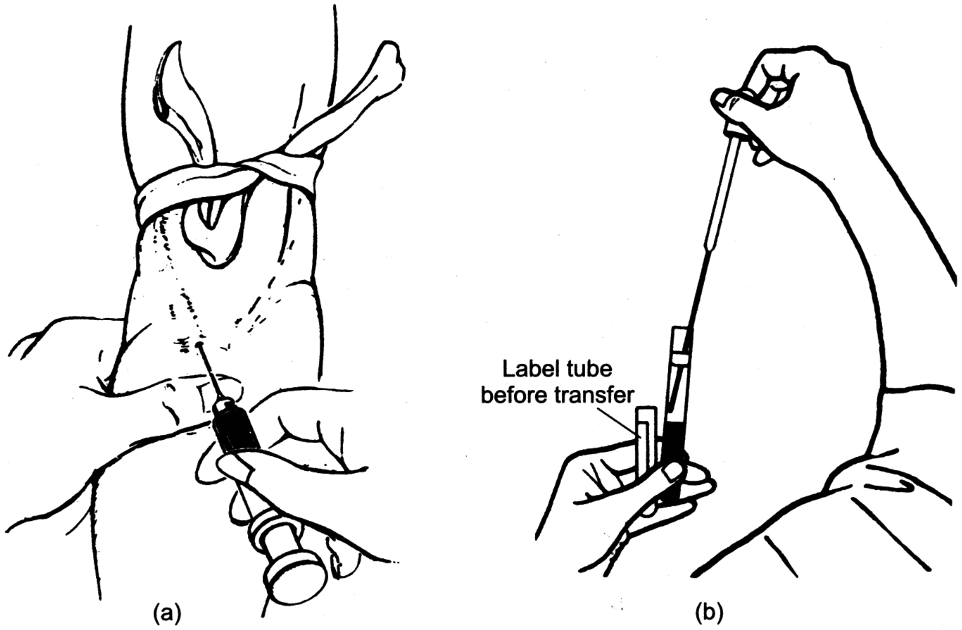

Figure 30.1 *Preparation of serum for biochemical analysis: (a-b) Collect blood by venepuncture in a container without anticoagulant. Allow the blood to clot at room temperature for 1 h and then separate the serum for biochemical tests.*

Within hospital premises, usually a distribution team transports the specimen to appropriate laboratories with minimum delay. Holding the specimen in leak-proof, impact-resistant containers is recommended in order to avoid biohazards. Occasionally, courier services are available to transport specimens to another laboratory, perhaps located in different city. Air transport is always desirable for prompt analysis. Regardless of the transport methods used, specimens must be packaged in secure containers to avoid contamination (use strong zippered plastic bags with biohazard signs printed outside). These must also be transported in an environment that meets biosafety regulations and protects the quality of the specimens.

For most routine tests to be performed **within an hour**, the specimen can remain at room temperature until testing. However, if the testing is to be delayed for a few hours, keep the specimens in the refrigerator at 4°C temperature. Do not freeze them unless instructed. Some specimens, however, should be immediately frozen after collection to prevent the loss of enzyme activity. Specimen for bilirubin determination should be stored in the dark as it degrades with exposure to light.

Collection and Processing of Blood Specimen

Blood is collected by **venepuncture** (Figure 30.1). Arterial blood is recommended for the analysis of blood gases and the specimen is collected by the physician. **Serum** specimen is mostly required for the biochemical analysis of blood; hence, anticoagulant is not added to the specimen container at the time of blood collection which is intended to yield serum. **Plasma** is submitted for the analysis of blood gases. **Heparin** (20 U/mL) is used as anticoagulant to prevent clotting. Specimen container must be **sealed** in order to avoid gaseous exchange between the specimen and its environment, and also it should be kept in ice. Heparin is least interfering in biochemical analyses, but is expensive. Hence, heparin should be used only when an alternative anticoagulant is not available, e.g., in the analysis of blood gases and pH. Heparin is the only anticoagulant which is neutral and does not affect the pH.

EDTA-anticoagulated whole blood is used for the determination of glucose-6-phosphate dehydrogenase of red blood cells. Plasma anticoagulated with fluoride-citrate can be used, instead of serum (recommended), for reporting blood glucose level as **'reducing substance'**. This is done when a delay is expected in glucose analysis. Fluoride inhibits glycolysis and thus prevents the loss of glucose during the lag period between specimen collection and glucose analysis. Fluoride is a weak anticoagulant and hence, it is combined with citrate (Chapter 5 in Vol. 1). Addition of fluoride, however, prevents the use of the enzymatic method for the glucose assay (e.g., glucose oxidase method), which is required for the reporting of **'true glucose'** value. Table 30.1 gives the list of additive or anticoagulant used for various biochemistry tests and those sent to other laboratories.

TABLE 30.1 *Use of additive or anticoagulant for various tests of biochemistry and for other laboratories*

Additive or anticoagulant	Usage
None (OR EDTA)	Serum, blood chemistries and blood banking
Polymer gel/silica activator	Serum separation, blood chemistries
Lithium heparin	Whole blood, plasma, blood chemistries
Glycolytic inhibitor + anticoagulant	Glucose determination
None or sodium heparin	Trace metals
EDTA	Whole blood, hematology and blood banking
3.2% or 3.8% sodium citrate	Coagulation tests
ACD solution	Blood banking studies (including blood transfusion)
Buffered sodium citrate	Westergren ESR

The specimen container used for blood collection in a biochemistry laboratory must be clean, free from detergent and bleach. After washing thoroughly with tap water, the containers should be finally washed with deionized water, and must be dried (preferably in an oven). The syringe used for blood collection should also be dry and the needle should be removed before transferring the blood held in the syringe. This prevents haemolysis. Haemolysed blood is unsuitable for most biochemical analyses and physical damage of red cells or presence of water may lead to their lysis. Many of these problems are now eliminated by the use of vacuum tubes as well as use of sterile disposable syringe with needle.

Specimen identification

Before collecting blood specimen, the patient must be identified using two identifiers. Patient's armbands now have bar codes. Devices incorporating a barcode reader and printer can be carried by the phlebotomist on rounds to scan the patient's armband and print matching bar-coded labels for the tubes of blood. Alternatively, barcoded labels can be generated at the same time the test is requisitioned.

Specimens must be labelled immediately after collection while the phlebotomist is still in the patient's presence. The label should include patient name and identification number, the date and collection of specimen, and the phlebotomist's initials.

Separation of blood components

Clinical biochemistry analysers can use whole blood, serum, or plasma for testing. Sometimes an analyser or method requires one particular type of specimen such as **heparinized plasma**. The laboratory standard operating procedure (SOP) manual will specify which type(s) of specimen and collection conditions are required for each test performed in the laboratory.

Whole blood is sometimes the specimen of choice for certain analyses. In modern biochemical analysers, whole blood is acceptable, which makes it easier and faster. Such specimens are ideal for **point-of-care testing** on the bedside. Whole blood is obtained by capillary puncture and immediately used. Heparinized capillary tubes are used to prevent clotting. If the blood is drawn by venepuncture, it must be collected in an anticoagulant tube, such as heparin or **EDTA**, to prevent clotting. The tube of whole blood **must be mixed well** immediately before testing.

Serum is the specimen used for most clinical biochemistry analyses. Serum is the fluid portion that remains after blood has been allowed to clot. Hence, blood is collected in a dry plain tube **without any anticoagulant**, allowing the blood to clot, centrifuging the clotted specimen, and removing the liquid (serum). Some blood collection tubes are especially made by the manufacturer to **speed up the clotting process**. Many modern laboratories are now using serum separator tubes that contain a special gel that forms a barrier between the serum and cells during centrifugation. Blood can also be collected in serum separator tubes, which contains special gel. In these tubes, during centrifugation, the red blood cells displace the gel in the bottom of the tube. This will be further discussed in detail (Figure 30.1).

Plasma, on the other hand, is obtained by **removing the liquid portion of anticoagulated blood** (whole blood) following centrifugation. The type of anticoagulant used is determined by the test that is ordered. If the laboratory is using a modern Vacutainer tube, the colour of the cap of the collection tube denotes the anticoagulant present in the tube as inner coating. **Plasma separating tubes** are available; these contain a gel that becomes displaced by red blood cells during centrifugation and produces a **barrier between red blood cells and plasma**.

The serum or the plasma to be used for testing **must be separated** as soon as possible from the blood's cellular portion. The separated liquid is then placed into another tube isolated from the blood's cellular portion which prevents the **exchange of substances**, such as ions and glucose, which could alter test results.

Preparation of serum specimen for biochemical analyses

Serum must be separated promptly from the clotted blood. This is easy when the blood is collected in a centrifuge tube.

1. Allow the blood to clot for 15 min at room temperature (do not refrigerate). If the clotting period is prolonged (1 h) it produces a greater quantity of serum and minimizes haemolysis.
2. Remove the clot that is adhering to the wall of the centrifuge tube by **'ringing'**. Ringing is a common laboratory term that means a gentle sweep around the inside walls of the tube with an applicator stick or glass rod in order to dislodge the clot from the wall. Excessive ringing is not necessary.
3. Centrifuge at 2500 rpm for 5 min.
4. Separate the supernatant (serum) within 2 h from the time of specimen collection. If the serum is not separated promptly, intracellular fluid is excreted, which produces erroneous findings for such analyses as potassium. Serum glucose level is also found to fall more rapidly due to glycolysis, if the cells are in contact with the serum. While separating the serum avoid contamination with red cells.
5. Keep the serum in labelled tubes at room temperature. If delay is anticipated, refrigerate the serum specimen. Protect the specimen from strong light (Sun) and heat which will lead to faster deterioration of the serum constituents sought in the analysis. Refrigerated specimens can be analysed within 24 h.

Precautions

- Before taking the aliquot for analysis, the serum specimen must be brought to room temperature; otherwise the volume of the aliquot will be inaccurate.
- Specimens for bilirubin analysis must be promptly processed and protected from direct light. Conjugated bilirubin is very sensitive towards light and leads to false low values if the specimens are not kept in the dark.
- Haemolysed serum is unsuitable for many biochemical analyses. As a result of haemolysis, constituents of red cells are released into the serum which leads to false

elevated values of serum for those constituents which are found in higher concentration inside the red cells, e.g., potassium, protein and cellular enzymes of red cells—acid phosphatases, lactic dehydrogenase and transaminases. False low values may also result due to dilution of the serum and the cellular contents of red cells—cholesterol, sodium, chloride and others. Haemoglobin may directly interfere in some of the analyses such as lipase, bilirubin and enzymatic, glucose determination.

- Before the results of the biochemical analysis are finally accepted by the physician, possible interference in the biochemical analyses by the drugs that the patient is taking, must be considered.

Removal of serum protein

Most new methods, however, are more sensitive and require small quantities of specimens. Thus, the interference due to precipitation is minimal and the serum protein need not be removed. In automated systems, the use of certain chemicals and dialysis has eliminated the protein removal step. In case of protein and enzyme determinations, or when the proteins do not interfere in the analysis, preparation of a protein filtrate is not necessary.

Sampling errors

It is repeatedly emphasized that reliable test results can only be obtained if the technologist has a proper specimen to work with. Improperly collected or handled specimen can cause misguiding erroneous test results. It is estimated that nearly 50% of the laboratory time is spent on specimen preparation and hence, it becomes extremely important that it is done correctly the first time. Some of the most important problems and the ways to avoid them are listed below.

Haemoconcentration: Difficulty in obtaining a blood specimen may lead to haemolysis with consequent release of potassium and other red cell constituents. Hence, results for these will be falsely elevated. This may happen when the tourniquet is left on too long (more than 1–2 min) during venepuncture. In case of prolonged stasis during venepuncture, plasma water diffuses into the interstitial space and the serum or plasma sample obtained will be concentrated. Proteins and protein-bound components of plasma such as calcium or thyroxine will be falsely elevated.

Haemolysis: Haemolysed blood cannot be used for most biochemical analyses. Intracellular enzymes and minerals are released (K^+, LDH, AST) which contaminates the serum sample. The result does not represent the true picture of patient's circulatory status. Haemolysis can be caused by over centrifugation, excessive shaking of the sample, freezing of red cells, poor venepuncture technique or contact with water.

Over centrifugation: During serum separation, blood specimen without anticoagulant should remain undisturbed for 20–30 min at room temperature for the formation of clot. It should then be centrifuged to separate the serum from blood cells. Serum should be removed from the clotted blood as soon as possible after centrifugation. This is now been done conveniently with the use of special gel (serum separator vacuum tube), but that may not be available in developing countries (Figure 30.2). To obtain plasma, the anticoagulated specimen can be centrifuged immediately after collection.

Remember

- Never freeze serum or plasma with the red cells. Freezing will cause cell lysis.
- Keep the cap on the blood collection container until the test is done. Evaporation may give false results. Gas exchange can also occur which will alter the pH and bicarbonate concentrations.

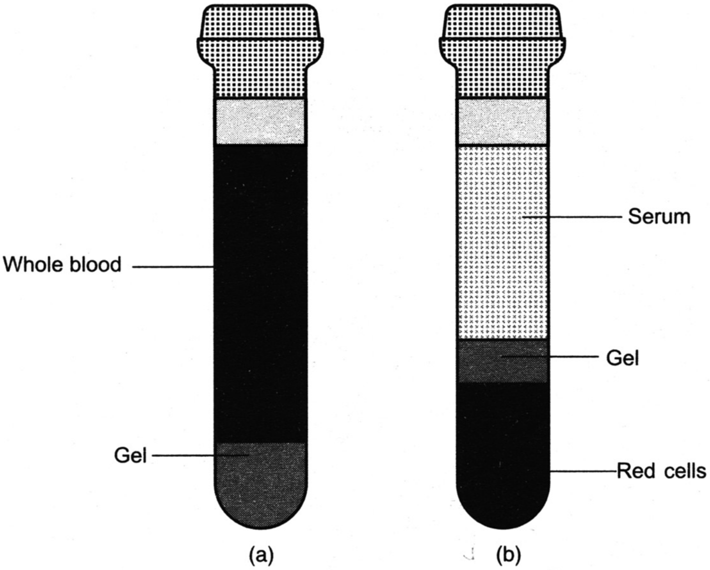

FIGURE 30.2 *Serum separator tube: (a) Before centrifugation, following collection of blood, (b) After centrifugation of the collected blood. Serum is at the top, ready to be taken out for testing. The gel separates red cells from serum.*

- Use clean glassware and pipettes for transferring specimens. The containers should be dry and clean in order to avoid contamination.
- Collect blood in the sequence of 'without anticoagulant' followed by 'with anticoagulant'. Accidental contamination of the needle with anticoagulant, during blood transfer, may affect the results.

Unacceptable blood specimen

Following are some of the examples of criteria that must be met for a specimen to be acceptable for laboratory testing:

- Label must be complete (name, date, time of collection, and name of person collecting the specimen) and attached in the proper place.
- Blood specimen must be free of haemolysis.
- Anticoagulated blood specimens must be free of clot. (Lack of mixing, immediately after collection, may cause this problem.)
- Specimen must be delivered to the laboratory within the specified time after collection.
- Outer surface of specimen container must have no visible contamination.
- Specimen must be stored properly until the time of testing.
- Blood specimens cannot be drawn from a site above an intravenous line.
- Specimens collected in anticoagulant must have the proper blood anticoagulant ratio.

Safety precautions

Technician should always be cautious in handling blood specimen. All specimens must be considered as infectious. Accidental exposure to blood and body fluids is more likely to occur during collection and processing of specimens than during specimen analysis. Standard precautions must be observed at all times. Some of the important precautions are as follows:

- Use appropriate protective equipment such as gloves, face protection (when needed), fluid resistant laboratory coat, frequent hand washing and changing of gloves.

- Use safety devices to avoid mouth pipetting, disposal needles, etc.
- Stay updated on specific procedures to handle patient with contagious disease.
- The technician should be aware of uncapping vacuum tubes containing specimens. This presents the potential hazards of aerosol creation, splatters and tube breakage.
- Modern laboratories use plastic tubes in order to prevent breakage and cleaning. The laboratory must have arrangement for their safe disposal.

Urine

Urine specimens are routinely analysed for the diagnosis of renal and metabolic disorders. They can also be used in toxicology investigation. Qualitative screening of a single discharge can provide an overall picture of renal disorder and diabetic condition (glucosuria), but quantitative biochemical analysis of a urine specimen is done with 24-h urine specimen for hormonal assays and diagnosis of endocrine-related problems.

As mentioned earlier, **urine biochemical testing** is performed as a part of routine random urinalysis with semiquantitative results reported that indicate a range for the analyte values. In some disease, it is necessary to obtain results that are more specific and quantitative. The physician is looking for a specific number reported for the chemical concentration rather than a range. This is not the routine and only performed by reference laboratories or large hospital laboratories. Some of the clinical situations that prompt to obtain quantitative report include kidney damage caused by diabetes, hypertension (high blood pressure), or related disease processes. Common quantitative tests performed on urine specimens include:

- Micro-albumin
- Total protein
- Creatinine

These tests require 24-h urine specimen.

A urine specimen is relatively easy to collect, but for quantitative analysis of a 24-h specimen, the patient's cooperation in collecting 24-h urine is very important along with the necessary attention of the nursing staff to refrigerate the specimen during collection. A composite sample of the urine discharged through the 24-h period is necessary because many of the constituents exhibit diurnal variation (e.g., most of the hormones). The 24-h urine gives a total picture which is fairly reproducible.

Collection of urine specimen

A **morning specimen** of urine, collected before breakfast, is considered to be ideal for the chemical screening of a **'single-specimen'**. A random urine specimen can also be used for screening. For urobilinogen analysis with a single-specimen, an afternoon specimen is preferred. The specimen should be collected between 2:00 pm and 4:00 pm. The laboratory should provide labelled clean containers for the collection of urine (Chapter 5 of Vol. 1).

For collecting a **24-h specimen**, the container should be of about 4-L capacity with appropriate preservative (Table 30.2). The bottle must have a proper label with the name of the patient, hospital, date of collection, starting time and finishing time. Write in bold letters; **DO NOT DISCARD** (24-h urine collection, refrigerate promptly). Refrigeration is needed during 24-h urine collection. Inadequate amount of preservative, loss of voided specimens or inclusion of two morning specimens in a 24-h period are common sources of error in specimen collection. The 24-h creatinine excretion stays constant for an individual and can be used as a guide to the adequacy of 24-h collection. This is particularly useful if several 24-h urine specimens are collected from the same person.

Errors in timing: The biggest source of error in the measurement of any analyte in a 24-h urine specimen is in the collection of an accurately timed volume of urine.

Table 30.2 *Collection of 24-h urine for routine biochemical analyses*

Test requested	Preservation procedure
Amylase, creatinine, uric acid, blood urea nitrogen, pregnanediol	Refrigerate after urine collection if testing delays
Aldosterone, calcium, catecholamines	Add one of the acids to make pH 4.5 (acetic, boric, HCl). Refrigerate after urine collection if testing delays
Corticosteroids, estrogens	Use boric acid (l g/L of urine); refrigerate after urine collection if testing delays
VMA (Vanillyl Mandelic Acid)	Acidify with HCl (5–10 mL); refrigerate after urine collection if testing delays
Porphobilinogen	Collect in a dark bottle, with 5 g sodium carbonate to maintain alkaline pH; refrigerate after urine collection if testing delays

Procedure of specimen collection for 24-h urine

1. Ask the patient to empty the bladder and discard the first morning specimen.
2. Take a clean urine collection bottle with preservative and label. Fill in the information regarding the patient on the label. Note the time when the patient emptied the bladder. From this point urine will be collected for a 24-h period. Record the starting time on the label.
3. Collect all the urine discharged from the recorded time until the following 24-h period. At the end of the 24-h collection period, the bladder is emptied and this urine specimen is added to those already collected. Refrigerate the specimen.
4. Measure the total volume and take a portion of well-mixed urine for biochemical analysis. Volume can be measured either from the markings on the bottle (if provided) or by pouring into a graduated cylinder. An easy way is by the weight method.
    (a) Weigh the container (W_c). Write the weight on the label.
    (b) Weigh the container with the 24-h urine specimen (W_{uc}).
    (c) Volume of the urine is $W_{uc} - W_c$. This method considers the specific gravity of urine to be 1, instead of greater than 1 (1.010 or higher), but the error is not very significant.

Stool

Presence of invisible blood in faeces is a condition referred as **occult blood**. Stool sample may be submitted to biochemistry laboratory for faecal blood test. Faecal occult blood can be a sign of a problem in your digestive system, such as a growth, or polyp, or cancer in the colon or rectum. If blood is detected, it is important for your doctor to determine the source of bleeding to properly diagnose and treat the problem.

If gastrointestinal bleeding may be easily seen as red blood, or black tar-like bowel movements, it is called **melena**. Occult blood test may not be needed in case of melena. It is identified physically by observation.

Preparation for stool collection

Please consult your doctor if certain food or drugs may cause in providing accurate results. Avoid them, if necessary.

Certain foods, vitamins and drugs (pain killers) have the potential to alter or affect the results of your occult blood sample. Therefore, before collecting stool specimens for occult blood, you should consult the doctor and discontinue them, if necessary.

Caution in collection of stool sample

Specimens must be collected properly to ensure accuracy in diagnosis. The following instructions must be followed:

1. Do not wrap specimen in absorbent material (such as toilet paper), which may absorb organisms your doctor needs to identify.
2. If your doctor has ordered multiple specimens, collect one every two or three days. No more than one per day.
3. Make sure the specimens are labelled with your name, date and time of collection.

Collection of stool for occult blood test

Do not collect samples during, or until three days after, your menstrual period, or while you have bleeding haemorrhoids or have blood in your urine. For your convenience, you can wait until completing all collections before returning them to the laboratory for examination.

1. Pass urine into toilet.
2. Place "hat" on the rim of the toilet and pass stool into the "hat."
3. Open the front flap of the haemoccult card.
4. Using the sticks provided, select a portion of the stool and apply it to one of the test areas on the card. Using another stick, select another portion of the stool and apply it to the other test area on the card.
5. Close the cover flap and keep it closed when not in use.
6. Discard the "hat" and its contents, unless it is needed for other stool procedures.
7. Label haemoccult cards with your name, date and time of collection.
8. Once complete, return cards to laboratory for testing within two days.

Collection of stool specimen from an infant

Do not submit a specimen that has been in direct contact with a diaper. Place the peds-bag on the child to prevent urine from coming into contact with the stool specimen.

It is best to **line the diaper** with plastic wrap to avoid absorption. You may then lift the specimen out of the diaper in the plastic wrap and place it in the appropriate container(s) as instructed.

Cerebrospinal Fluid (CSF)

Cerebrospinal fluid (CSF) acts as a cushion, protecting the **brain and spine** from injury. CSF specimen is normally clear with the same consistency as water. CSF test looks at the fluid that surrounds the brain and spinal cord. The test results may be used to diagnose brain or spinal cord injury or establish certain **neurologic disorders** (meningitis) such as infection or development of a tumour. A spinal tap may also be done to establish the diagnosis of **normal pressure hydrocephalus**.

Collection of CSF is only **done by the physician** because of the risks involved during specimen collection. There are different ways to get a sample of CSF and **lumbar puncture** (spinal tap) is the most common method. Details of specimen collection are discussed in Chapter 26 of Vol. 2.

Routine chemical analysis of CSF is usually restricted to **protein** (globulin and total) and **glucose**. CSF specimen is always taken in three tubes. Of the three tubes, the third tube is taken for biochemical analysis. This is least contaminated with blood cells. Presence of blood cells, especially haemoglobin, will lead to false high values of protein. One way to avoid contamination of red cells is to centrifuge the CSF (third tube) before taking the aliquot for **protein assay**.

Glucose analysis must be done promptly in order to avoid loss of glucose due to glycolysis. You can add sodium fluoride (0.5 mg/mL) to a portion of the CSF fluid, and then refrigerate. Toluidine or glucose oxidase method, as described under serum glucose analysis (Chapter 33 of this volume), can be used where proteinfree filtrate is not necessary. Use 1 mL of diluted specimen (dilute 1:10, with distilled water) for the glucose assay.

The process of handling other body fluids such as **pleural, synovial, ascetic and pericardial** fluids is same as that of CSF. They are submitted to the biochemistry laboratory for their biochemical assays.

REVIEW QUESTIONS

1. How do you prepare the serum specimen for biochemical analyses? Why are the haemolysed and lipaemic sera unsuitable for biochemical analyses?
2. What are the precautions taken for the specimens requested for bilirubin assay?
3. Which are the normal proteins present in the serum specimen? Why is it necessary to remove them prior to the biochemical analyses? How can this be avoided? Would you remove these proteins for the determination of total protein in serum?
4. List some of the chemicals present in plasma but not in serum.
5. When is the best time for collecting blood and urine specimens for routine biochemical analyses?
6. List some of the clinical conditions when the physician wants a "quantitative report" of urinalysis.
7. Why is it necessary to acidify a 24-h urine specimen collected for VMA analysis?
8. What is the recommended procedure for collecting a 24-h urine specimen?
9. What is the clinical significance of collecting a 24-h urine specimen?
10. What are the common biochemical analyses done with CSF specimen? If three tubes are used in CSF collection, which one should be used for biochemical analysis? Give reasons.

Chapter

31

Techniques of Analytical Chemistry

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Chapter Outline

- Introduction to Analytical Chemistry
- Analytical Chemistry and Clinical Chemistry
  - Basic Steps of Clinical Biochemistry
- Applications of the Principles of Analytical Chemistry
  - Photometry
  - Chromatography
  - Electrochemistry
  - Immunochemistry
- Instrumentation for Proteomics
- Osmometry
- Analytic Techniques for Point-of-Care Testing (POCT)
- Review Questions

INTRODUCTION TO ANALYTICAL CHEMISTRY

Analytical chemistry, as applied to clinical laboratories, is a science dedicated to the quantification of the chemical compounds found in the body in health and sickness. It tries to quantitate certain components in the body that the physician seeks for diagnosis.

Analytical methods can be separated into **classical** and **instrumental.** The classical wet chemistry (precipitation, extraction, distillation, etc.) is no longer practiced in routine analytical chemistry laboratory, and qualitative analysis by colour, odor, or melting point is now obsolete. Although classical quantitative analysis is achieved by measurement of weight or volume, this too has been eliminated by the current practice of buying a kit providing all the chemicals. Thus, the clinical laboratory focuses on instrumental analytical chemistry which is engaged to measure physical quantities of the analyte such as light absorption (colorimetry, spectrophotometry), fluorescence, or conductivity (ion selective electrode, potentiometer).

The **separation of materials** is another aspect of clinical biochemistry in which techniques such as **chromatography** and **electrophoresis** made positive contributions. In recent years, analytical chemistry is also participating in improving experimental design, in **chemometrics,** and in the creation of measurement tools to provide better chemical information. Chemometrics is the chemical discipline that uses mathematical and statistical methods to model **intelligent laboratory systems,** including robotics, self-optimizing instruments, and create new software for better imaging techniques and graphic presentation of chemical data.

ANALYTICAL CHEMISTRY AND CLINICAL CHEMISTRY

Basic techniques such as centrifugation, filtration, heating, mixing, quantitative transfers and gravimetric procedures are described in Chapter 4 of Vol. I. In this chapter, we will concentrate on specific analytical procedures and instrumentation as applied to clinical biochemistry.

Nearly 25 tests are routinely done in clinical biochemistry and majority of these analyses are based on the principles of **photometry,** or the measurement of light. This includes colorimetry or absorption photometry, spectrophotometry, flame emission photometry, fluorometry, turbidimetry and nephelometry. The technique of **electrochemistry** is used for the analysis of inorganic ions, pH and blood gases. Currently the principles of electrochemistry have found new applications in various small instruments used as point-of-care or POC services on bed side of patients. The field of **immunochemistry** has expanded widely in recent years and has become an essential tool for the analysis of microquantities of drugs (drug monitoring), toxic compounds and in the assay of hormones. We also have included **electrophoresis,** a technique used in separating closely related organic compounds such as proteins, haemoglobins and lipids.

Our discussions in this chapter will focus on the specific technology and instrumentation that is best suited for non-urban laboratories in developing countries. We have also tried to predict the oncoming technology which may be favourable to the current prevailing conditions of the developing countries.

Basic Steps of Clinical Biochemistry

The basic steps in analytic chemistry consist of:

1. **Specimen processing:** Examples include separating serum from a whole blood specimen or the removal of a specific serum protein before running the test by a technique such as affinity chromatography.
2. **Chemical reaction:** A chemical reaction may be undertaken in order to measure the amount of the analytes present in the specimen. In case of colorimetric procedure, a chemical reaction with the analyte (the substance sought) generates a colour, the intensity of which is proportional to the quantity of the analyte.
3. **A standard to compare:** A standard solution is an artificially made reagent solution of the substance to be assayed, which includes a known concentration of that substance. It is a reference solution to compare with the unknown, or the specimen. A control, however, is different from the standard solution. It is a specimen with known value of the substance to be analysed. This is required for an internal quality **control.** Based on the derived standard curve for the assay, your internal control sample should have a reading equivalent to its known content of the substance. A standard curve is illustrated in Figure 31.1.

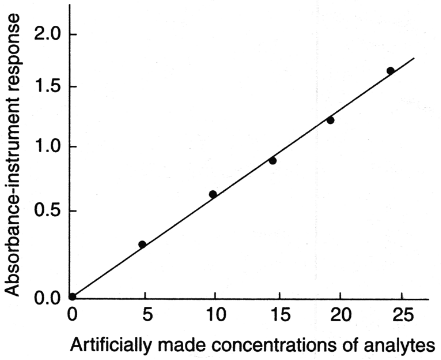


FIGURE 31.1 *A standard curve*

4. **Calculation:** Calculate the concentration of unknown in the specimen against the concentration of the standard (known). Usually an instrument is involved, which provides a numerical figure as a measure of the amount of analyte under given conditions, such as a specific wavelength. This value is used in the following formula for determining the concentration of the unknown.

$$C_u = \frac{IR_u}{IR_s} \times C_s$$

where, C_u is the concentration of unknown, C_S is the concentration of standard, IR_U is the instrument response of unknown, and IR_S is the instrument response of standard.

APPLICATIONS OF THE PRINCIPLES OF ANALYTICAL CHEMISTRY

Basic principles of analytical chemistry are now widely applied in clinical biochemistry. Following will be elaborated in the subsequent pages:

- Spectrophotometry and photometry: Spectrophotometer, reflectance photometry, flame photometry, fluorometry, turbidimetry and nephelometry.
- Electrochemistry: Ion-selective electrodes, pH electrodes (potentiometry), gas-sensing electrodes, enzyme electrodes, coulometric chloridometers
- Electrophoresis: Isoelectric focusing capillary electrophoresis
- Chromatography: High performance liquid chromatography, gas chromatography
- Mass spectrometry
- Analytic techniques for point-of-care testing.

The devices like **"Lab on a chip"** integrate multiple laboratory functions on a single chip of only millimeters to a few square centimeters in size and that are capable of handling extremely small fluid volumes down to less than picoliters.

Photometry

Photometry (photo = light; metry = measurement) is the most common analytic technique used in clinical biochemistry. The principle of photometry is based on the physical laws of radiant energy or light. In this method, the intensity of absorbed, transmitted, reflected or emitted light is measured and related to the concentration of the test substance. As colorimeters and spectrophotometers are widely used in clinical biochemistry laboratories, they are discussed separately. Photometric instruments measure light intensity without consideration of wavelength. Most instruments today use filters (photometers), prisms, or gratings (spectrometers) to select (isolate) a narrow range of the incident wavelength. Radiant energy that passes through an object will be partially reflected, absorbed, and transmitted.

Physical properties of light The visible range of the spectrum covers from 700 to 400 nm (Figure 31.2). The wavelengths beyond the red end of 700 nm are the infrared and heat waves. The wavelengths below 400 nm are in the UV region and carry high energy and also deep penetrating actions. Hence, these are harmful for biological objects.

Light source The light source provides the radiant energy for photometric determinations. In case of colorimeter, where a visible range of light is required, a tungsten lamp is the best choice. It emits a continuous spectrum of light in the visible range (420–760 nm). If the desired wavelength is in UV region, a hydrogen lamp is used, which also gives a continuous spectrum in a range that extends from the lower visible to the lower UV range (600–250 nm),

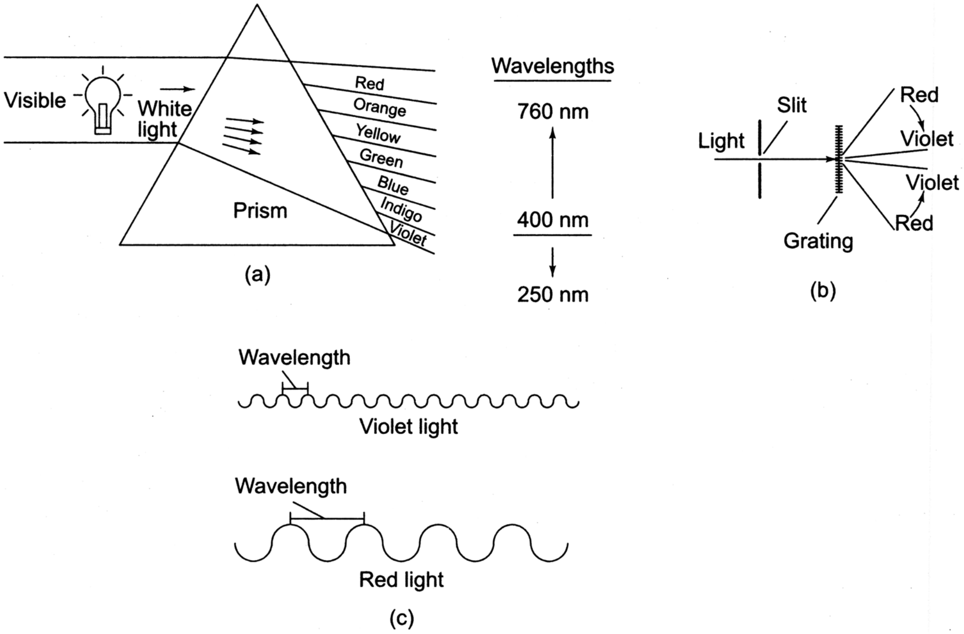


FIGURE 31.2 *Components of white light: (a-b) White light can be split by prism or diffraction grating, (c) Violet light has shorter wavelength than the red light*

going down in the 'beyond-visible range'. The continuous spectrum gives the advantage of choosing any specific wavelength for the entire region—visible or UV. The xenon lamp emits high-energy UV rays and the mercury lamp gives discrete bands in the lower visible and UV range. These are mostly used in fluorometry where high energy is needed to excite an organic compound subjected to analysis. The hollow cathode lamp is another kind of lamp used in atomic absorption photometry, which emits only a single wavelength of light from a glowing metal. The wavelength emitted is specific for the metal (copper, selenium, zinc, lead and others) to be analysed.

Quality of light In order to increase the specificity and sensitivity of an optical measurement, a specific quality of light is allowed to enter the coloured solution. The quality of light is regulated by a monochromator in the case of spectrophotometers, which allows one to select a specific wavelength of light to pass through the test solution. The monochromator may be a prism or a diffraction grating (Figure 31.3). Special coloured filters are used in a colorimeter, each of which has the limitation of providing a range of wavelengths near the desired wavelength (Table 31.1). The filter chosen for the colorimeter is usually complementary to the colour

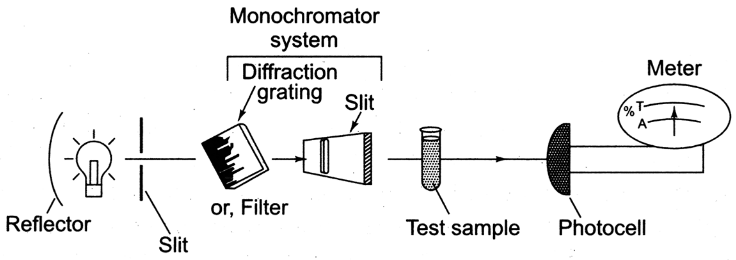


FIGURE 31.3 *Components of a spectrophotometer/colorimeter*

TABLE 31.1 *Use of complementary filter*

Colour of solution	Filter used	Peak transmission (nm)
Bluish-green	Red	680
Blue	Yellow	580
Purple	Green	520
Red	Blue-green	490
Yellow	Blue	470
Yellowish-green	Violet	430

of the solution to be measured. The chosen wavelength should give the highest absorbance reading for the analysis. The height of the peak is proportional to the concentration of the substance in solution. The required wavelength is provided within the assay instructions.

Recently, in some automated systems, a dual wavelength measurement is introduced in order to eliminate the error due to the presence of protein, turbidity (e.g., lipaemic) or colour of the serum specimen (e.g., jaundice).

Absorption photometry

If a pencil of light is allowed to pass through a coloured solution, some amount of light will be absorbed while the rest of the light will be transmitted through the solution (Figure 31.4a).

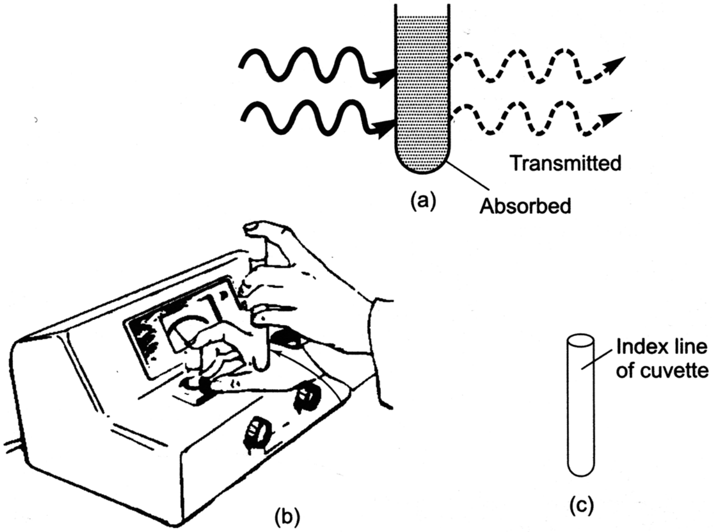


FIGURE 31.4 *Principle of absorption photometry: (a) When light passes through a coloured solution, a part of it is absorbed and part of it is transmitted, (b) A modern spectrophotometer, (c) The index line on the cuvette which should be aligned with the marking given on the photometer so that the configuration of the cuvette does not change*

The amount of light absorbed is proportional to the nature of the solution (expressed as extinction coefficient or 'ε', which is a constant), the concentration of the solute or colour, and the distance of the path of light. This is known as **Beer's law** and can be expressed as follows:

$$A = \varepsilon \, L \, c$$

Where,

A = absorbance

ε = molar extinction coefficient (this is constant for the same analyte)

L = path length of the cell holder or distance of the path of light (which is constant 1 cm)

c = concentration of the solution

Thus, following the Beer's law, the direct relationship between absorbance and concentration, the transmittance reading is first converted to the absorbance scale (Figure 31.5) for preparing the standard curve. Modern colorimeters, however, provide the absorbance reading along with the transmittance reading on the scale provided. Note that the absorbance is read on a logarithmic scale, which is wider at low values and narrower at higher values. The highest reading of the absorbance is 2 (or infinity) while that of transmittance is 100. The '0' reading of the absorbance coincides with the 100% transmittance reading. An absorbance reading beyond unity is used with caution as the error of reading increases due to the compressed gradation of the scale in this region (Figure 31.6).

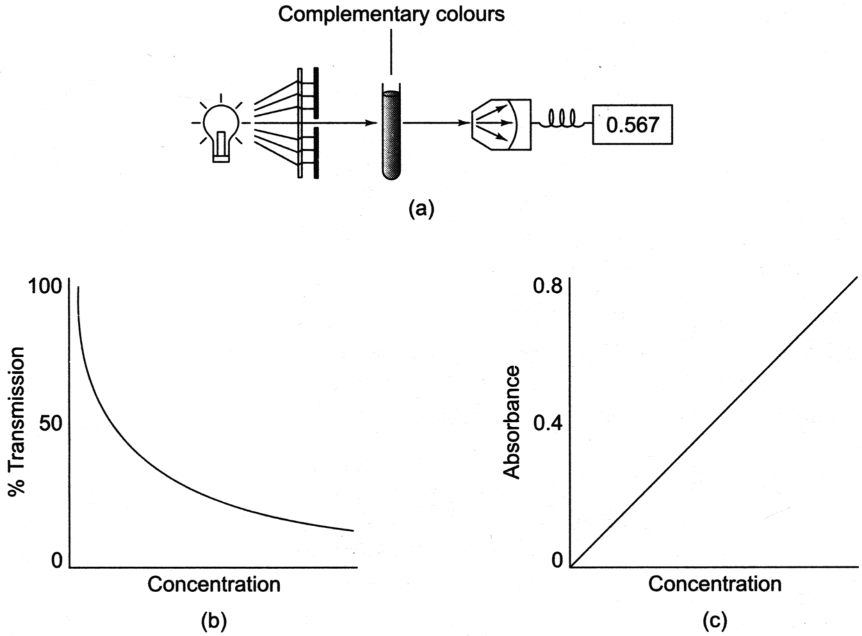


FIGURE 31.5 *Application of Beer's law in absorption photometry: (a) Use of complementary filter or wavelength of light to pass through the solution (red filter for blue solution) gives the best response, (b-c) Concentration of the coloured solution is inversely proportional to percent transmittance, but directly proportional to absorbance. Hence standard curve on the linear graph should be made with the values of absorbance.*

As ε and L (1 cm) are constant, the new relationship can be expressed as

$$A \propto c$$

This means that absorbance is a direct measure of concentration.

Since absorbance (A) is inversely related to transmittance (T)—more absorbed means less transmitted—the relationship of A and T (Figure 31.4) is expressed as in the following equation

$$A = 2 - \log\%T$$

The photo detector system, however, can only measure the transmitted light (Figure 31.5a). If the scale of a photoelectric colorimeter is in %T, it should first be converted to absorbance (A), before using the values for the calculation of concentration of the unknown (Figures 31.5b, c and 31.6).

$$\text{Concentration of unknown } (C_u) = \frac{A_u}{A_s} \times C_s$$

where, C_u is the concentration of the unknown, A_u is the absorbance of unknown (test solution), A_s is the absorbance of the standard (known), and C_s is the concentration of the standard.

Note The unit of the concentration (g/dL or mmol/L or mg/dL) will be the same as given for the standard.

Modern photoelectric colorimeters and spectrophotometers, however, provide the logarithmic scale of absorbance *(A)* along with the % transmittance, which is linear (Figure 31.6). Thus, in setting the blank (e.g., water), the instrument must read 100% transmittance and have a '0' (zero) reading for absorbance.

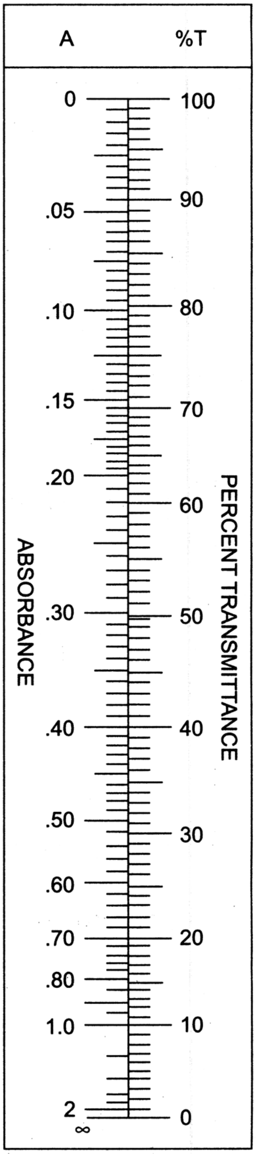


Figure 31.6 *Conversion scales of transmittance (%) and absorbance*

Colorimeters and spectrophotometers As colorimeters and spectrophotometers are the most common instruments in clinical laboratories of developing countries, it is appropriate to discuss these two instruments separately.

Colorimeters and spectrophotometers are closely comparable except that colorimeters use filters while spectrophotometers use prisms or a diffraction grating that yields a single selected wavelength of light. So, we can say that spectrophotometers are only sophisticated colorimeters. These instruments can be standalone or part of an analyser that does multiple tests. As spectrophotometers are the single most important equipment in a clinical biochemistry laboratory, we will concentrate on the use and care of these instruments in the following pages.

Components of photometers The components of a modern spectrophotometer are shown in Figure 31.3. A technician should understand the functions of each component in order to maintain them properly.

Light source The most common source of light for work in the **visible and near-infrared region** is the incandescent tungsten or tungsten-iodide lamp. Only about 15% of radiant energy emitted falls in the visible region, with most emitted as near-infrared. Often, a heat-absorbing filter is inserted between the lamp and sample to absorb the infrared radiation.

The lamps most commonly used for **ultraviolet** (UV) work are the deuterium-discharge lamp and the mercury arc lamp. Deuterium provides continuous emission down to 165 nm. Low-pressure mercury lamps emit a sharp-line spectrum, with both UV and visible lines. Medium and high-pressure mercury lamps emit a continuum from UV to the mid visible region. The most important factors for a light source are range, spectral distribution within the range, the source of radiant production, stability of the radiant energy, and temperature.

Monochromator Isolation of individual wavelengths of light is an important and necessary function of a monochromator. The degree of wavelength isolation is a function of the type of device used and the width of entrance and exit slits. The band pass of a monochromator defines the range of wavelengths transmitted and is calculated as width at more than half the maximum transmittance. Numerous devices are used for obtaining monochromatic light. The least expensive are coloured-glass filters. These filters usually pass a relatively wide band of radiant energy and have a low transmittance of the selected wavelength. Although not precise, they are simple, inexpensive, and useful. Most of the analyses, however, are done by only a few pre-set wavelengths. For getting the best response for an absorbance reading, only complimentary colours are chosen to pass through the coloured solution in the cuvette (Figure 31.5a)—such as a red filter that is complimentary to a blue solution. Table 31.1 gives some of the most commonly chosen wavelengths of light. Interference filters are special filters that transmit multiples of the desired wavelengths. They require accessory filters to eliminate these harmonic wavelengths. Interference filters can be constructed to pass a very narrow range of wavelengths with good efficiency. The prism is another type of monochromator. Diffraction gratings are most commonly used as monochromators. A diffraction grating consists of many parallel grooves (15,000 or 30,000 per inch) etched onto a polished surface. Gratings with very fine line rulings produce a **widely-dispersed spectrum.** Because the multiple spectra have a tendency to cause stray light problems, accessory filters are used.

Slit The light emerging from the monochromator is then passed through a narrow slit that improves the quality of light by choosing a single wavelength. This is found in all spectrophotometers.

Cuvette (sample cell) The cuvette holds the solution whose absorbance is to be measured. The cuvette is a part of the optical system; hence, extreme care must be taken in handling the cuvettes. The light path must be kept constant to have absorbance proportional to concentration. The first and foremost job is to have matched cuvettes. They are often sold in matched sets. Otherwise test the matching as described. The cuvettes must be matched. Fill each cuvette to be tested, take readings, and save those that match within an acceptable tolerance (e.g., ±0.25% T). Cuvettes are sold as round or square. Because it is difficult to manufacture round tubes with uniform diameters, they should be etched to indicate the position for use. Square cuvettes have plane-parallel optical surfaces and a constant light path. They have an advantage over round cuvettes in that there is less error from the lens effect, orientation in the spectrophotometer, and refraction. Cuvettes with scratched optical surfaces scatter light and should be discarded. Inexpensive glass cuvettes can be used for applications in the visible range, but they absorb light in the UV region. Quartz cuvettes must, therefore, be used for applications requiring UV radiation. The cuvette must be optically transparent, scrupulously clean, devoid of any scratch and free from contamination. The latter is of special significance when one is working in the UV range. Cleaning of the cuvette must be done with lens paper or soft tissue paper or toilet paper. Store the cuvette in a dry covered place; dust will scratch the cuvette. Never clean the cuvette in chromic acid; use only a mild, good quality detergent.

Photodetector and galvanometer The **photodetector** generates an electric current when light falls on it and then transmits it to the galvanometer. The needle of the galvanometer deflects as a consequence of the electric impulse and the deflection is proportional to the light intensity. The detector measures the transmitted light, which is reciprocally related to the absorbance. There are several kinds of photodetectors. The simplest of them is the **barrier-layer or photocell.** It is inexpensive and durable. It requires no external voltage source but relies on internal electron transfer to produce a current in an external circuit. But it requires high level illumination and used in wide band path. **Phototube** is the alternate photodetector. Like the photocells, it has photosensitive material that gives off electrons when light energy strikes it but differs in that an outside voltage is required for its operation. The photocurrent is linear in phototube. The third major type of light detector is the **photomultiplier** (PM) tube, which detects and amplifies radiant energy. Finally, in this system the analog signal received from the phototube is converted first to a voltage and then to a digital signal through the use of an analog to digital (A/D) converter. Digital signals are processed electronically to produce absorbance readings. The new innovation of **photodiode** brings excellent linearity, speed and small size, but is not as sensitive as the phototubes because of lack of internal amplification.

Single beam and double beam spectrophotometers For single-beam spectrophotometers, the absorbance reading from the sample must be blanked using an appropriate reference solution that does not contain the compound of interest. Double-beam spectrophotometers permit automatic correction of sample and reference absorbance. Because the intensities of light sources vary as a function of wavelength, double-beam spectrophotometers are necessary when the absorption spectrum for a sample is to be obtained. Computerized, continuous zeroing, single-beam spectrophotometers have replaced most double-beam spectrophotometers.

Quality assurance in spectrophotometry

In order to get reliable data, the spectrophotometer must function properly. To validate the function of the instrument, three things must be checked:

- Wavelength accuracy
- Stray light
- Linearity

A routine system should be devised for each instrument to check and record each parameter. The probable cause of a problem and the maintenance required to eliminate it are generally described in the instrument's manual.

Wavelength accuracy Wavelength accuracy means that the wavelength indicated on the control dial is the actual wavelength of light passed by the monochromator. It is most commonly checked using standard absorbing solutions or filters with the absorbance maxima of a known wavelength. The filter is placed in the light path and the wavelength control is set at the wavelength at which maximal absorbance is expected. The wavelength control is then rotated in either direction to locate the actual wavelength that has maximal absorbance. If these two wavelengths do not match, the optics must be adjusted to calibrate the monochromator correctly. Some instruments with narrow band pass use a mercury vapour lamp to verify wavelength accuracy. When mercury vapour lamp is not available, it is substituted for the usual light source, and the spectrum is scanned to locate mercury emission lines. The wavelength indicated on the control is compared with known mercury emission peaks to determine the accuracy of the wavelength indicator control.

Stray light Stray light refers to any wavelengths outside the band transmitted by the monochromator. The most common causes of stray light are reflection of light from scratches on optical surfaces or from dust particles anywhere in the light path and higher-order spectra produced by diffraction gratings. This causes absorbance error, especially in the high-absorbance range. Stray light is detected by using cut off filters, which eliminate all radiation at wavelengths beyond the one of interest. To check for stray light in the near-UV region, for example, insert a filter that does not transmit in the region of 200 to 400 nm. If the instrument reading is greater than 0% T, stray light is present.

Linearity Linearity is demonstrated when a change in concentration results in a straight-line calibration curve, as discussed under Beer's law. Coloured solutions may be carefully diluted and used to check linearity, using the wavelength of maximal absorbance for that colour. Sealed sets of different colours and concentrations are available commercially. They should be labelled with expected absorbance for a given band pass instrument. Less than expected absorbance is an indication of stray light or of a band pass that is wider than specified. Sets of neutral density filters to check linearity over a range of wavelengths are also commercially available.

Basic technique of colorimetry

The three basic steps of colorimetry or visual spectrophotometry are as follows:

1. ***Chemical reaction***: This leads to the development of colour through a chemical reaction. If the test compound is a chromogenic substance, a chemical reaction may not be necessary.

2. ***Physical measurement***: The detector measures the transmitted light (Figure 31.3). The intensity of the colour is measured (*A* or %T), both for the test solution as well as for the standard. The latter, with known concentration of the test compound, is treated identically as the specimen or test solution.
   Note Use an appropriate filter to yield the desired quality of light.
3. ***Calculation***: Results are either calculated by the mathematical formula or read from a calibration curve prepared from varying concentrations of the standard and the corresponding absorbance readings. If the readings are taken in %T, first convert the values to absorbance *(A)* before calculating the results (Figure 31.6).

Some commonly used terms

Blank solution: This is used to set the photometer to '0' absorbance *(A)* or' 100%' transmittance (%T). This can be water or the reagent solution (if coloured), also referred to as a reagent blank.

Standard solutions: These are solutions of known concentrations, which range within the limits found in the specimen (normal and abnormal).

Test solution: This is the solution into which the specimen was added in order to develop a colour.

Control: This is identical to the specimen but, unlike the (unknown) specimen, the concentration of the analyte is known. A control is used to determine the accuracy and dependability of the test.

Preparation of calibration curve

As with any other analytical technique, colorimetry requires the preparation of a calibration curve. The calibration curve shows the relationship between varying concentrations of the analytical compound sought and the instrument reading. In the case of photometry, the curve confirms that Beer's law is obeyed. A slope of 45° is ideal for the calibration curve. It shows the balanced relationship between absorbance and concentration (Figures 31.7a and b). In other words, for each increase in concentration, a satisfactory proportional increase in the absorbance reading is found. The same cannot be said for the other two curves shown (Figures 31.7c and d) against the ideal standard curve (Figure 31.7a). If the curve is at an angle less than 45°, the sensitivity of the method is poor. In other words, a large change in concentration brings about only a small change in absorbance. On the contrary, if the calibration curve is at an angle greater than 45°, it indicates that the method cannot work on a wide range of concentrations of the substance to be analysed as the readings will vary too widely with a slight change in concentration. If the linear relationship is not observed (Figure 31.7d) in the calibration curve, the technician must establish the limit of the linear relationship. It is a good policy to check the linear relationship and redraw the calibration curve whenever there is any change in the procedure or equipment performance, such as the changing of a bulb, a new standard, a new reagent, etc.

Procedure of taking colorimeter readings

The following procedure is taken from one of the manufacturer's instructions (Beckman Instrument Company, USA). Such instructions from similar photometers are available from other manufacturers as well (see Appendix at the end of this Volume).

Note Read the manufacturer's instructions carefully before handling the instrument.

1. Set the instrument to 0%T setting (or $\propto$ setting of absorbance) with the help of the 'zero setting knob'. During the zero setting, light is not allowed to pass through the cuvette. In some instruments, this is called 'dark current setting'.
2. Match two cuvettes after filling them with water and taking their absorbance readings. Record the error, if there is any. If the manufacturer supplies matched cuvettes, this will not be necessary.
   Note The marking on the cuvette must be in line with the marking on the instrument so that the configuration of the cuvette remains identical while taking each reading (Figure 31.4c).

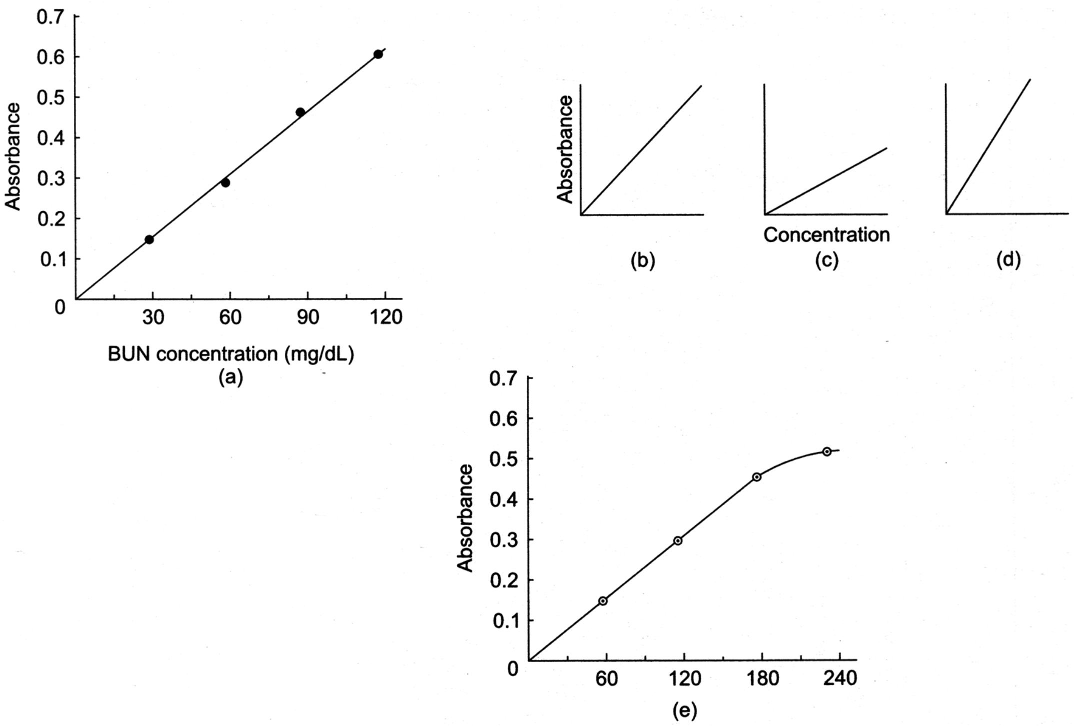


FIGURE 31.7 *Significance of calibration curve: (a) Typical calibration curve of BUN, (b) Ideal calibration curve should have a slope of 45° between the absorbance and transmittance, (c-d) Any deviation from the 45° slope will result in an increased error, (e) The calibration curve is good only up to the point where it shows linearity*

3. Pour the blank into one of the cuvettes after taking out the water.
4. Set the instrument to 100%T (or 0 absorbance) for the blank. Use the blank setting knob to set the other extreme of the galvanometer while light is passing through the blank.
5. Perform the required analytical procedures using various concentrations of the standard solutions.
6. Take the absorbance reading of each solution (variable concentrations). If a matched cuvette is used, rinse the cuvette each time and always start from the lowest concentration.
7. Record the readings in a tabular form—absorbance readings against the sequential concentrations of standards used.
8. Plot the calibration curve on linear graph paper for the absorbance readings (shown on the *Y*-axis) against the various concentrations of the standard (shown on the *X*-axis). (See Chapter 4 of Vol. 1 for details.)
9. The calibration curve should be at an angle of 45° and must be well labelled. Record the instrument and exact wavelength used. This is because the calibration curve varies between instruments and at different wavelengths.

Additional information

- If the calibration curve is not linear beyond a certain concentration (Figure 31.7), the absorbance reading of the non-linear region should not be considered. The specimen should be diluted and rerun until the absorbance is in the linear region.

- In preparing serial dilutions of the standard, try to use larger quantities of a more dilute standard than very small quantities of a concentrated standard in the assay. The latter tends to increase the sample delivery error.
- Linear relationship of the calibration curve establishes the following mathematical formula

$$C_u = \frac{A_u}{A_s} \times C_s$$

where C_u is concentrations of unknown, C_s is concentration of standard, A_u is absorbance of unknown and A_s is absorbance of the standard.

The formula for calculating the concentration of the unknown (specimen) is applicable only when the relationship between absorbance and concentration is linear. Hence, the calibration curve should first be drawn before using the formula. If the curve is not linear and does not have a 45° slope, the results will not be reliable.

Note The unit of concentration for the specimen will be the same as the standard.

Reflectance photometry

With the popularization of POCT, reflectance photometry is becoming handy as a part of solid-phase technology. The instrument does not need any liquid reagent and there are fewer breakdowns. The reagents are, however, more expensive but the results are reliable.

Reflectance photometers measure light intensity of a specific wavelength that is **reflected by a coloured product** (Figure 31.8b). This reflected light is detected by a photocell, and the information is converted into the appropriate units. Instruments using solid-phase technology install reflectance photometry in their system, which is ideal for small laboratories. Technicians with limited training can handle most of these.

In **solid-phase chemistry analysers** (Figure 31.8a), the reagents are in a dried form embedded in the test spot. One can use whole blood as the specimen. The blood sample is applied directly to the reagent strip, slide or cartridge that contains all the reagents needed for the analysis in the test area or reagent pad. The reagents are in multiple layers in a cartridge, with each layer having a specific function. The layers are capable of filtering the red cells, leaving only plasma to mix with the test reagents. The resulting colour of the final product is detected by reflectance photometry. The colour intensity is measured and converted to appropriate units for the test being performed. Different slides (or cartridges) are used for different tests. Nearly 30 different tests can be performed on a small desktop analyser.

Urine strip analysers also operate by reflectance photometry. The reagent strip pads change colours depending on the composition of the urine. These colour changes are detected by the instrument and the results are displayed or printed.

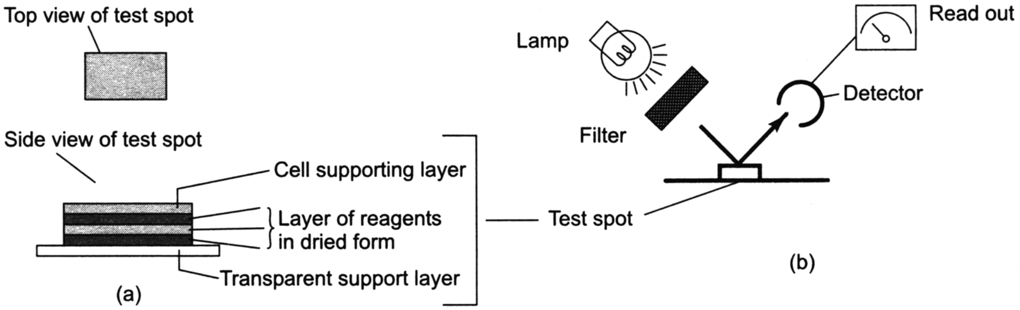


FIGURE 31.8 *Solid phase technology: (a) Manufacturer provides reagents stacked on a spot, (b) specimen soaked in and the colour intensity measured by reflectance photometry*

Turbidimetry and nephelometry

Turbidimetry (Figure 31.9a) is classified under absorption photometry, but the radiant energy of light is intercepted by the solid particles, which are in a state of suspension in the liquid medium, rather than absorbed by the system. The machine measures the transmitted light. The method is difficult to standardize because the particle size is critical. This method is in use for several semiquantitative assays, such as protein determination by a precipitation method and estimating the bacterial population before running an antibiotic sensitivity test (see Chapter 20 of Vol. 2).

On the other hand, the method of **nephelometry** (Figure 31.9b) measures the intensity of radiation scattered by a suspension. The procedure can be highly sensitive and has found its application in immunochemistry. If the antigen–antibody complex forms a fine suspension, this method of photometric assay can be applied in quantifying the immunochemical reaction.

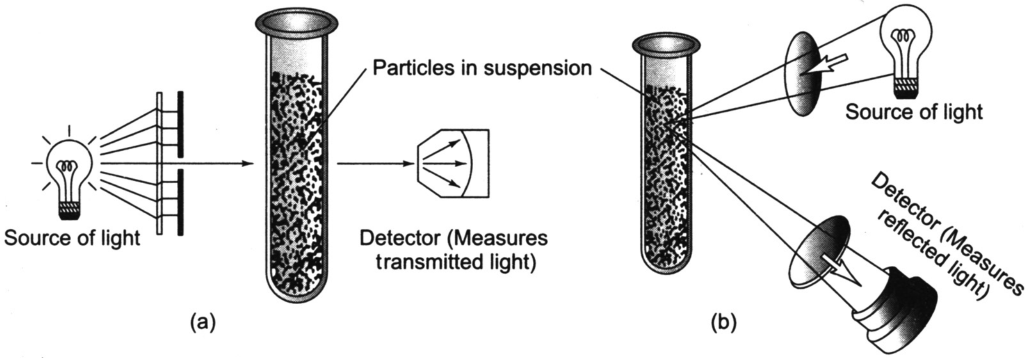


FIGURE 31.9 *Principles of (a) turbidimetry and (b) nephelometry*

Flame photometry

Flame-emission photometer which measures light emitted by excited atoms is widely used to determine concentration of Na^+, K^+, or Li^+. With the development of ion selective electrodes for these analytes, flame photometers are no longer routinely used in clinical chemistry.

Fluorometer

Unlike photometry where the light is absorbed by the analyte in solution and we measure the % transmittance, in fluorometry we measure the emitted light coming out of the analyte due to fluorescence phenomena. For example, if quinine is dissolved in 0.1 N sulphuric acid, it will fluoresce in short wavelength of light and one can measure the degree of fluorescence that will be proportional to the amount of quinine in 0.1 N sulphuric acid.

Basic instrumentation

The source emits short-wavelength high energy excitation light. A mechanical attenuator controls light intensity. The primary filter, placed between the radiation source and the sample, selects the wavelength that is best absorbed by the solution to be measured. The fluorescing sample in the cuvette emits radiant energy in all directions. The detector (placed at right angle to the sample cell or cuvette) and a secondary filter that passes the longer wavelengths of fluorescent light prevent incident light from striking the photodetector. The electrical output of the photodetector is proportional to intensity of fluorescent energy. In spectrofluorometers, the filters are replaced by prisms of grating monochromators. Various gas-discharge lamps (mercury and xenon-arc) are used, but the light detectors are almost exclusively PM tubes because of their higher sensitivity to low light intensities.

Fluorescence concentration measurements are related to molar absorptivity of the compound and other factors. As with all quantitative measurements, a standard curve must be prepared to demonstrate that the concentration used falls in a linear range.

Chromatography

Chromatography refers to the group of techniques used to separate complex mixtures on the basis of different physical interactions between the individual compounds and the stationary phase of the system. The basic components in any chromatographic technique are the mobile phase (gas or liquid), which carries the complex mixture (sample); the stationary phase (solid or liquid), through which the mobile phase flows; the column, holding the stationary phase; and the separated components (eluate).

Modes of separation

Only selected modes of separation, widely used in clinical laboratories, will be discussed here.

Adsorption

Adsorption chromatography, also known as liquid-solid chromatography, is based on the competition between sample and the mobile phase for adsorptive sites on the solid stationary phase. There is an equilibrium of solute molecules being adsorbed to the solid surface and desorbed and dissolved in the mobile phase. The molecules that are most soluble in the mobile phase, move fastest; the least soluble, move slowest. Thus, a mixture is typically separated into classes according to polar functional groups.

Partition

Partition chromatography is also referred to as liquid-liquid chromatography. Separation of solute is based on relative solubility in an organic (nonpolar) solvent and an aqueous (polar) solvent.

Ion-exchange chromatography

In ion-exchange chromatography, solute mixtures are separated by virtue of the magnitude and charge of ionic species. Anion and cation resins mixed together (mixed-bed resin) are used to deionize water. Ion-exchange chromatography is used to remove interfering substances from a solution, to concentrate dilute ion solutions, and to separate mixtures of charged molecules, such as amino acids. Changing pH and ionic concentration of the mobile phase allows separation of mixtures of organic and inorganic ions.

Chromatographic procedures

Thin-layer chromatography

Thin-layer chromatography (TLC) is a variant of column chromatography. A thin layer of sorbent, such as alumina, silica gel, cellulose, or cross-linked dextran, is uniformly coated on a glass or plastic plate. Each sample to be analysed is applied as a spot near one edge of the plate, as shown in Figure 31.10. The mobile phase (solvent) is usually placed in a closed container until the atmosphere is saturated with solvent vapour. One edge of the plate is placed in the solvent, as shown. The solvent migrates up the thin layer by capillary action, dissolving and carrying sample molecules. Separation can be achieved by any of the four processes previously described, depending on the sorbent (thin layer) and solvent chosen. After the solvent reaches a predetermined height, the plate is removed and dried. Sample components are identified by comparison with standards on the same plate. The distance a component migrates, compared with the distance the solvent front moves, is called the retention factor or R_f

$$R_f = \frac{\text{Distance moved by leading edge of component}}{\text{Total distance moved by solvent front}}$$

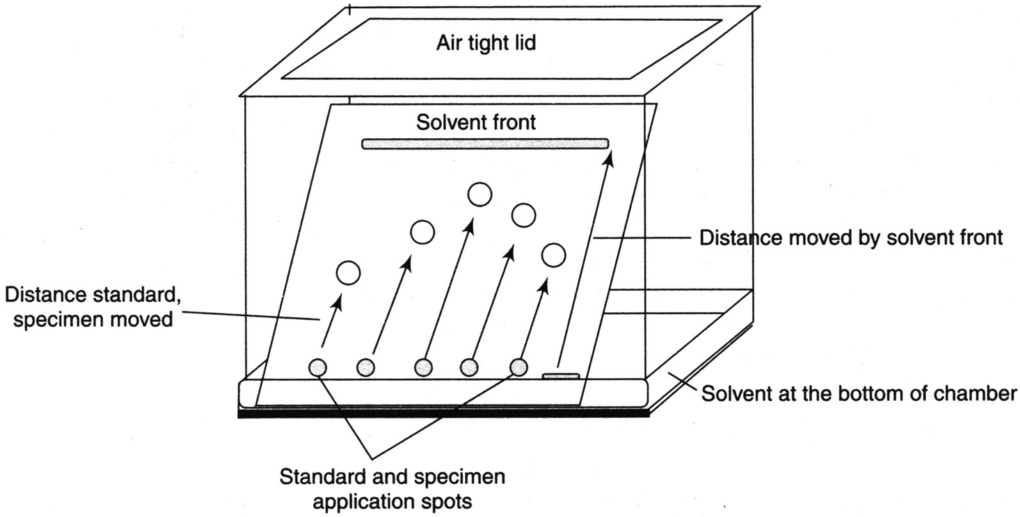


FIGURE 31.10 *TLC plate in a chromatographic chamber*

Each sample-component R_f is compared with the R_f of the standards. R_f values may overlap for some components, further identifying information is obtained by spraying different stains on the dried plate and then comparing colours of the standards. TLC is most commonly used as a semiquantitative screening test. Absorbance of each developed spot is measured by a densitometer and the concentration is calculated by comparison with a reference standard chromatographed under identical conditions.

High-performance liquid chromatography

Modern liquid chromatography uses pressure for fast separations, controlled temperature, in-line detectors, and gradient elution techniques. Basic components of HPLC are shown in Figure 31.11 given below.

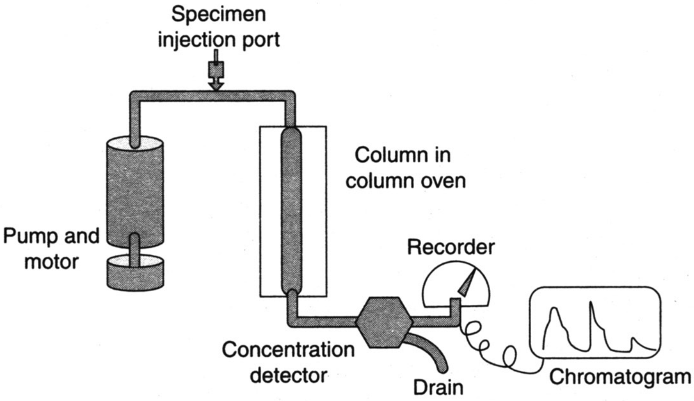


FIGURE 31.11 *Basic components of HPLC (high-pressure liquid chromatograph)*

A hydraulic amplifier pump forces the mobile liquid phase through a column at a much greater velocity than that accomplished by gravity-flow columns. The specimen is injected within the line before it enters the stationary phase, which are the long stainless steel columns inside a jacket-oven to attain desired temperature of the stationary phase in which the mobile phase moves in gaseous form. The most common material used for column packing is silica gel. The mobile phase commonly used is acetonitrile, methanol, water, or any combination of solvents. Mixture of analytes falls apart as they are forced through the column and purge out through the external port which can be sensed by the detector and quantitatively determined from the chromatogram.

Gas chromatography

Gas chromatography is used to separate mixtures of compounds that are volatile or can be made volatile. Commonly used in clinical laboratories is gas-liquid chromatography (GLC), with a non-volatile liquid stationary phase. The setup is similar to HPLC, except that the mobile phase is a gas and samples are partitioned between a gaseous mobile phase and a liquid stationary phase. The carrier gas can be nitrogen, helium, or argon. The instrument can be operated at a constant temperature or programmed to run at different temperatures if a sample has components with different volatilities. This is analogous to gradient elution described for HPLC. Compounds with higher boiling points will move slowly through the column. The effluent passes through a detector that produces an electric signal proportional to the concentration of the volatile components. As in HPLC, the chromatogram is used both to identify the compounds by the retention time and to determine their concentration by the area under the peak (Figure 31.12).

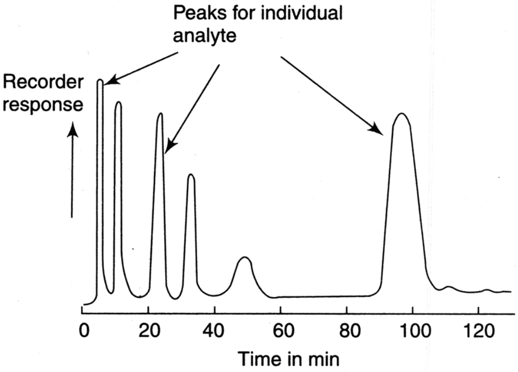


Figure 31.12 *Typical chromatogram from high performance liquid chromatography*

Electrochemistry

Electrochemical technology has suddenly become popular with the advent of hand-held glucose meters, which make it easy to monitor the serum glucose level of diabetic patients. Based on the same principle of measuring electrical energy—flow of current **(amperometry),** potential difference between electrodes **(potentiometry)** and others—various tests are now performed at ease. In some of the glucose meters, special electrodes measure the electrons generated when the sample and reagents react. Instruments based on the principle of amperometry measure glucose with the help of a disposable biosensor strip containing the electrode. When the sample interacts with the reagents in the biosensor strip, the current (electrons) generated is detected by the meter and converted into glucose units. It takes only a few seconds to complete the entire test.

Measurement of pH and various electrolytes are based on the principle of **potentiometry** (Figure 31.13). In simple words, it measures the potential difference between two electrodes that act as probes that are connected by the flow of ions in solution. A common use of electrodes in clinical biochemistry is to measure the hydrogen ion concentration using a pH meter. **Ion-selective electrodes** selectively measure a particular ion in the presence of other ions. So, a pH electrode is an ion-selective electrode for hydrogen (H^+). The pH meters are extensively used in clinical biochemistry laboratories for preparing various reagents and buffer solutions (Figure 31.14).

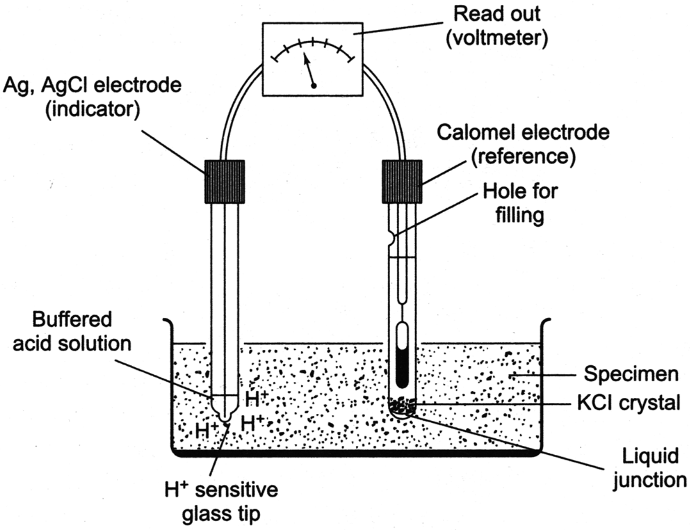


FIGURE 31.13 *Principle of potentiometry*

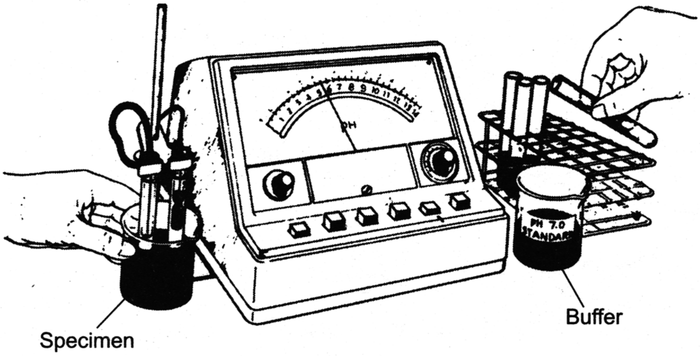


FIGURE 31.14 *The pH meter is widely used in clinical biochemistry laboratories for preparing reagent solutions and buffers*

Two electrodes are required for potentiometric analyses. One electrode contains a known concentration of the ion to be measured and is called the **reference electrode**. The other electrode, which is responsive only to the flow of current (amperometry) through the ion being measured, is exposed to the unknown solution. The difference between the concentration of ions in the reference electrode and the ions in the unknown solution causes an electrical potential to develop. This potential across a membrane in the electrode is proportional to the difference between the two concentrations. The potential, measured in voltage, is converted by the microprocessor into a number representing the concentrations of the ion in the unknown solution. Because each ion-selective electrode is responsive to a specific ion, the sodium (Na^+) electrode, for example, will measure only Na^+ ions in a sample. The technology of ion-selective electrodes is used in many clinical instruments and is particularly useful for measuring electrolytes.

Electrophoresis

Electrophoresis is the migration of charged solutes or particles in an electrical field. Iontophoresis refers to the migration of small ions, whereas zone electrophoresis is the migration of charged macromolecules in a porous support medium such as paper, cellulose acetate, or agarose gel film. An electrophoretogram is the result of zone electrophoresis and consists of sharply separated zones of a macromolecule. In a clinical laboratory, the macromolecules of interest are proteins in serum, urine, cerebrospinal fluid (CSF), and other biologic body fluids and erythrocytes and tissue. Electrophoresis consists of five components: the driving force (electrical power), the support medium, the buffer, the sample, and the detecting system. A typical electrophoretic apparatus is illustrated in Figure 31.15. Charged particles migrate towards the opposite charged electrode. The velocity of migration is controlled by the net charge of the particle, the size and shape of the particle, the strength of the electric field, chemical and physical properties of the supporting medium, and the electrophoretic temperature.

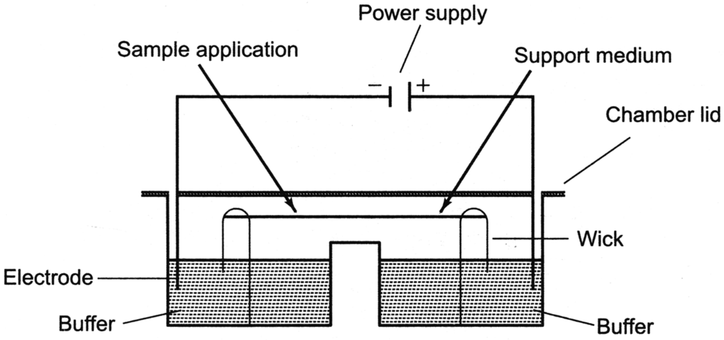


FIGURE 31.15 *Basic components of electrophoresis apparatus*

Procedure

The sample is soaked in hydrated support for approximately 5 min. The support is put into the electrophoresis chamber, which was previously filled with the buffer. Sufficient buffer must be added to the chamber to maintain contact with the support. Electrophoresis is carried out by applying a constant voltage or constant current for a specific time. The support is then removed and placed in a fixative or rapidly dried to prevent diffusion of the sample. This is followed by staining the zones with appropriate dye. The uptake of dye by the sample is proportional to sample concentration. After excess dye is washed away, the supporting medium may need to be placed in a clearing agent. Otherwise, it is completely dried.

Basic components

Power supply

Power supplies operating at either constant current or constant voltage are available commercially. In electrophoresis, heat is produced when current flows through a medium that has resistance, resulting in an increase in thermal agitation of the dissolved solute (ions) and leading to a decrease in resistance and an increase in current. The increase leads to increase in heat and evaporation of water from the buffer, increasing the ionic concentration of the buffer and subsequent further increase in the current. The migration rate can be kept constant by using a power supply with constant current. This is true because, as electrophoresis progresses, a decrease in resistance as a result of heat produced also decreases the voltage.

Buffers

Two buffer properties that affect the charge of ampholytes (compound sought) are **pH** and **ionic strength.** An ampholyte (compound sought) is a molecule, such as protein, whose net charge can be either positive or negative. The ions carry the applied electric current and allow the buffer to maintain constant pH during electrophoresis. If the buffer is more acidic (pH below 7.0) than the isoelectric point of the ampholyte, then it binds H^+ ion and becomes positively charged (cation$^+$); the positively charged cations then migrate towards the **cathode.** If the buffer is more basic (pH above 7.0) than the isoelectric point of the ampholyte, then the ampholyte loses H^+ ion and becomes negatively charged (anion$^-$); these anions then migrate towards the **anode**. A particle without net charge will not migrate and remains at the point of application. The rate of migration depends on the size of the molecule. During electrophoresis, these ions migrate towards their destination, *i.e.,* the electrode over the support material. The rate of migration depends on the size of the molecule. As a result, the ampholyte molecules (like protein) get separated. They are then located for identification by the use of appropriate methods like staining or when put under UV (ultra violet) light.

(***Note*** The **isoelectric point** is the pH at which a particular molecule carries no net electric charge and will not migrate towards its opposite pole.)

Support materials

Use of cellulose acetate paper in electrophoresis has been replaced by cellulose acetate or agarose gel in clinical laboratories. Cellulose is acetylated to form cellulose acetate by treating it with acetic anhydride. Cellulose acetate, a dry, brittle film composed of about 80% air space, is produced commercially. When the film is soaked in buffer, the air spaces fill with electrolytes and the film becomes pliable. After electrophoresis and staining, cellulose acetate can be made transparent for densitometer quantitation. The dried transparent film can be stored for long periods. Cellulose acetate prepared to reduce electroendosmosis is available commercially. Cellulose acetate is also used in isoelectric focusing.

Agarose gel Agarose gel is another widely used supporting medium. Used as a purified fraction of agar, it is neutral and, therefore, does not produce electroendosmosis. After electrophoresis and staining, it is detained (cleared), dried, and scanned with a densitometer. The dried gel can be stored indefinitely. Agarose gel electrophoresis requires small amounts of sample (approximately 2 mL); it does not bind protein and, therefore, migration is not affected.

Polyacrylamide gel Polyacrylamide gel electrophoresis involves separation of protein on the basis of charge and molecular size. Layers of gel with different pore sizes are used. The gel is prepared before electrophoresis in a tube-shaped electrophoresis cell. The small-pore separation gel is at the bottom, followed by a large-pore spacer gel and, finally, another large-pore gel containing the sample. Each layer of gel is allowed to form gelatin before the next gel is poured over it. At the start of electrophoresis, the protein molecules move freely through the spacer gel to its boundary with the separation gel which slows their movement. This allows for concentration of the sample before separation by the small-pore gel. Polyacrylamide gel electrophoresis separates serum proteins into 20 or more fractions rather than the usual five fractions separated by cellulose acetate or agarose. It is widely used to study individual proteins (e.g., isoenzymes).

Starch gel Starch gel electrophoresis separates proteins on the basis of surface charge and molecular size, as does polyacrylamide gel. The procedure is not widely used because of technical difficulty in preparing the gel.

Treatment and application of sample

Serum contains a high concentration of protein, especially albumin and, therefore, serum specimens are routinely diluted with buffer before electrophoresis. In contrast, urine and CSF are usually concentrated. Haemoglobin haemolysate is used without further concentration. Generally, preparation of a sample is done according to the suggestion of the manufacturer of the electrophoretic supplies. Cellulose acetate and agarose gel electrophoresis require approximately 2 to 5 mL of sample. These are the most common routine electrophoreses performed in clinical laboratories. Because most commercially manufactured plates come with a thin plastic template that has small slots through which samples are applied, overloading of agarose gel with sample is not a frequent problem. After serum is allowed to diffuse into the gel for approximately 5 min, the template is blotted to remove excess serum before being removed from the gel surface. Sample is applied to cellulose acetate with a twin-wire applicator designed to transfer a small amount.

Detection and Quantitation

Separated protein fractions are stained to reveal their locations. Different stains come with different plates from different manufacturers. The simplest way to accomplish detection is visualization under UV light, whereas densitometry is the most common and reliable way for quantitation. Most densitometers will automatically integrate the area under a peak, and the result is printed as percentage of the total. A schematic illustration of a densitometer is shown in Figure 31.16.

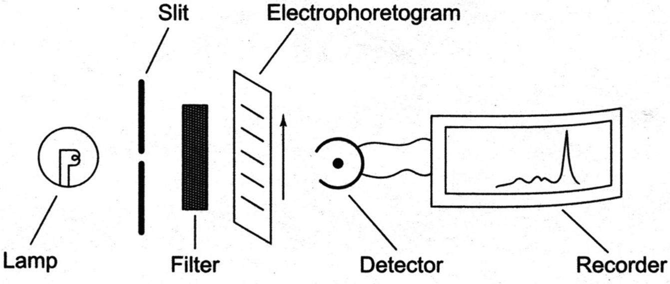


FIGURE 31.16 *Reading of electrophoretogram through transparent support medium*

In the case of a serum protein study, the serum specimen, which is a mixture of various proteins, is placed as a spot on a support medium. The medium is wetted with the buffer used for the free flow of the electric current. As the electric current passes through the sample, different proteins (albumin, globulins—alpha$_1$ alpha$_{2,}$ beta and gamma) migrate at different rates based on charge. Albumin has the highest rate of migration and gamma globulin is the slowest. The location of each separated protein-spot is determined by staining with a protein-staining dye (Ponceau S). Photometric measurements (Figure 31.16) can also track the migration of proteins on an electrophoretogram. Figure 31.17 shows several patterns associated with normal and abnormal clinical conditions.

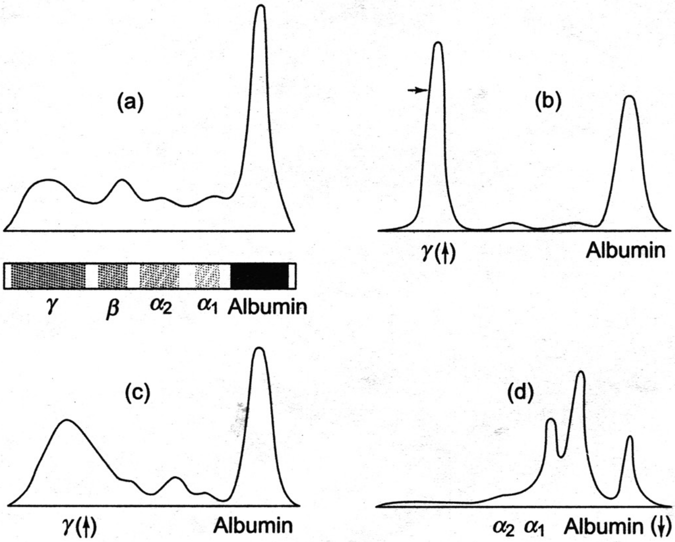


FIGURE 31.17 *Separation of serum proteins by electrophoresis. (a) Serum electrophoretogram of normal subject, (b) of patient with multiple myeloma showing monoclonal peak of Bence Jones protein, (c) of patient with chronic infection and increased gamma globulin and (d) of patient with nephrotic syndrome showing fall of albumin.*

Electrophoresis of haemoglobin is done with the goal of identifying abnormal haemoglobin. The migration rate of abnormal haemoglobins varies from the normal haemoglobin (HbA) and thus they are identified based on their location. Staining is not necessary for locating haemoglobin spots due to the inherent colour of these proteins.

Immunochemistry

Assay methods based on immunological principles are used in essentially all departments. In simple words, antibodies develop within our body through our defensive immune system as a result of invading foreign antigens (pathogens). The antibodies generated react with the antigens and make them inactive. This can be demonstrated in the laboratory through the agglutination of red cells, which carry the antigens on their surface. This has been discussed in Chapter 15 of Vol. 1. The same principles are applied in the identification of various infectious agents, as will be presented in the chapter on immunology. Numerous other applications of immunology in the clinical laboratory include: tissue compatibility testing for grafting, identification of cells markers and evaluation of patients' immune function in the cases such as recurring infections, poor wound healing or other symptoms that indicate a possible problem with the immune response. We will confine ourselves here to the application of the same principles used in a chemical assay. Here the immunochemical methodology detects substances unrelated to the immune system, such as drugs or hormones, using antigen-antibody reactions.

While trying to focus on the application of immunochemical analytical procedures we will postpone our detailed discussion on the nature and types of antibodies, which are immunoglobulins, their structures and functions. This has been discussed in Chapter 23 of Vol. II and will be further elaborated in immunochemistry in the following sections. Whether the laboratory is looking for the antibody in the patient's serum or circulating antigen (drug or hormone), the laboratory must be able to detect and quantitate the immunologic reaction.

Commercially available latex particles carry the antigen or the antibody and the resulting immunologic reaction between the two can be visible (Figure 31.18a). The lattice formation allows the precipitate to be visible to the naked eye (Figure 31.18b). This process of agglutination leads to visible clumping or aggregation of cells or particles due to reaction with a specific antibody. Similar reactions are seen in blood typing. The test for rheumatoid arthritis is also based on this principle. Slide agglutination tests can give a qualitative or semiquantitative result.

Application of nephelometry in immunochemistry

When a specific antibody reacts with a soluble antigen, a suspension of very small particles forms, the concentration of which can be measured by a nephelometer. The use of nephelometry principles allows immunological tests to be automated. This is done by passing a beam of light through the suspension (Figure 31.9) resulting from both the antigen–antibody reaction and the resultant precipitate formation. The light gets scattered; the scattered light is then collected electronically and measured for a quantitative report. The greater the amount of light scattered, the more concentrated is the suspension of the particles. This method has largely replaced the RID technique.

Radial immunodiffusion

The precipitation reaction of antigen and antibody is applied in a radial immunodiffusion (RID) test (Figure 31.19) that measures the concentrations of various types of immunoglobulins (IgG, IgM or IgA) in patient's serum. Agar plates, about the size of a microscope slide, are commercially available and contain antihuman globulin or IgG (or antihuman IgA or IgM) diffused throughout the agar. The agar also contains small wells. Each well is filled with

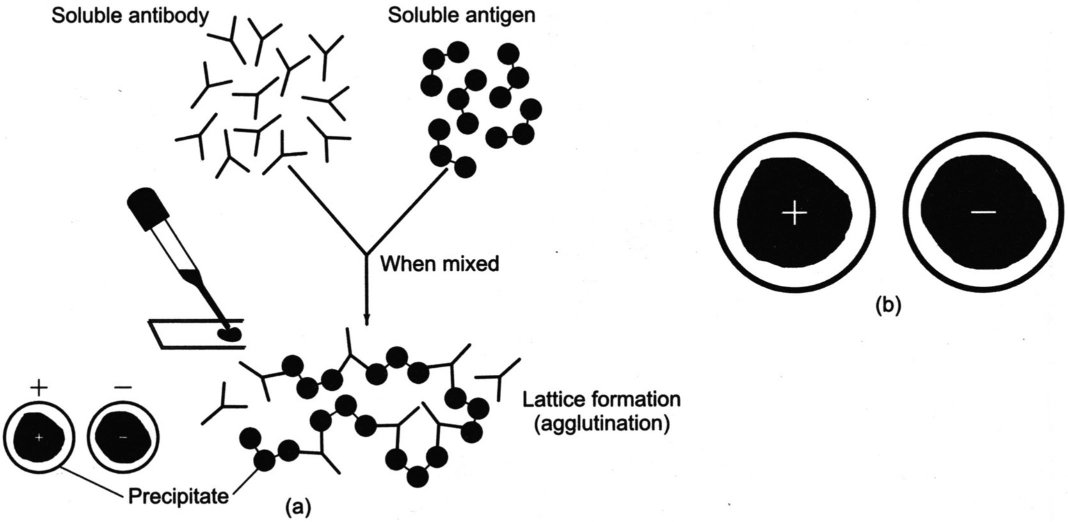


Figure 31.18 *Immunological reaction of soluble antigen and soluble antibody may be visible by the clumping effect (agglutination) as a result of lattice formation of the antigen and antibody. The results could be used as a semiquantitative assay.*

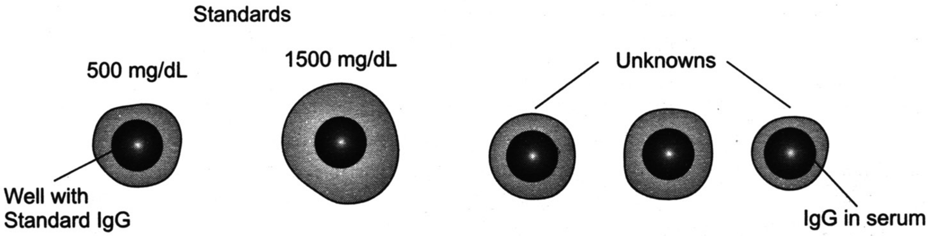


Figure 31.19 *Agar precipitation technique (radial immunodiffusion) where rings of precipitation form when serum diffuses out of wells and reacts with specific antibody in agar. The size of the ring could provide quantitative data for diagnosis.*

a patient's serum, a serum dilution, or a standard of a known amount of IgG. After setting up the plate, it is incubated for several hours. As the IgG in the serum or standard (kept in the well) diffuses out of the well and into the agar, it reacts with the anti-IgG in the agar and forms a white ring of precipitation around the well (Figure 31.19). The diameter of the ring is proportional in the concentration of IgG in the sample. A standard curve is constructed using the diameters of the rings produced by the IgG standards. The concentration of the unknown is determined by comparing its precipitation diameter with the standard curve.

Labelled antibody techniques

Several types of immunological tests use labelled (or 'tagged') antibodies. These labelled antibodies are mostly antihuman globulins, which will recognize any antibody in the serum reacted with its corresponding antigen. Hence, they are also referred to as anti-antibodies. The labelling can be done by the use of fluorescent dyes, enzymes or radioisotopes (Figures 31.20–31.22). The elements of an enzyme immunoassay (EIA) are depicted in Figure 31.20. This figure shows the principles behind the application of both EIA and radioimmunoassay (RIA) in the determination of the concentration of various analytes. The fluorescent antibody

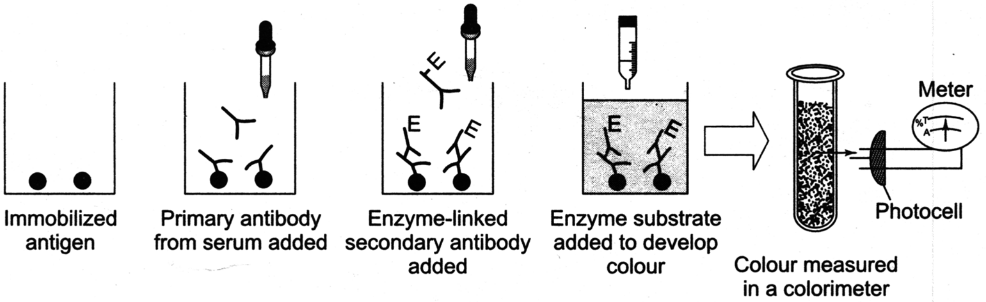


Figure 31.20 *The technique of antibody labelling uses tagged-enzymes in the quantitative assay of the amount of antibody present in the serum. The amount of antibody is detected by colorimetric method using substrate that yield colour. The non-labile antigen adhered to the wall of the container is available commercially.*

(FA) technique, Western blotting, and flow cytometry are sophisticated versions of the labelled antibody technique. These methods are used in hormone assays, drug monitoring, and to detect the presence of viral antigens (e.g., HAA) in serum.

Enzyme immunoassay

EIAs use enzyme-labelled antibodies that result in a visible reaction (Figure 31.20). This method utilizes less expensive equipment and therefore is becoming popular in developing countries. The test can be designed to detect either an antibody in a patient's serum, or an antigen (e.g., viral antigen) in circulation in a patient's body.

The process goes through four major steps. In the first step, commercially available antigen-coated beads are taken in a container and incubated with the serum specimen of the patient. The antigen on the bead is sought as the infectious agent. A patient's serum may have a primary antibody against the antigen sought. If the patient has the antibody against the antigen coated on the bead, it will become attached to the antigen. Subsequent washings in the following step do not take away the attached antibody on the bead. In the second step, a secondary antibody, linked with an appropriate enzyme, is laid on the bead holding the primary antibody. The secondary antibody is an antihuman globulin which reacts with any human antibody and thus gets stuck to the bead. The beads are again washed to remove any free secondary antibody. Now, we have the beads in the tube with the antigen of the pathogen, its primary antibody and the secondary antibody with the linked enzyme, all laid one over the other. Now in the third and final step, an appropriate amount of a substrate is added upon which the enzyme yields a visible colour. The colour will be proportional to the amount of primary antibody present in the patient's serum, and therefore, the reported results are considered quantitative in nature.

Radioimmunoassay

RIAs represent some of the earliest additions to the modern generation of immunoassays. They can be divided into either **solid phase RIA** (Figure 31.21) or **liquid phase RIA** (Figure 31.22). The illustrations provided will give you an idea of this sensitive analytical technique. The methodology requires expensive equipment for the measurement of radioactivity. This limits their popularity in developing countries.

Fluorescent antibody technique

In FA tests, a fluorescent dye is conjugated to the antibody. These dyes fluoresce when excited by a beam of UV light and epifluorescence microscopes must be used to read and interpret the reactions (Figure 31.23).

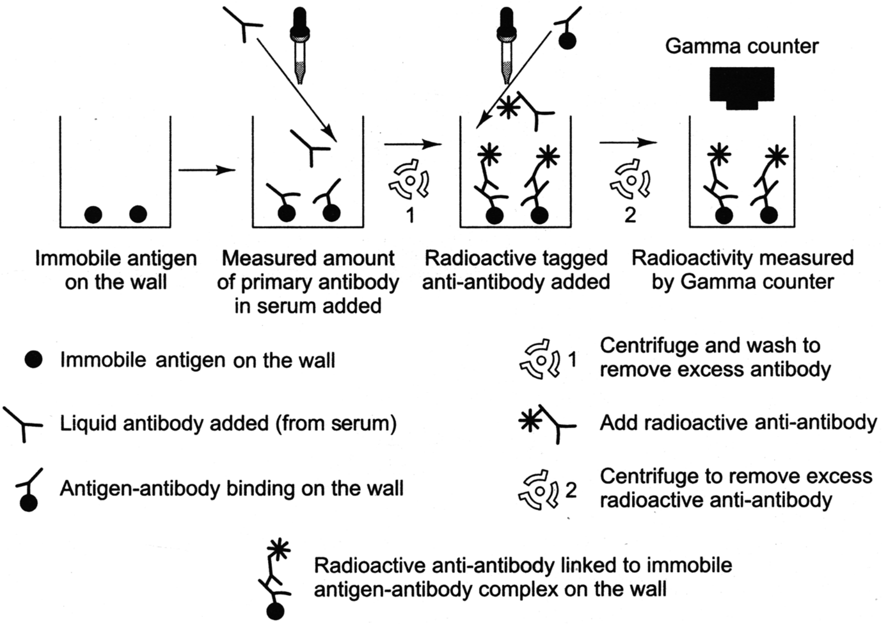


Figure 31.21 *Solid phase radioimmunoassay: Plastic tubes (or beads) are coated with non-radioactive antigen (or antibody) which reacts with the corresponding antibody (or antigen, Hepatitis virus HAA) present in patient's serum. The antibody (or antigen) gets fixed and the excess antibody (or antigen) is washed away by the buffer. In subsequent steps, radioactive anti-antibody (or antibody) is added which sticks to the antibody (or antigen, HAA) and the excess is washed away. The radioactive tubes (or beads) are then subjected to the counting of radioactivity. In case of absence of antigen (normal serum), radioactive antibody does not stick and the tubes (or beads) stay non-radioactive.*

An FA test can be direct or indirect. In the case of a direct test, there is no secondary antibody (anti-antibody) used (like tagged human globulin) to react with patient's antibody. The method allows the laboratory to detect antibody in a patient's serum or antigen in a patient specimen. FA tests are used in microbiology and parasitology. It is also used to detect auto-antibodies present in autoimmune diseases such as lupus.

The FA technique is successfully used in the diagnosis of syphilis. Syphilis is the venereal disease caused by *Treponema pallidum,* a spirochete or spiral bacterium. Because of the difficulty of growing *Treponema* in culture, the screening method of choice has been the detection of serum antibody. Since several non-syphilitic conditions can cause biologically false positive (BFP) reactions using other tests, including tuberculosis, hepatitis, pneumonia, pregnancy and rheumatoid arthritis, the fluorescent treponemal antibody test is considered both specific and confirmatory of a diagnosis of syphilis. A prepared slide of the laboratory strain of *Treponema* is incubated with a patient's serum, if the patient is suspected to have circulating antibodies associated with *T. pallidum* infection (syphilis). After incubation, the slide is gently washed to remove unbound non-specific antibody, and the specimen is then incubated with fluorescently labelled antihuman globulin. The slide is washed again and then examined under a fluorescent microscope. The presence of fluorescent-stained spirochetes indicates that the patient's serum is positive for antitreponemal antibodies. The method is also applied in the diagnosis of infection by *Mycobacterium* or *Cryptosporidium.*

A newer method called a *T. pallidum* microhaemagglutination assay (MHA-TP) is more suitable for underdeveloped countries, as it does not require the expensive fluorescent microscope. The method is discussed in Chapter 23 of Vol. 2.

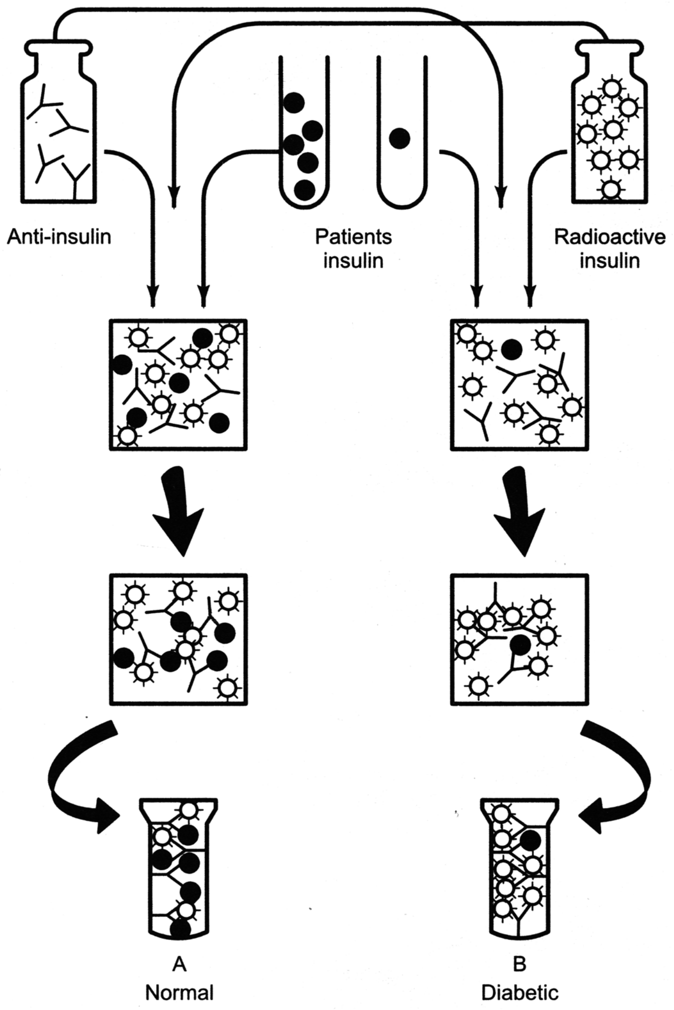


FIGURE 31.22 *Liquid phase radioimmunoassay: In this system, a competition is set up between the antibody (e.g., anti-insulin) and two kinds of the same antigen—radioactive (commercially available) and non-radioactive which comes from the patient. At the end, binding of the radioactive antigen is inversely proportional to the amount of antigen present in the serum. The method is applicable in the diagnosis of diabetic patient. The serum of a normal person has a higher concentration of insulin and thus less radioactive Insulin is attached to the antibody (series A), than in the diabetic patient (series B).*

INSTRUMENTATION FOR PROTEOMICS

The next generation of biomarkers for human diseases will be discovered using techniques found within the research fields of genomics and proteomics. **Genomics** use the known sequences of the entire human genome for determining the role of genetics in certain human diseases. **Proteomics** is the investigation of the protein products encoded by these genes. Protein expression is equal to and, in many cases, more important for disease detection than genomics because these products determine what is currently occurring within a cell, rather

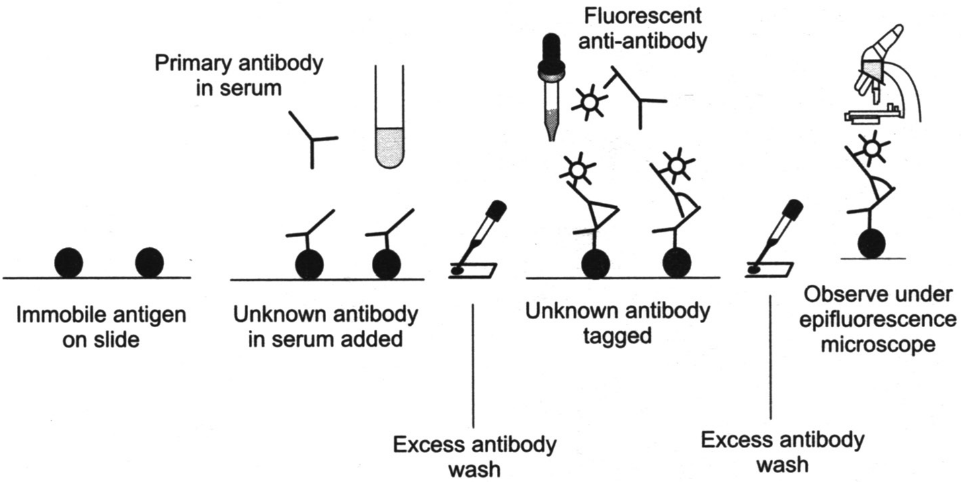


FIGURE 31.23 *Antigen (e.g., Treponema sp.) from laboratory culture is laid on the slide. This is reacted with its corresponding antibody, suspected to be present in the serum. The excess antibody is washed away after a while and then overlaid by fluorescent anti-antibody (anti-human globulin). This is followed by a second wash in order to remove the excess fluorescent anti-antibody. The slide is then observed under an epifluorescence microscope. If the patient's serum has the antibody against the antigen of interest, the antigens will glow and test will be reported as positive.*

than the genes, which indicate what a cell might be capable of performing. Moreover, many (posttranslational) changes can occur to the protein, as influenced by other proteins and enzymes that cannot be easily predicted by knowledge at the genomic level.

A "shotgun" approach is often used in the discovery of new biochemical markers. The proteins from samples (e.g., serum, urine, tissue extract) of normal individuals are compared with those derived from patients with the disease being studied. Techniques such as **two-dimensional electrophoresis** can be used to separate proteins into individual spots or bands. Proteins that only appear in either the normal or diseased specimens are further studied. Computer programs are available that digitally compare gels to determine spots or areas that are different. When candidate proteins are found, the spots can be isolated and subjected to sophisticated **Mass spectrometric (MS)** analysis. MS is an analytical chemistry technique that helps identify the amount and type of chemicals present in a sample.

OSMOMETRY

An osmometer is an advanced instrument used to measure the concentration of solute particles in a solution. The four physical properties of a solution that change with variations in the number of dissolved particles in the solvent are osmotic pressure, vapour pressure, boiling point, and freezing point. Osmometers measure osmolality indirectly by measuring one of these colligative properties, which change proportionally with osmotic pressure. Osmometers in clinical use measure either freezing-point depression or vapour-pressure depression; results are expressed in milliosmolal per kilogram (mOsm/kg) units. Measurement of osmolality and calculation of osmolar gap are useful diagnostic tools in pathological situations such as hyponatremia, or intoxication by methanol or ethylene glycol. It is necessary to handle reliable systems of osmolality measurement. As it is not done in routine laboratories, no further details are presented here.

ANALYTIC TECHNIQUES FOR POINT-OF-CARE TESTING (POCT)

Point-of-care testing (POCT) devices are widely used for a variety of clinical applications, including physician offices, emergency departments, intensive care units, and even for self-testing. Because analyses can be done at patient-side by primary caregivers, the major attraction of POCT is the reduced turnaround time needed to deliver results. In some cases, total costs can be reduced if the devices eliminate the need for laboratory-based instrumentation or if increased turnaround times lead to shorter hospital stays. POCT relies on the same analytic techniques as laboratory-based instrumentation: spectrometry, electroanalytic techniques, and chromatography. As such, the same steps needed to perform an analysis from the central laboratory are needed for POCT, including instrument validation, periodic assay calibration, quality control testing, operator training, and proficiency testing. The analytic techniques used in these devices are given in this section.

The most commonly used POCT devices used at bedside, in physician offices, and at home are the finger stick blood glucose monitors. The first-generation devices use a photometric approach, whereby glucose produces hydrogen peroxide (H_2O_2) with glucose oxidase immobilized onto test strips. The H_2O_2 is coupled to peroxidase to produce a colour whose intensity is measured as a function of concentration and measured using reflectance photometry. A schematic of this technique is shown in Figure 31.24. These strips are measured for glucose concentration without the need to wipe the blood off the strips.

The strip technology in a POCT platform can also be used to measure proteins and enzymes, such as cardiac markers. The separation of analytes from the matrix is accomplished by paper chromatography, in which specific antibodies immobilized onto the chromatographic surface capture the target analyte as it passes through. For qualitative analysis, detection is made by visual means. Reflectance meters similar to those used for glucose are also available for quantitative measurements.

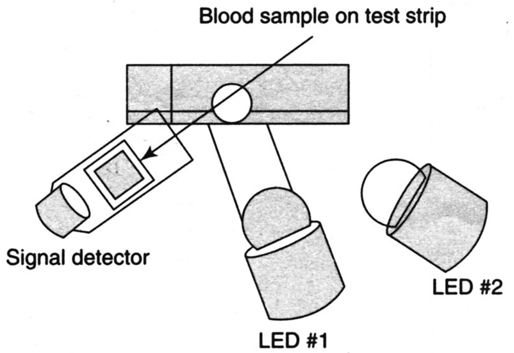


FIGURE 31.24 *Dual-wavelength reflectance photometry used in point-of-care glucose monitors. A microporous hydrophilic membrane is used as a reservoir of the sample to filter out solid cellular material from the reservoir and provide a smooth optical surface for reflectance measurements.*

The next generation of POCT devices uses biosensors. A biosensor couples a specific biodetector, such as an enzyme, antibody, or nucleic acid probe, to a transducer for direct measurement of a target analyte without the need to separate it from the matrix (Figure 31.25). The field has exploded in recent years with the development of microsilicon chip fabrication because biosensors can be miniaturized and made available at low costs. An array of biosensors can be produced onto a single silicon wafer to produce a multipanel of results, such as an electrolyte profile. Commercial POCT devices use electrochemical (e.g., microion-selective electrodes) and optical biosensors for the measurement of glucose, electrolytes, and arterial blood gases. With the immobilization of antibodies and specific DNA sequences, biosensor probes will soon be available for detection of hormones, drugs and drugs of abuse, and hard-to-culture bacteria and viruses such as Chlamydia, tuberculosis, or human immunodeficiency virus.

Electrochemical principles are also applied in wider range. This developing new technology has suddenly captured the market and has proved to be of great value for POC service, which may take place at home or in the physician's office. Several hand-held analysers such as

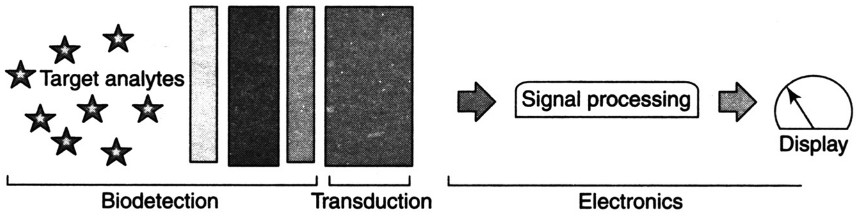


Figure 31.25 *Schematic diagram of a biosensor*

glucose meters are based on electrochemical technology. Other terms used for this technology include amperometry and coulometry. Patient samples are applied to disposable biosensors, strips that look similar to other reagent strips. These biosensors, in addition to containing reagent for the chemical reactions, also contain an electrode called electrochemical sensor. When the sample interacts with the reagents in the biosensor strip, the current (electrons) generated is detected by the meter and converted into glucose units.

In case of haemoglobin A1c (also termed glycated haemoglobin or GHb) determination, which is crucial for diabetic patients, the reports are available within 5 min using a table-top HbA1c analyser. The technology employed by the manufacturer is called **'affinity chromatography'** and requires only 10 µL of blood.

Affinity chromatography is almost exclusively used for the purification of biological molecules such as proteins (haemoglobin A1c) and other macromolecules. Biotech research has used affinity chromatography because of its ability to separate one desired species from a host of other biological molecules. It operates on the principle that ligands, attached to a matrix made up of an inert substance, bind to the desired molecule within the solution to be analysed (Figure 31.26a). Ideally, the ligand will interact only with the desired molecule and form a permanent bond. All other compounds (impurities) in the solution will be

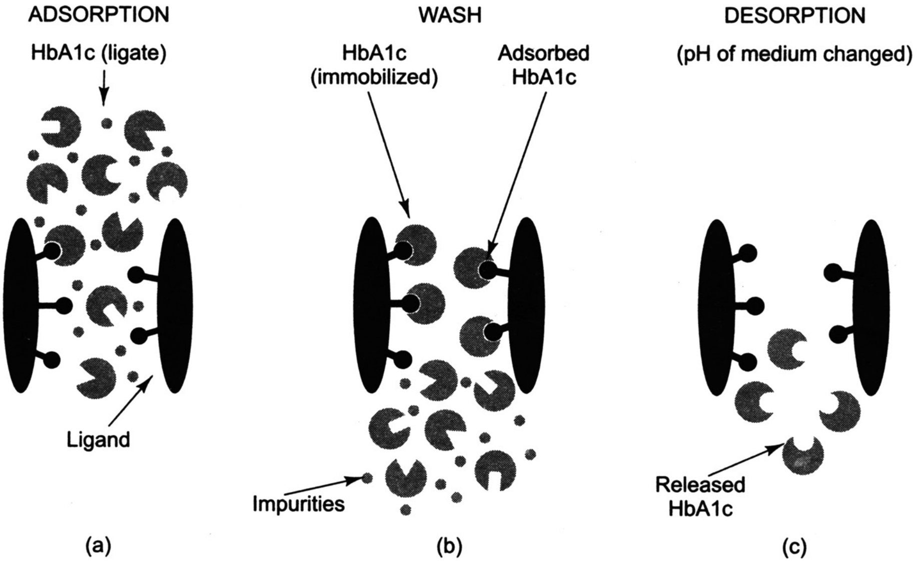


Figure 31.26 *Determination of haemoglobin A1c by affinity chromatography: (a) Blood specimen with HbA1c ligate is laid on the ligand that results in the adsorption of the HbA1c on the ligand due to its nature of selective affinity, (b) In the following step, the washing process removes the impurities but not the ligate, (c) The ligate (HbA1c) is then finally released by desorption for its measurement following change in the pH of the medium*

eluted (Figure 31.26b), leaving the desired product in the column. The desired molecule is then removed from the column by using a wash (typically changing the pH) that lowers the dissociation constant and allows recovery of a nearly pure sample (Figure 31.26c).

REVIEW QUESTIONS

1. What are the basic steps of analytical chemistry?
2. Define the following.
   (a) Standard solution (b) Control serum (c) Reagent blank (d) Water blank
3. What is Beer's law? Discuss its significance to analytical chemistry.
4. What is the principle of colorimetry? How does it differ from spectrophotometry?
5. How does absorption photometry differ from reflectance photometry?
6. State the basic difference between a colorimeter and a spectrophotometer.
7. What is the basis of solid phase technology? What is its future in developing countries?
8. Discuss the principle of electrophoresis and its application in the separation of haemoglobin and serum proteins.
9. Explain affinity chromatography with example.
10. How is gas liquid chromatography different from thin layer chromatography?
11. Show the basic components of a high pressure liquid chromatograph (HPLC).
12. List the components of an electrophoresis apparatus and state the function of each component.
13. Which of the following techniques is most suitable for developing countries? Enzyme immunoassay, radioimmunoassay, or fluorescent immunoassay
14. Which instrument will detect the immunologic reaction in an enzyme immunoassay?
15. What is the basic difference between nephelometry and turbidimetry?
16. What is the principle of the latex agglutination test?
17. How does the immunofluorescent assay confirm the diagnosis of syphilis?
18. What is a biologically false-positive report?
19. What is the principle of affinity chromatography? Give an example of its application.
20. How is glucose determined by hand-held glucometers?
21. What is the significance of an HbA1c determination?
22. What is the difference between the terms ligate and ligand?

Chapter
32

Automation in Clinical Biochemistry

Aurin Chakravarty, Dipto Chakravarty and Chhotelaal Pande

Chapter Outline

- Introduction
- History of Laboratory Automation
- Present State of Laboratory Automation
- Benefits of Automation in Clinical Laboratories
    - Layout for an automated Clinical Laboratory
- Classification of Automated Systems
    - Continuous Flow Analysis
    - Discrete Analysis
    - Centrifugal Analysis
- Steps of Automation in Biochemical Analysis
    - Sample Handling and Sample Measurement
    - Overcoming Interference
    - Transport and Delivery of Specimen and Reagent
    - Reagent Handling
    - Reaction Conditions
    - Reaction Measurement
    - Calculation of Results
- Quality Control and Preventive Maintenance
- Automated 'Stat' Testing
- Computers in Clinical Laboratories
    - Challenges of Computerization
    - Glossary of Terms
    - Operation of a Computer
    - Computerization of Clinical Laboratory Instruments
    - Conclusion

- Automation in the Clinical Laboratories of Developing Countries
  - AutoAnalyzers
  - Clinical Corona
  - Auto Pacer
  - SEAC (AMES)
- Point-of-Care Testing: A New Approach
- Time-Saving Devices and Kits
- Conclusion
- Review Questions

INTRODUCTION

Computer automation in clinical laboratories is the mechanization of the steps in a procedure. In this chapter, the reasons and rationales for automation are considered, including the ways to achieve it. Examples from the major instrument categories often seen in developing countries are also examined.

Contrary to common belief, automation is not intended merely to save labour, which may not be a limiting factor for the developing countries, but it allows reliable **quality control** (QC) measures to be imposed strongly. Other advantages include increased accuracy, less discrepancy in results, better QC, reduced subjective errors and the use of smaller quantities of samples and reagents, which may ultimately prove to be economical. More and more laboratories in the developing countries are thinking in terms of at least semi-automation because of poor quality of technicians, frequent labour problems, reduction of cost per test and increased reliability. The problems that the developing countries are facing include the repair and maintenance of equipment, and difficulty in obtaining spare parts. Alternate manual methods must be available in order to save the laboratory from complete breakdown.

HISTORY OF LABORATORY AUTOMATION

Laboratory testing has grown from a manual "hands-on" process providing a simple test menu to an instrument-centric, high-volume clinical engine inside the modern healthcare enterprise. The lines automation have grown both in size and scale during past seven decades with the advent and proliferation of computers. The "transport belts" for laboratory samples bring fast and accurate routing of specimens to specific points in the laboratory workflow. With the fast increase of laboratory requests, now we see the automation a necessity and not a luxury.

Laboratory automation began in 1950s and its history includes the evolution of laboratory instruments and growth of the laboratory information (management) systems (LIS/LIMS). The first automated instrument was introduced in 1957. This instrument, the Autoanalyzer I, utilized continuous flow analysis (CFA) to dramatically increase the instrument throughput (20 samples at a time) to leap past the manual processes of the time. This instrument started the drive to automate the clinical chemistry laboratory workflow at the level of the assay. Following the Autoanalyzer I, instruments like SMA (Sequential Multiple Analyzer, 1969) and SMAC (Sequential Multiple Analyzer with Computer, 1974) came to market with larger test menus and higher throughputs. This trend continues today with each new generation of instruments that elevate the volume of work and menu of assays.

The laboratory information systems/laboratory information management systems began to take shape in the 1970s. Thus came the electronic data management to the laboratory to manage the workflow and electronic interfaces to the instruments. This helped to eliminate successfully the paper-based logs and capturing data electronically, directly from laboratory instruments. Coupled with automated instruments, the throughput of the clinical laboratory dramatically increased around the instruments. Simultaneously the increased speed of automation could handle more specimens at a rate greater than what a laboratorian could provide. Despite having automated ordering, resulting, and test performance, the pre- and post-analytic workflows were highly manual processes at the end of 1970s. Thus came automation in specimen identification (perhaps accessioning and labelling, if not already done) followed by routing to the correct part of the laboratory. The pre-analytic phase alone is estimated to have consumed 60% of the time and effort in the total specimen workflow. The estimates of the contribution of pre-analytic error to total laboratory error range from 30% to 86%.

PRESENT STATE OF LABORATORY AUTOMATION

With the three components of automation growing within the clinical laboratory in late 1980s — instrument-level automation, growth of the laboratory information (management) systems, and early pre- and post-analytic automation—the strategies for laboratory automation have taken a variety of routes. First, the degree of automation at the level of instrument has grown to handle higher throughputs as the demands of laboratory have grown. Second, improvement in data management and information technology (IT), went hand-in-hand to effect end-to-end platforms that govern the entire business process and workflow of the laboratory in order to make automation for a laboratory to be successful.

In developing countries, slow progress of automation in laboratories is due to various reasons but two of those stand foremost—poor economy and readily available technical service.

BENEFITS OF AUTOMATION IN CLINICAL LABORATORIES

Given below are some of the benefits of automation in clinical laboratories:

1. Cut healthcare costs.
2. Maximize efficiency and minimize errors by integrating mechanical, electronic.
3. Informatics tools to perform an ever expanding variety of laboratory tasks.

Statistics from developed countries

Following the installation of automation error reduction rates exceed 70%, while staff time per specimen collection is reduced by over 10%. Patient safety is increased by an average 50% reduction in specimen turnaround time directly attributable to automation.

Conclusion

Laboratory automation has become a well-accepted technology that allows high quality, efficient, and patient-centric operation with low operating costs. Automation should also be the foundation on which a Six Sigma program can be built and maintained. Technological advances will increase the number of laboratory unit operations that can be either partially or fully automated. Near patient or personal wellness testing should be considered an extension of the laboratory automation enterprise.

Layout for an Automated Clinical Laboratory

- Specimen arrivals should take place at the proximal end of the laboratory where accessioning can occur immediately after unpacking, and the automated sorter should be readily accessible at the end of the accessioning line.
- Each analytical automation line should run in parallel so that bench scientists can service several analytical areas with as few steps as possible.
- Completed specimens should be automatically stored at the distal end of the laboratory conveyor belt so that they may participate in reflex, repeat, or add-on testing without human intervention.
- Delivery and storage of analytical reagents is ideally accomplished in a bank of refrigerators/freezers with doors on both sides of the laboratory installed parallel to the analytical systems they serve. Stocking occurs from the back of each unit and retrieval of reagents from the front.

CLASSIFICATION OF AUTOMATED SYSTEMS

Automated systems are seen only in the urban settings of both advanced as well as in the developing countries. They can be grouped under three basic categories.

Continuous Flow Analysis

This is the first automated system introduced into the market in the 1960s by Technicon (USA) that has since been followed by other systems. In this system, liquids such as specimen, reagents and diluents are pumped through a continuous tubing system leading to continuous flow. Samples are introduced in a sequential manner, following each other through the same network. A series of air bubbles at regular intervals serve as separating and cleaning media. The system is most profitable for larger laboratories. The commonly used models SMA 6/60 and 12/60 process 60 specimens per hour and, respectively, report the results of 6 and 12 diagnostic tests simultaneously. This automated system suffers from the disadvantage that the physician may not be interested in all the tests reported by the instrument. Conversely, he may be interested in some other tests, which the instrument does not report.

Discrete Analysis

In this system each sample is separated along with the accompanying reagent. This can be used both for special tests as well as for routine tests. It is ideal for smaller laboratories. Some of the popular ones that fall in this category are Clinicon (Boehringer Mannheim) and Auto Pacer (Miles of India Ltd.).

Centrifugal Analysis

This is the most recent introduction in the markets of developed countries that can be broadly classified under the discrete system. After placing the specimens and the corresponding reagents in their places on the special centrifuge head, the machine is run and the force generated by centrifugation transfers the reagents and the specimen to a cuvette for chemical reaction. Optical measurements are made while the cuvette is in motion (2500 rpm). The method is most useful in performing enzyme assays.

In the following sections, we present the basic principles of these technologies without going into many details.

STEPS OF AUTOMATION IN BIOCHEMICAL ANALYSIS

The steps of biochemical analyses can be broadly divided under the following headings—sample handling, sampling processing, reagent handling, analytical procedures (e.g., mixing, heating, etc.), reaction analysis (e.g., reading the absorbance) and calculation. The approach of various automated systems to expedite these steps is discussed here. The goal of this description is to give an idea that automation does not replace our classical approach but it only makes adjustments to speed up the manual process.

Sample Handling and Sample Measurement

Very little automation is applied in this step. By and large, the specimens are manually labelled, centrifuged and divided into aliquots if tests for more than one work station have been requested. This manual processing of the specimen is also important from the point of view of rejecting those specimens, which are not acceptable for the analysis (e.g., haemolysed or lipaemic specimens, presence of debris), and review the request form received with the specimen. Extreme care is needed in this step to avoid mislabelling of patient samples or their aliquots.

In automated systems, the instrument must be fed with the specimen in an **identifiable manner.** Therefore, the sample-holding containers must be placed in the same order and the technician must write down clearly the order of the arrangement and should never rely on his memory. One should anticipate the problem involved when there is a mix up. Some laboratories prefer to number the sample cups so that if they are misplaced, they can be put into the proper slot. In most automated systems, the samples are held in small cups which are placed on a circular tray. The slots are sequentially numbered. One can use an **automatic pipetter** for taking aliquots of specimens into the sample cups. Use a fresh tip for each sample which may be disposed of or recovered after thorough cleaning and drying. In case of continuous flow analysis, the requisite amount of specimen is picked up by the sampler while in others an aliquot of the specimen may have to be manually placed. Probes used in the automated systems for picking up specimen must be thoroughly washed, preferably with a diluent, before the following specimen is drawn. One convenient way to avoid **carry over effect** is to dispense the specimen first and then the diluent.

While handling the sample one must avoid the exposure of the aliquot to the air for a prolonged period. This will increase the concentration of sample constituents due to evaporation and aliquots taken after evaporation will lead to false high results. Decay of the constituents should also be considered.

Overcoming Interference

Protein causes major interference in many analyses. In the continuous flow system, use of a **dialyser** eliminates the protein in order to facilitate the determination of other components. The dialyser is, however not used for total protein determination. In other systems, there is no such step as protein removal. They either use such a method as will be least interfered with by protein; or they use a more sensitive method, or they add appropriate chemicals to remove the protein. Some discrete analysers read absorbance with two wavelengths (bichromatic) which also help to reduce the effect of protein.

Transport and Delivery of Specimen and Reagent

The volume of reagent required for the determination of a given analyte is related directly to the volume of sample used. Most chemical reactions rely on the combination of reagents and sample in exact proportions to yield accurate results. For most instruments, the manufacturer has predetermined these proportions. However, some manufacturer allows the instrument users to develop and use their own test applications.

In unit test systems, the volume of reagent has been pre-measured. The **'dose'** of reagent, already in the reaction vessel, needs only to be delivered to the area where the sample will be added. This is usually done mechanically by pushing the reaction container to the sampling station or loading zone.

In continuous flow systems, **peristaltic pumps** and plastic tubing transport the specimen and reagent through the system. The volume of reagent is determined by the inside diameter of the rubbing, just as the volume of sample is determined. As the samples flow continuously through the tubing, reagents are added at different points, reaction conditions are provided around the tubing, and timing is determined by the distance of the coil to travel through the reaction chamber.

Positive displacement syringes are another method to deliver specific volumes of reagent to a reaction chamber. These syringes operate in the same manner as the positive displacement syringes used for sample aspiration and delivery. The speed of the driving motor should be controlled as closely as possible to prevent sudden changes in velocity and the accuracy should be verified.

Reagent Handling

Most chemical reactions require the combining of the reagent and sample in exact amounts. This is called proportioning. In continuous flow analysis, the diameter of the tube regulates the volume of the reagent fluid picked up; for the amount of specimen, the dwelling time of the probe inside the specimen container determines the amount of specimen picked up. The rate of flow for all fluids is the same. A single peristaltic pump is used for drawing the fluids. For the discrete system, a single probe may measure the volumes of specimens and reagents but the dwelling time in them varies. Individual automatic dispensers are also used in, some discrete systems where syringes and volumetric overflow devices are used to dispense requisite quantities of sample and reagent into test tubes or containers. A single-reagent assay is ideal for automated biochemical analyses. This, however, is not possible and single-reagent assay is not as accurate.

Reagents can be dispensed directly from bulk containers supplied by the manufacturer, or reconstituted in the laboratory. Reagents prepared by the manufacturer are expensive and under the conditions of most developing countries with transportation difficulties and cold storage problems, the preparation of reagents in the laboratory becomes a necessity. Hence those manufacturers, who are able to supply dry reagents or provide the necessary formula for the preparation of reagents within the laboratory, will be most successful in marketing their goods in the developing countries.

Specimen carry-over is a common problem in continuous flow analysis. Thus, if a low-concentration specimen follows a high-concentration specimen, the former will have carry-over effects due to contamination. Either in such a case the specimen should be re-run or water blank introduced to flush out the system. Alternatively, increase the wash time between specimens.

Reaction Conditions

Mixing of reagents with the specimen is a vital component of biochemical analysis. This is accomplished in several ways in the automated systems. In the continuous flow, it is done through glass coils where the liquid rotates and the liquids (specimen and reagents) fall through one another during their rise and fall through the loop. In the discrete systems, other methods are adopted—vibration, slewing action, centrifugal rotation (in centrifugal analysers), pressing and releasing of plastic bags which receive the fluids and ultrasonic waves.

Automated incubation is merely a **delay station** where the test mixture is allowed to react. The chamber where the incubation is held is heated to the desired temperature by the use of a heating block, air, water bath or oil bath. Time is a definite limitation. To sustain the advantage of speedy multiple analyses, the reaction is not taken to completion, as is required in the manual procedures. Rather, the rate of the reaction can be measured and the values after the completion of the reaction are extrapolated. The instrument may also delay the measurement for a pre-determined length of time or present the reaction mixtures for measurement at constant intervals of time. In case of continuous flow analysis, all measurements are made against a standard and as the procedures are precisely timed, the result of the standard is highly reproducible even if the measurement is made much before the completion of the reaction. In the discrete system, there are a number of delay stations before the readings are taken.

Dry slide methods accomplish mixing of the sample with premixed, pre-measured reagents through diffusion. As the sample comes into contact with the top layer of the slide, it is drawn by capillary action into the porous layer. As the reagents hydrate, components of interest diffuse into the reagent layers.

Discrete system mix reaction components by means of stirring paddles of sticks, motion of the reaction vessel, forceful addition of reagents and agitation by air bubbles or ultrasonic waves. Some system use magnetic stir bars.

Mention should be made of the use of **ion-selective electrodes** for the measurement of sodium, potassium, chloride and occasionally carbon dioxide. The reagent handling systems and reaction conditions for ion-selective electrodes are typically separate from those previous described with colorimetry or enzymatic measurement. Most ion-selective electrodes are of the flow-through variety in which the sample and reference solutions are moved via peristaltic pumps through chambers containing fixed indicator and reference electrodes. The specimen must remain in contact with the electrodes long enough to reach steady state. The electrodes are designed to minimize response time so that a steady state can be reached rapidly, maximizing the throughput of the system.

Reaction Measurement

After the reaction is completed, there must be a sensing device to measure the change like development of colour. Traditionally, **absorbance photometry** has been used for the measurement of analytes. This method has three basic components—light source, spectral isolation and a detector. **Light** sources commonly include tungsten, quartz-halogen, deuterium, mercury and xenon lamps. Each has advantages and disadvantages. Interference filters, monochromators, such as diffraction gratings help in **spectral isolation.** Diffraction gratings provide a continuous spectrum and therefore a great choice of wavelengths for use. A diffraction grating also allows the use of two or more wavelengths (bichromatics) at the same time for correction purposes, to eliminate the absorbance contribution of interfering substances. Photomultiplier tubes (PMTs) are the most prevalent detectors in automated systems. They are sensitive and give a very rapid response.

In recent years, methods other than absorbance photometry have been developed for the measurement of analytes. Reflectance photometry is one of them. In reflectance photometry, diffuse, reflected light rather than absorbed light is measured. This is used in many equipment that employ **'dry chemistry'.** One drawback to reflectance photometry is that intensities are not linear with concentration of the analytes of interest; they do not follow Beer's law. To correct for this deviation, mathematical algorithms are used to linearize the relationship between the intensity and reflected light and the analyte concentration. Other photometric methods include turbidimetry, nephelometry and fluorometry. Many of these techniques are used in automated immunoassays.

The **sensing** can be done on the original site where the reaction occurred (internal) or taken into another vessel (external) to make the measurements. In the continuous flow analysis, the reagent stream under analysis flows continuously through the flow cell which acts like a cuvette. The air bubbles are removed by the de-bubbler before the reacted solution enters the flow cell for photometric measurements. The sensing device converts the optical response into electrical impulses which are then sent to the read out device. The read out device can be the strip chart on which the results are traced or on the digital display.

Chemical reactions can be monitored either at one time point or at many. Commonly, **single-point** monitoring is used for **end-point** (or midpoint) analysis in which the reaction has gone to completion or the instrument extrapolates the value, as mentioned earlier. Multiple-point monitoring is done in case of kinetic studies (enzyme activity).

Calculation of Results

In the automated system, the results are automatically computed from the response of the sensor and finally printed out in appropriate units. The printing can be done directly on the patient's request slip (or reporting slip) or on the laboratory form. In order to avoid transcription errors, it is advisable that multiple copies of the print out should be made so that different copies of the same report can be routed to different destinations—financial office, physician, patient's record, laboratory record and others.

QUALITY CONTROL AND PREVENTIVE MAINTENANCE

Automated systems are not free from errors. In fact, they frequently render **a false sense of security** among the users and the results may be far from reliable. The system must be frequently subjected to **QC procedures** using control sera. Control runs should be done in the beginning of each day and the results plotted on the QC chart (Chapter 7 of Vol. 1).

Preventive maintenance is done to ensure the analyser continues to function properly. Keeping an analyser clean may be the most important maintenance procedure, regardless of the instrument. Cleaning up spilled specimens and reagents will help prevent future malfunctions. Other maintenance procedures include discarding waste, cleaning water baths, cleaning reaction vessels (if reusable), replacing reagents, replacing worn or damaged parts (e.g., filters, rubbing syringes, probes and lamps), and readjusting components to ensure proper functioning.

AUTOMATED 'STAT' TESTING

The word 'stat' is an abbreviation of the Latin word **statin,** meaning immediately. Many clinical conditions require **immediate** reporting of results. If the automated system cannot be adapted towards such a condition of changing the priorities, it will be a handicap. In some systems, such as the continuous flow analysis, the interruption in the processing of the current samples and the time required to change to the proper reagents may not be acceptable or practical. In such cases discrete analysers are handy. The other requirement for automated stat procedures is to have a short dwell time. The machine may not have a high **throughput.** Throughput is an expression that indicates the maximum number of individual samples or test analyses that can be practically performed per hour by an assay system with the required dwell time taken into account.

COMPUTERS IN CLINICAL LABORATORIES

Computing and use of computers has become pervasive in our society. With commoditization of personal computers, and availability of broad spectrum of software applications, computers are used in every scientific field, including the field of laboratory medicine. The era of computerization has effectively driven high levels of QC, while increasing the laboratory output, and expediting the effectiveness of diagnosis.

Computer application in the clinical laboratory can be classified into five groups:

1. ***Specimen handling:*** Identification and recording
2. ***Operation of instruments:*** Automatic and semiautomatic

3. ***Storage of information:*** Results of laboratory findings
4. ***Communication:*** Facilitates information transfers between laboratories and the physician
5. ***Robotics:*** Robot-driven automation (commonly referred to as *robotics),* further streamlines the processes in clinical laboratories. Robotics is applied in two ways: to reduce the risk of humans in handling contaminated samples, as well as to achieve higher level of precision

Computer systems have many definite advantages over other laboratory management systems. Not only they are capable of processing vast amounts of data with higher accuracy; they are also able to store and retrieve information in real-time, thus making the end-to-end performance evaluation (QC) of laboratory instruments with new degrees of efficiency.

Computers have become a pervasive part of the human society. The usage and applications of computers and computer-assisted systems have proliferated across the advanced nations as well as third world countries. Given the widespread application of computers, it is desirable to understand WHAT types of computers exist and HOW basics of computers work.

A **computer** in its simplest term is expressed as a machine that manipulates data according to a list of **instructions.** The ability to store and execute instructions called **programs** makes computers extremely versatile and distinguishes them from calculators. The heart of a computer is its central processing unit (CPU), which is essentially a sophisticated integrated circuit, which enables it to carry out arithmetic functions, logic functions and to move data in and out of memory. Memories are of two types: one is referred to as random-access-memory or RAM, which is programmable and erasable, and the other is called read-only-memory or ROM, which normally cannot be modified. This part of the memory allows the computer, in conjunction with the CPU, to function independently of human input, a feature that allows for almost complete automation in the laboratory, which is the focus of this chapter.

The aforementioned parts of the computer, along with a variety of input devices (such as keyboard, mouse, scanner or a tablet) have become a very useful tool in the laboratory. Although it may not be obvious at times, several automated laboratory instruments have incorporated these devices into their designs in a pervasive way. Add to it, a variety of output devices (such as screen, printer, plotter or speaker), which has made data retrieval very simple. Via this modernization of technology, every laboratory has benefited in terms of effectiveness and efficiency.

Computers come in many different sizes and complexity depending on their use.

1. **Supercomputers** and mainframes are the most powerful machines, and tend to have no use in the average clinical laboratory setting. These machines are equivalent of multiple high performance computers working in parallel as a single system.
2. **Servers** are computers that have been optimized to provide services to other computers over a network. Servers usually have powerful processors, lots of memory and large hard drives. This class of machines used to be referred to as mini-computers.
3. **Workstations** are high-end desktop computers that typically have powerful processor, and enhanced capabilities for performing a special group of task, such as 3D graphics or game development.
4. **Desktops** or personal computers are the most common and widely used, and have proliferated in office and laboratories due to their small portable size and versatility. The personal computer defines a computer designed for general use by a single person. While a Mac is a PC, most people relate the term with systems that run the Windows operating system. PCs were first known as microcomputers because they were a complete computer but built on a smaller scale than the huge systems in use by most businesses. A PC that is not designed for portability is a desktop computer. The expectations with desktop systems are that you will set the computer up in a permanent location.
5. **Laptops** or Notebooks are portable computers that integrate the display, keyboard, a pointing device or trackball, processor, memory and hard drive all in a battery-operated package slightly larger than an average hardcover book.

6. **Handhelds** are palm-top computers (commonly called Personal Digital Assistants or PDAs) are tightly integrated computers that often use flash memory instead of a hard drive for storage. These computers usually do not have keyboards but rely on touch-screen technology for user input. PDAs are typically smaller than a paperback book, very lightweight with a reasonable battery life.
7. **Wearables** are ultraportable computers that can be integrated into watches, cell phones, visors and clothing. This class of computers support common consumer applications such as email, camera, database, multimedia, calendar and phonebook.

Challenges of Computerization

Automation and computerization are enablers for efficiency and effectiveness. However, the steps of computerization in the laboratory are not necessarily a panacea. As with all machineries, the computer is subject to upkeep and breakdown. Environmental conditions can adversely affect the computer and its peripheral equipment. Excessive heat, humidity, dust, magnetic fields, smoke and transient loss or changes (even minor) in electrical line currents, can cause adverse results, such as data loss or data corruption. Therefore, wherever computers are deployed, it is imperative that:

- Electrical voltage stabilization is provided, with preferably uninterrupted power supply (UPS) backup units.
- Backup copies of all data (raw or otherwise) are kept in an off-line storage so that the information is intact and the data can be re-created in the event of a problem.
- Automated procedures can be bypassed manually or alternative procedures be made available in the laboratory, particularly those procedures which are vital in the emergency situation. In cases where electricity is a frequently interrupted, supplemental unit such as voltage stabilizer, battery backup (UPS unit) or inverter is essential, depending on the extent of power fluctuation and/or the duration of power loss. A typical UPS unit usually keeps the computer running until data gets saved to the desk and the computer has had a chance to shut itself down gracefully.
- In developing countries, a fully redundant computer system that can function as a back-up is desirable due to the frequent scarcity of technical labour to expedite the repair of the primary computer in the event of a failure.

In the following section, an attempt has been made to provide a primer to orient the reader's general understanding of computers. The concepts include hardware, software, interfacing, storage, input, output and computer compatibility.

Glossary of Terms

Before beginning the discussion of the computer, familiarization with some of the terms commonly used in computer technology is necessary:

Address: The physical location of a piece of information within the computer's memory.

Algorithm: The series of operations required to do a simple task. For example, algorithm might be set up to calculate the mean from a list of numbers.

Binary code: It is a numeration system having a base of 2. It has only 0's and l's (or 'ON' and 'OFF' switches), which are expressed in powers of the number 2. The word 'bit' evolved from binary digit which happens to be the computer's smallest unit of data representation. A bit is like a toggle switch, which can have only two possible states, either 'on' or 'off'.

Booting: The process of starting a program of the computer after it has been turned off. It is derived from the phrase 'pulling oneself up by one's bootstraps'.

Browser: An 'easy to use' standard user interface which is the most popular way to interact (or browse) with the interconnected computers on the Internet.

Bug: An error or problem in the code of a program. It can be a logical or a typographical error.

Byte: A set of 8 bits makes a 'byte' that represents a character to the digital computer (e.g., one alphabetical or numerical character is represented by a byte).

Code: The set of instructions written to perform a task in the computer. A code may be written in any language compatible with the computer.

CD: Compact Disc or CD is a low-cost analogue optical storage medium that has essentially replaced the floppy disk. There is a variety of CD-s available, depending on user's need. Some common variations of CD (also called CD-ROM) are, write-once audio and data storage CD-R, rewritable media CD-RW, Super Audio CD (SACD), Video Compact Discs (VCD), Photo CD, Picture CD and Enhanced CD. Of these media types, CD-ROMs and CD-Rs remain widely used technologies in the computer industry.

Database: This is the file or series of files in which all the data of a project are stored. From the database other files can be made to suit various applications and produce various reports.

Directory: The index of file names and locations in a data-storage device such as a disk; it corresponds to the contents of a book.

Disk: An internal or external device that stores data.

DSL: It is a data communications technology that enables faster data transmission over copper telephone lines than a conventional voiceband modem can provide. It does this by utilizing frequencies that are not used by a voice telephone call. The acronym DSL or xDSL, stands for Digital Subscriber Line.

DVD: Digital Video Disc or DVD is a low-cost digital optical storage medium that has essentially replaced the floppy disk and CD-s. There is a variety of DVD-s available, depending on user's need. Variations of the term DVD often describe the way data is stored on the discs: DVD-ROM (Read Only Memory), has data that can only be read and not written, DVD-R and DVD+R can record data only once and then function as a DVD-ROM. DVD-RW, DVD+RW and DVD-RAM can both record and erase data multiple times.

File: A set of data blocks, which corresponds to a chapter in a book. In a laboratory computer, there might be a file of all the tests offered by the laboratory along with their names, costs and reference ranges.

Disk: A device that stress the computer data. It may be internal or external to the computer. Also, it may be removable or fixed, depending on how it is hooked up. Popular storage devices are USB drives, Media Cards and portable drives, which have replaced the floppy drives in the last decade.

Hardware: This is a general term for the physical equipment used in a computer system. Hardware includes the CPU, the monitor, keyboard, mouse, printer, etc.

HTML: HTML or Hyper Text Markup Language is the predominant mark-up language for web pages. It provides a means to describe the structure of text-based information in a document and to supplement that text with interactive forms and embedded images. HTML is written in the form of tags, surrounded by angle brackets.

Instruction: Commands given to the computer that specify a set of operations that the computer has to do.

Interface: The connection between different components of a computer system or between different computers.

Internet: A global network of interconnected computers worldwide, linked via a variety of connections, which may be via modem, cable, ADSL or satellite link.

I/O: Acronym for Input/Output

Magnetic tape: A data storage medium which closely resembles the tape used to record music. These devices used as low-cost, high-capacity storage medium for data, but have been phased out given the advent of new modern technology.

Memory: The part of a computer system which stores data and programs for use by the computer.

Microcomputer: A computer who's CPU is a microprocessor. It is usually a desk top or portable machine having a substantial amount of memory, and a limited number of I/O ports or peripherals.

Microchip: A semiconductor device with the property to hold transient or permanent data. It has extensive use in electronic industries. Integrated electronic circuits are embedded on silicon wafers to construct these microchips.

Microprocessor: A processing unit built in a single silicon chip. They are used to control and execute tasks involved in the operation of electrical equipment.

Modem: An acronym for modulator-demodulator. It is a device used for communication facilitation connecting two computers or computer systems through a telephone line. Modems have become less common, given the advent of the Internet and 'on-line' systems.

Program: A set of step-by-step instructions designed to perform designated tasks on a computer.

RAM (random-access memory): Memory to which one can both read and write, changing the contents many times during processing. RAM is used to store data temporarily in the sense that these data are retained only as long as the power is on *(refer ROM).*

Random access: The ability to have access to a piece of information from an array without having to look at all the elements from the beginning. An example of random access is the ability to play a particular selection on a phonograph record without having to play all the record up to that point.

ROM (read only memory): Storage whose contents cannot be changed by stored program instructions; it generally contains non-alterable programs and built-in functions of the computer *(see RAM).*

Software: The generic name for the programs, routines and the operational procedures for computers.

Monitor: The most common display device for computers. It usually consists of a keyboard and mouse that helps interact with the computer.

USB: Universal Serial Bus is a serial bus and I/O standard to connect devices to a host computer. It was designed to allow several peripherals to be connected using a single standardized interface socket and to improve plug and play capabilities of multifarious computer peripherals such as mouse, keyboards, PDAs, gamepads, joysticks, scanners, digital cameras, printers, flash drives and external hard drives.

Word: Group of bits which the computer processes at a time. The size of a word ranges from 8–64 bits and is defined by the hardware and software used in any particular system.

World Wide Web: A 'web' or network of networks, which links millions of computers together, and allows the user to interact with the information via an 'easy to use' standardized universal user interface, called the 'browser'.

XML: XML or eXtensible Markup Language is a general-purpose specification for creating custom markup languages, and its most common purpose is to aid information systems in sharing structured data, primarily over the Internet.

Operation of a Computer

A computer is made operational by combination of two major components—the hardware and the software.

Hardware

The hardware is the physical structure of the computer which has three major components—CPU, Input/Output and Memory, as outlined in form of a conceptual view of the computer in Figure 32.1.

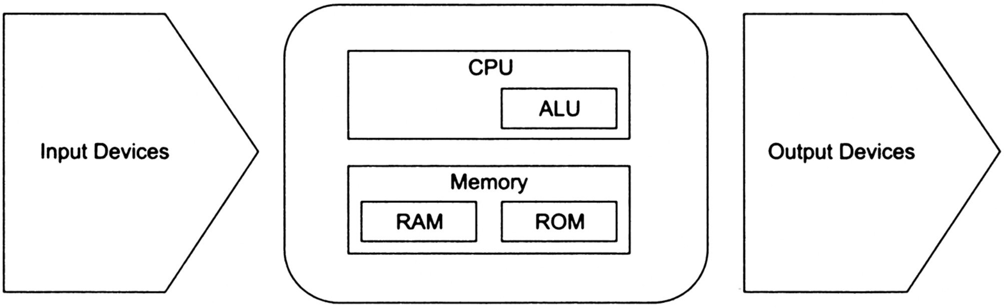


FIGURE 32.1 *Conceptual view of primary parts of a computer*

Central processing unit This unit coordinates and directs all the operations of a computer system. The CPU has two components, the arithmetic-logic unit (ALU) that performs numerical computations, and the control unit (CU) that regulates the timing and coordination of machine activities.

Physically, the CPU is a unit of electronic circuitry (integrated circuit). Miniaturization of technology has made large-scale integrated circuits fit in tiny footprint, thus shrinking the size of the CPU. A computer can have a single or multiple CPUs, which functions as a cluster, and supervises the operations within the computer. The other components such as ROM and RAM are under the control and governance of the CPU.

When a software program is executed, it is the CPU that orchestrates its execution by performing the operations specified by the encoded instructions (Figure 32.2). When a mathematical calculation is involved the ALU is activated; if storage of data in memory or secondary storage is required, the CPU sets up and supervises the storage of data. If a printout of data is called for by the programs, the printer is activated and the data from the memory is passed through the CPU and transmitted to the printer. The connectors which indirectly link the CPU with its peripherals are referred to as the **interface cards.** In summary, the CPU is the brain and **'command centre'** of the computer.

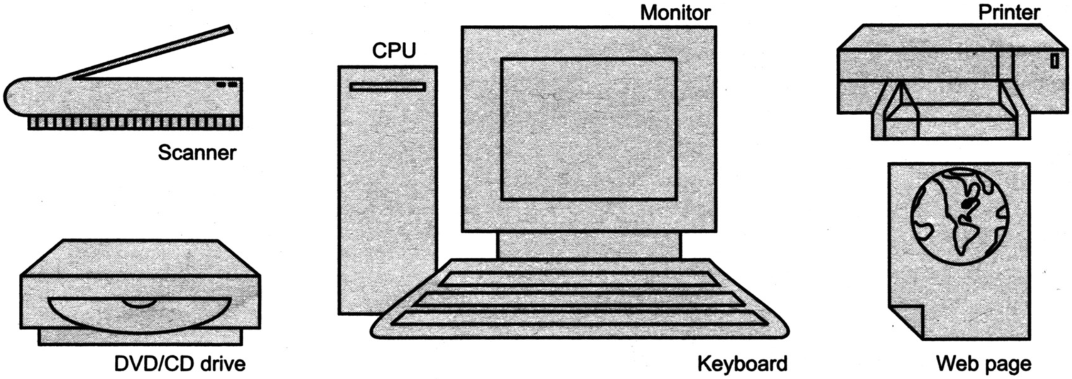


FIGURE 32.2 *Components and primary parts of a computer*

Input/Output (I/O) This interface unit transfers information into and out of the computer. It may include a monitor, keyboard, mouse, scanner, printer, bar-code reader, speaker or a laboratory instrument. Some of the common components of the computer are shown in Figure 32.2.

Memory/storage unit The memory unit stores data for use by the computer. There are two kinds of storage units—the primary storage unit and the secondary storage unit. The primary storage unit is built into the computer system. Part of primary storage contains permanent information, which never gets erased or altered. This is called ROM. It contains pre-defined tasks or perhaps a built in computer language. The other part is called RAM. This is designed to contain temporary information and is addressable and erasable. It may contain the user's computer program, program data and its computed results.

Secondary storage units (also called external memory) can be thought of as being **'accessory'.** Its usage is not indispensable for a computer. Disk drives, tape drives or removable media are some of the most widely used secondary storage units. Their purpose is to expand the memory and to store data for later usage, accessing the data as needed by the program.

Conceptually, the memory is a large array of electronic circuits. Each individual circuit may be switched **'on'** (coded as 1) or **'off'** (coded as 0). As long as there is an electric current, the condition of being on or off codes the memory with bits of data. The ability to manipulate data in the form of an on/off code is the fundamental task of the digital computer's CPU. The on/off property of microchips makes them **binary machines.** As a result, many coding attributes are in powers of 2. The simplest memory element, the on/off (I/O) condition, is termed a bit and groups of 8 bits are called **bytes.** Memory is generally expressed in terms of megabytes (MB), which is approximately a million bytes. A single character, like the letter *'A'*, takes up 1 byte (or 8 bits) of memory. It follows that in one line of 80 characters, 80 bytes will be required. ***(Note*** Each space should also be considered as a character).

The discussion of computer memory would be incomplete without mentioning two terms—the address and the content. In order to logically store data in the computer's memory, data must be placed in an orderly way into locations within the computer's memory array. The location of the data within the array is called the address and the information stored at the address is referred to as content. The content may be determined by addressing the memory location. A **programmer** prepares the program (software) using a suitable computer or programming language. On booting up the program, the stored codes get converted by a translating unit into binary language. It then gets loaded into the RAM and the program is ready for execution. Pre-written programs, which are saved on disk, card or tape, can be re-loaded time and again into RAM.

The laboratory technician need not know anything about the 'program' or computer to accomplish his goals. He only needs to be familiar with the program's purpose and the user's commands.

Software

The programs and routines involved with the operational procedures are broadly referred to as software. The computer does not understand the human language. Hence, the instructions must be interpreted into a language that the computer understands. To facilitate programming, various computer languages have been developed which resemble the English language. The process of programming is relatively complex and requires a set of specific skills; rules must be followed, and the computer must be instructed every step which is to be performed in order. There are a variety of programming languages, and programmers usually select the language best suited for the application. The program code written is translated into machine language (binary code) before execution.

The programming task can be simplified through the use of a flowchart, which is a symbolic presentation of the steps required to solve the problem. Typically, the programmer and laboratory technician collaborate closely in order to achieve the desired results.

Computerization of Clinical Laboratory Instruments

Computer applications in laboratory instrumentation are relatively new. The development of the microprocessor has revolutionized the very concept of analytical chemistry and instrumentation. In automated systems, the computer controls each step, including identification of specimen, aspiration of specimen and reagents, reading of results after periodic time intervals, standardization, collection of data, tallying of information, comparing and writing to databases, calculation, and output of results. One of the major role of laboratory computers is to regulate the QC of data. **Quality control** is a natural application for the CPU because of the ease with which large amounts of data can be collected and compared, calculated, interpreted or standardized. In some cases, the computer can be interfaced directly to a laboratory instrument so that the QC data and patient's results may be easily collected and stored in a database for later use. In less sophisticated programs, the computer can simply compare the patient's laboratory values to standards with the acceptable range supplied by the user. If the result is outside this range, it can be identified automatically with an asterisk or other mark so that the user may choose to reject the run, collect another sample or notify the physician of the results.

Storage of laboratory information and the easy access to the stored data have proved to be most helpful in modern laboratory operations, particularly with increased number of tests available. This has reduced the time and space needed for filing of papers and in addition, has made it much easier to provide the previous history of the patient's laboratory findings.

Machine failure

The computer is a blessing for the modern era as long it is working. Its failure is always possible and this can paralyse the entire system, if contingency planning is not in place. This may occur because of failure of electronic components or occasional abuse and misuse by the users. The components most likely to fail are those that involve mechanical parts, such as are found in disk drives and printers. If an electronic component does fail, the usual way to approach repair is to replace the computer circuit boards that might be involved. It is a good idea to keep spare parts or backup computers, if at all possible. It is equally wise to identify a source of technical assistance in the event that mechanical or electronic problems cannot be serviced promptly by the user.

Computer housing

Computer equipment must be kept at a cool temperature (24°C) and at low humidity. The level of humidity should be low enough so that it is non-condensing but high enough so that static electricity is not a problem. These environmental problems are likely to appear in the form of a memory error or a disk read error.

Conclusion

Computerization is an integral part of laboratory automation and operation. As the numbers of laboratory tests, patients and diagnostic techniques increase, it becomes imperative that data be processed and managed more efficiently and effectively.

Despite the drawbacks noted in this chapter regarding computerization (including computer failure), careful and thoughtful selection of the computer system together with the identification of specific purposes for utilization of such a system will resolve most, if not all of the potential problems before they occur. A common obstacle that must be quickly overcome is fear and apprehension of computers-assisted technology. As explained in this chapter, the user of the computer need not have any prior knowledge of computers to get high-quality results, as long as the individual follows instructions.

The following can serve as guidelines in taking the efforts out of the process of computerization:

- Identify tasks that are best suited for the computer in the laboratory.
- Evaluate pre-packaged software available for performing these functions.
- Investigate available computer models that can run the packaged software per its specifications, keeping in mind that expandability and flexibility in the system are important (both in terms of data storage and future applications).
- Prepare laboratory personnel for the new technology, and reducing anxiety or 'technophobia'. The staff should be assured of the productivity gains to be achieved with the new machinery.
- Arrange for appropriate end-user technical training, diagnostic training as well as repair assistance procedures.
- Setup backup procedures (preferably in manual form) for anticipated problems (lost data, machinery failure, power failure, etc.) well in advance.
- Create backups often, and make it a part of the prescriptive steps so that backup copies of data files are always available.
- Assign a resource to champion the computer operations and function as the laboratory's on-site staff to run initial set of diagnostics in the event of a malfunction.
- Periodically repeat the aforementioned tasks of computerization upkeep, revise the operational guidelines and retrain the staff on any new extensions or software, as needed to ensure a smooth and uninterrupted operation in the laboratory.

AUTOMATION IN THE CLINICAL LABORATORIES OF DEVELOPING COUNTRIES

The first automated system was introduced into the market by Technicon under the name of **AutoAnalyzer.** The company is now owned by Bayer. It is a continuous flow system where the specimens flow in a continuous stream while separated by air bubbles. At the final stage, the air bubbles are released and colour of the mixture is measured by absorption photometry. Acid, phosphatases and bicarbonate—any of these can be chosen according to the need of the laboratory.

Centrifugal fast analyser is the next generation of automation and has many unique features. It is a discrete system where reagents and specimens are placed in the innermost discrete compartments in a rotor using positive displacement syringes. The reagents and samples get mixed and delivered to outer compartment by centrifugal force. The outer chamber provides appropriate reaction conditions and read the change in optical density at quick sequence each time the cuvette passes over the optical device during rotation. The instrument is very useful for the determinations in rate reactions.

The dry slide technology has become quite popular not only in large laboratories but also serve the needs of POC in the physician's office. The colour reactions in dry slide technology are read through reflectance photometry.

Dade Behring (formerly Dupont) is marketing a different kind of discrete analyser. All the reagents are put in different pouches of a plastic bag where they are mixed with the specimen and the mixture undergo the required chemical reaction conditions. Finally, the optical absorption of the reaction fluid is read at specific wavelength through the plastic bag. A bar code marked at the top of the bag prompts the machine to respond to specific test.

We will focus here on three automated analysers which are commonly seen in various laboratories of India and other developing countries—AutoAnalyzer (Bayer), Clinical Corona (Boehringer Mannheim) and Auto Pacer (Miles of India Ltd.). We will focus here the operation of these analyzers.

AutoAnalyzers

This is grouped under the 'continuous flow' where the specimens move in a stream, separated by air bubbles. In discrete system all specimens are independent of each other and one can have multiple choice of tests and they can have random access. Three unique approaches of the discrete system include centrifugal fast analysers, dry slide technology and compartmental pre-packaged reagents that move on a belt, meeting all the reaction conditions and ultimately subjected to optical measurements before packages are disposed off. Bulk reagents are available and spare parts are becoming easier to obtain through the Internet.

Components of AutoAnalyzer

The five major components of the AutoAnalyzer are shown in Figure 32.3.

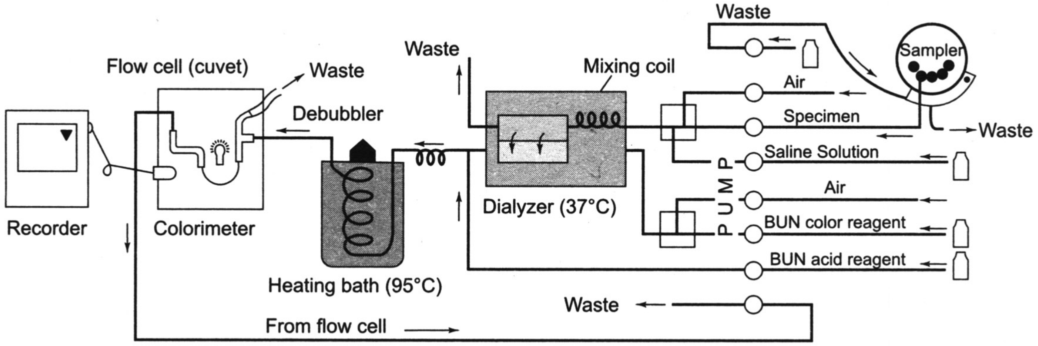


FIGURE 32.3 *Continuous flow automated system. Components and working of AutoAnalyzer for the analysis of blood urea nitrogen (BUN) by diacetyl monoxime method.*

Sampler and Cam The sampler is a circular platform that holds the cups containing the standards and specimens for analysis. As the sampler rotates, it brings each cup in turn under the sampling probe, which aspirates for a FT, decides sample volume, and alternates with the wash cycle. The dwell time in the specimen and the sample-to-wash ratio are governed by the cam. An appropriate cam is selected to determine the rate of analysis. The most common sample-to-wash ratio is 2 : 1.

Pumps and Manifolds The pump is the heart of the AutoAnalyzer, and the manifolds are the arteries. The proportionating pump has a peristaltic action that moves the fluids inside the tube in one direction at a constant speed, rendering a uniform rate of delivery throughout the system. The advancing movement accomplishes sample aspiration reagent pickup, mixing, and all other actions. The diameter of the manifold tube determines the volume of the fluid. Before the sample stream enters the dialyser, **air bubbles** are introduced in both specimen and reagent streams. The air bubbles help in cleaning the manifold tubing and avoid carry-over effects. In an enzyme study, substrate and the serum specimen (containing enzymes) are first mixed in an incubation chamber kept at 37°C for a certain period. The extended coil determines the time delay. After incubation, the specimen stream with products of the substrate enters the upper chamber of the dialyser.

Dialyser The dialyser is a double compartment separated by a semi-permeable membrane. A diluted sample stream **('donor stream')** circulates on one side of the membrane while the recipient stream (generally one of the reagents or saline solution) circulates through the other side. The membrane allows part of the sample constituents of low-molecular mass (the analyte) to pass through while it holds back, the compounds with high-molecular mass (protein) that flow into the waste. The amount of solute that passes through the membrane is influenced by the instrumental factors, which are kept constant and the variable concentrations gradient, which is the basis of flow analysis. Although only a fraction of the total amount of the analyte present in a unit volume of specimen passes through the membrane, the ratio of diffusion remains constant. The AutoAnalyzer operates on the accurate measurement of the sample-standard ratio. It is not important to have the total amount of compound to be taken for analysis, nor is it necessary to take the chemical reaction to completion. As long as a photometric measurement is possible at the final step, there is no loss of accuracy.

Reaction Chamber or Heating Bath This provides elevated temperature and a time delay which are required for the development of a coloured reaction product. The temperature is usually maintained at about 95°C, and occasionally at 37°C, depending on the analysis. The time delay is accomplished by introducing a long glass coil inside the chamber in continuation with the manifold tubing.

Detector and Recorder The basic analytic procedure of the AutoAnalyzer is colorimetry. Thus the colour of the reagent stream, following the chemical reaction, is proportional to the amount of reacting compound. A small amount of coloured reagent is drawn by the continuous flow system into a microcuvette. The air bubbles are discarded by the 'F' tube or **debubbler.** To avoid fluctuation of the light source caused by voltage fluctuation, the colorimeter employs a dual-beam system. Light from a single tungsten filament lamp is split and **collimated** into two beams, one of which acts as a reference (null balance) while the other goes through the cuvette. Initial baseline conditions are achieved by controlling the light intensity of the reference beam through the introduction of a suitable aperture plate. The photocell of the colorimeter reads the light energy that is transmitted through the coloured solution (%*T*) and converts it to electrical energy. The electrical impulse is finally communicated to the mechanical device of the recorder or directly to the computer.

The recorder chart provides a continuous measurement of the intensity of the light that has passed through the **flow cell** and is received by the **detector** (%7). The response is shown as **peaks** indicating the concentrations of the analytes. The highest concentration is at the centre of the peak with adjoining slopes that indicate a decreasing concentration of the analyte in the wash cycle phase. The peaks obtained from the specimen on the recorder chart are compared against the peaks of the standards. It is therefore important to **calibrate** the AutoAnalyzer prior to the running of specimens. Variation from a smooth shape of the peak frequently indicates many internal problems. For example, a sharp spike in the middle of a peak or between peaks indicates the presence of air bubbles at the time of colour measurement; trailing indicates possible obstruction in continuous flow; and overlapping peaks are indicative of poor wash.

Clinical Corona

The **Clinical Corona** (or Corona) made by Boehringer Mannheim is a discrete, compact and fully automated clinical batch analyser (Figure 32.4). It is a bench-top model that requires minimal space. A microcomputer system controls the whole analytical process via its programs. The program to be used in a specific assay is' selected by the operator, who enters an analysis code through the keyboard.

Corona performs methods for routine biochemistry and is also capable of handling special tests with appropriate reagents. Its throughput (maximum number of tests that can be performed per hour) is 200 samples per hour for end point analysis (single observation) and 140 samples per hour in kinetic mode (multiple; observations) for enzyme assays. The required serum volume for a set of 20 chemistries is as small as 500 µL, and the reagent consumption per test is typically 400 µL (Figure 32.5).

Substances analysed (kinetic or end point) in the Corona are: Glucose (hexokinase), total protein (biuret), albumin (bromocresol green), bilirubin (dichloro diazophenyl), creatinine (Jaffe), triglyceride (ATP method), urea (UV method), uric acid (UV method), calcium (o-cresolphthalein complexone), cholesterol (p-aminophenazone) and iron (bathophenantrolin). Other tests can be introduced according to the need of the laboratory.

The enzyme assays (kinetic) include—SCOT, SGPT, LD, CK, ALP and GGT.

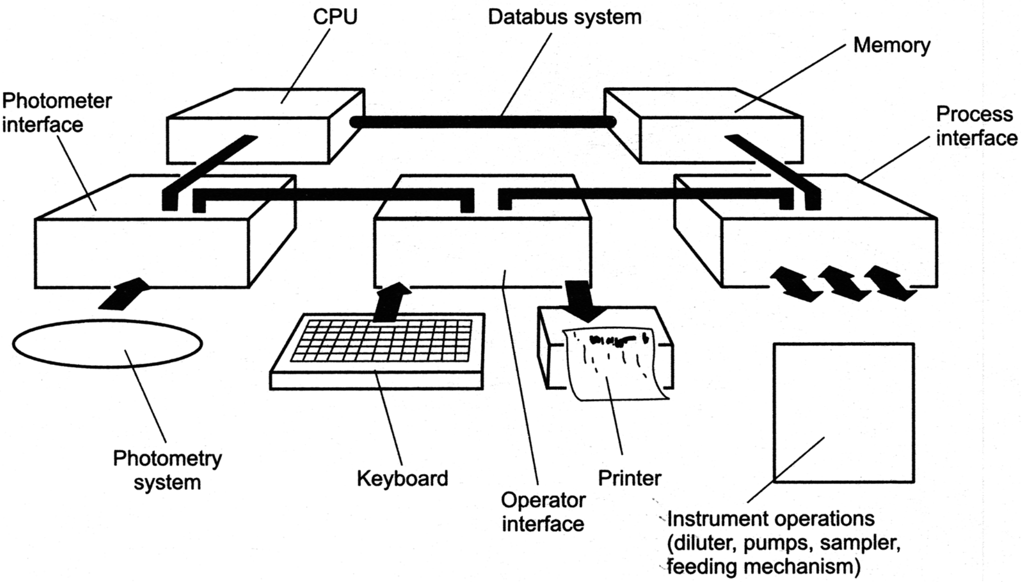


FIGURE 32.4 *Use of microprocessor in the operation of laboratory instruments. The communication between different modules in the operation of Clinicon (Boehringer Mannheim, Sweden).*

Components of Corona

Various components of Corona and their functions are described here (Figure 32.5). It is important that the operator understands the function of each component in order to trouble shoot.

Sampler The sampler is a 40-position carousel that carries the specimen. Specimens must have clear identification, and the sequence must be noted in a register. Movement of the sampler is controlled by the microcomputer. The slots are numbered and it is possible to run replicates and standards simultaneously by choosing the slots.

Diluter This prepares the specimen for the assay, mixes the reagent and the sample, and delivers it into the reaction chamber. It is fully integrated to function with a single syringe and one valve whose movements are totally controlled by the microcomputer. The diluter has a small dead volume as only one syringe is involved in the whole dilution process. The dilution cycle begins by sucking the reagent into the syringe. The valve is switched on and thereafter the sample is aspirated and separated from the reagent by air. When the pipette has left the sample cup, another segment of air is sucked in and the outside of the pipette is wiped in order to minimize carry-over. The pipette is then transferred to the cuvette into which the sample and reagent are delivered. The volumes of the fluids are regulated by the length of the stroke, and pickup deliveries of the fluids are regulated by the valve. The syringe is capable of delivering 5–1000 μL of the specimen.

Reaction Chamber The main chemical reaction for the test is in the reaction chamber which is temperature controlled and newer chemicals are added. The instrument uses disposable **cuvettes** (which add to the cost of its running) which are placed in racks in the in-feed area of the instrument, and end up in the out-feed area when the analytical process is complete. Just before entering the thermostat the cuvette receives the reagent and sample previously picked up by the diluter from a cup in the sampler. The racks travel through the thermostat and during this travel the reaction mixture is gradually heated to the set temperature. At any point in time during this feeding through the thermostat it is possible to add two additional

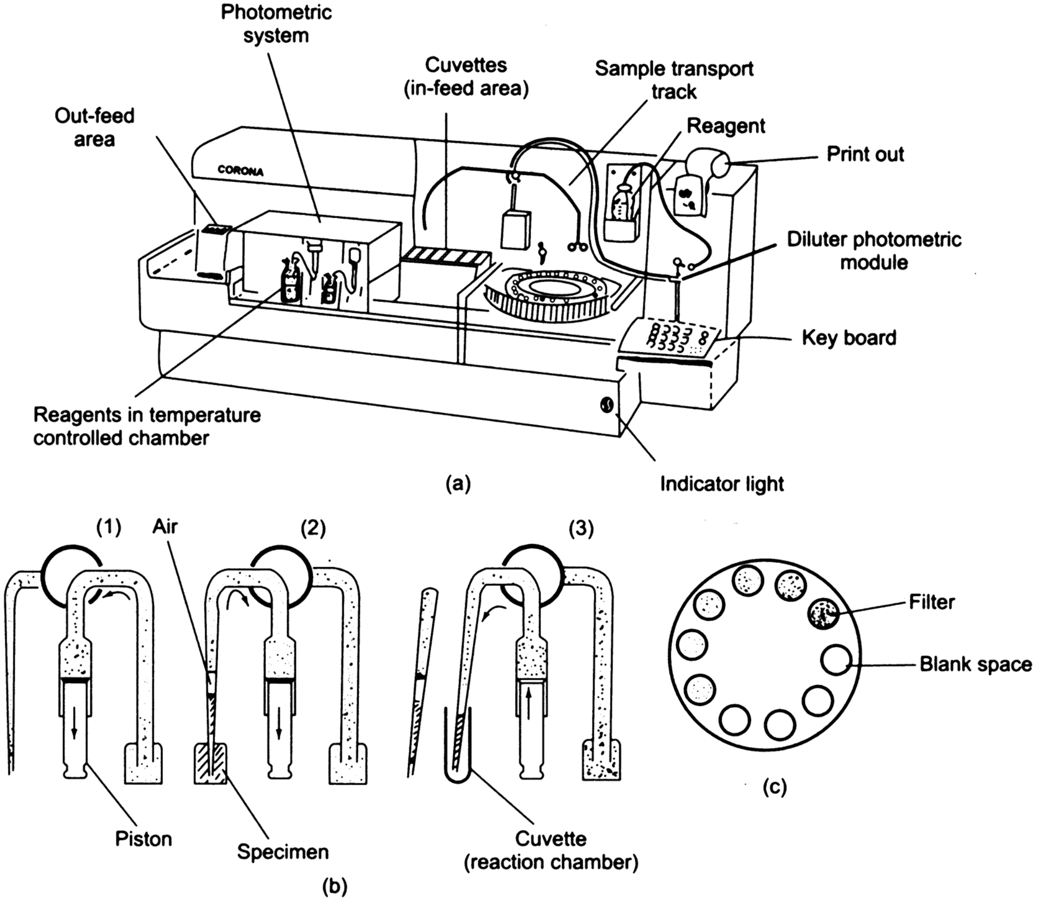


FIGURE 32.5 *Discrete automated system—Corona (Boehringer Mannheim, Sweden): (a) Principle components of Corona; (b) Operation of diluter: (1) Filling of reagent, (2) Aspiration of specimen, and (3) Transfer and delivery of specimen and reager; (c) Filter disc used in the photometric system (visible range)*

reagents. These reagents are preheated in order not to upset the temperature of the reaction mixture already in the cuvette. The pipette, tubing and heating block are in one piece which is easy to position and remove from the top of the thermostat. The reagent pumps are controlled by the microcomputer.

Detector The detector is built-in within the reaction chamber. The detector is an optical system which is typical of any photometer. Contents of the cuvettes are well mixed before taking **optical measurements.** Most observations are made in the visible range, using a tungsten lamp but the instrument is capable of working in the ultraviolet range as well. **Radiant energy** from the light source passes through two lenses and the emerging parallel beams of light then enter the interference filter in order to choose the desired wavelength of light to go through the cuvette with the test solution. The parallel beam of light is then focussed by another lens in order to concentrate it at the middle of the cuvette. The light is absorbed by the test solution and the non-absorbed light is focussed by another series of lenses onto a **silicon photodetector.** The electrical signal sent by the photodetector is amplified, converted into logarithmic and digital form, and finally transferred to the microprocessor.

Printer After receiving the signal from the CPU the microprocessor then performs the necessary calculations and transfers the final information to the output printer.

Microcomputer System The microcomputer is the brain of the machine and controls the whole instrument through the CPU. The microprocessor receives instructions from the software (program) which is operated by the user of the machine. The memory of the computer has two components, the user file (comparable with random access memory, RAM) and the master file which is provided by the manufacturer. The operator, by a few commands via the keyboard can make a selection of 16 analyses which are automatically loaded into the user file. These analyses are immediately accessible for routine use. Only one analysis should be run for a batch. For different analyses, different chemicals and reaction conditions will be needed.

There are three assay modes—constant rate (CR), fixed time (FT) and end point (EP). Depending on the chosen mode, the arithmetic-logic unit of the CPU makes the necessary mathematical calculations. CR mode is used for enzymes; the slope is determined by the best fit to measured data. The accuracy is checked and compared to a pre-set value (RMS) to yield a factor. The concentration results are obtained by multiplication by the factor. **Fixed time mode** is used for substrates and immunoglobulins. The primary value is determined by integration of the measured data forming the midpoint *(t/*2*)* of the reaction rate until the plateaux is reached. The EP mode is applied for routine chemistry—albumin, bilirubin, calcium and others. In the EP mode, the absorbance level is determined by the best fit to measured data, and the concentration result is obtained by comparison with standards.

Before starting the machine load the carousel with specimens, check the identifications of the specimens, place the standards, record the sequence, orime the diluter with the reagent, check the temperature light (it must be on), place the empty cuvettes in position, and enter the analysis code, date and sequence number on the keyboard. Then press the 'start' key provided on the keyboard.

The Corona is programmed to check the QC. If a result does not fulfil a pre-set QC parameter or unit value the result will be accompanied by a text or a symbol which depends on the type of error. Error messages are also given when the standard curve of an assay is unacceptable.

When the analysis code is entered by the user, all the settings are completed. The Clinicon Corona, however, is flexible enough to adapt to newer methods to meet the needs of the individual laboratory. In order to adapt to newer methods, 38 parameters have to be fixed such as temperature, wavelength, concentration unit, etc.

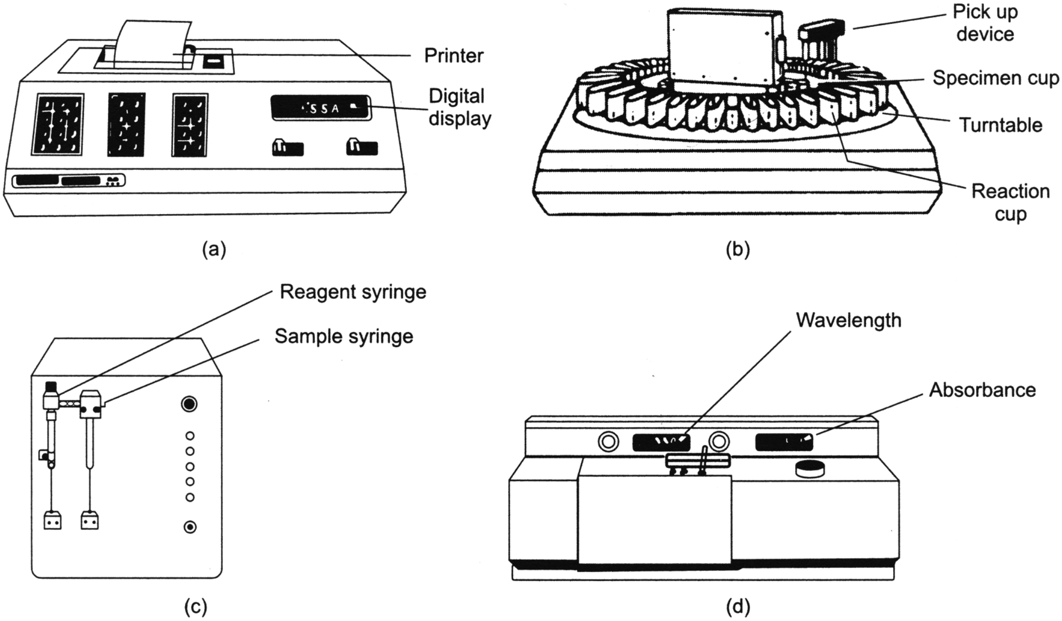
Auto Pacer

The Auto Pacer (Chemetrics Analyser–I) made by Ames, USA (Miles of India Ltd.) is a computerized discrete analyser of modular design. It is a bench top model which is fully automated right from the sample dispensing to the final printout of the results. The technician has to load the machine with samples, provide appropriate reagents, instruct the computer through the computer keyboard and set the parameters as required by the tests. It is capable of doing 26 biochemical tests. Although the analyser is pre-programmed for 26 tests, 37 programs are available and it is open for the addition of new tests as they are developed in future. There are also two open programs available to the user (one kinetic for enzyme study and one EP for routine chemistry). Over and above all these programs, a built-in statistical program for QC, i.e., for calculating standard deviations, coefficients of variation, and means, is also available. It is faster than Clinicon Corona and can handle 300 EP reactions per hour (throughput) and 40 kinetic enzyme assays per hour. The analyser processes the sample in batches and it is possible to interrupt a batch run to do a 'Stat' test. The reagents are available from the manufacturer and can also be prepared in the laboratory.

The program provided by the manufacturer is not totally inaccessible. The user can change the test parameters, if needed, such as the number of data points, range of normal and others. The instrument is also designed for handling enzyme immunoassays, and has proved to be an important tool in drug analysis.

Components of Auto Pacer

The modular design provides the following components (Figure 32.6):



(a) (b) (c) (d)

Figure 32.6 *Various components of Auto Pacer: (a) Digital display and printer, (b) Specimen pickup module, (c) Specimen and reagent pickup syringes, and (d) Photometric module*

Sampler It is a turntable carrying 60 reaction cups and 60 sample cups per tray. The carousel can be separately loaded with fresh specimens while the tests are in progress with one batch.

Automatic Dispenser This component functions in the automatic dispensing and diluting of sample and reagents. Pump and syringes are housed in this module.

Detector This consists of a spectrophotometer for photometric measurements. The spectrophotometer is of high precision and narrowband (8 nm) that incorporates a digital readout. It works in the entire range of photometric determinations for routine biochemical analyses (335–850 nm).

Computer This is the brain of the machine. The keyboard is the communication link between the user, the manufacturer's software and the microprocessor. The temperature of the reaction chamber and flow cell is controlled by an internal thermostat (25–37°C). It is attached to the instrument and is programmed to function according to the test requirement. It has a built-in printer that gives impact paper printouts. If the results are not normal, the printout gives an automatic flag of abnormal, non-linear, invalid or out of range values.

The instrument is capable of performing EP, kinetic or initial rate reactions as the need be. It is designed for continuous batch operation round the clock.

SEAC (Ames)

This is a semi-automated analyzer (Ames, USA; Miles of India Ltd.) where the technician and the instrument works like a sophisticated spectrophotometer do the initial preparations. The advantage of this machine is that the use of separate cuvettes is avoided, and the conditions of the test can be programmed. Boehringer Mannheim and other manufacturers have also marketed similar products. This instrument is highly effective in measuring enzyme kinetics and is a necessary tool for enzyme-linked immunoassays.

POINT-OF-CARE TESTING: A NEW APPROACH

In recent years, there is a growing demand of **'Point-of-care testing (POCT)'** that led to the rapid advancements in technology that will enable the physician to make decision on the bedside. This is making rapid changes in all aspects of health care. One of the major changes in the clinical laboratory has been the implementation and increased use of POCT. This brings the laboratory test to the patient rather than obtaining a specimen from the patient and transporting it to the laboratory for testing. This makes laboratory test results available more rapidly, providing improved patient care. In advanced countries it is applied in numerous situations—nursing homes, physician's office, emergency rooms, intensive care units and for bedside testing in hospital wards. The evolution of small, simple-to-use analysers that require only one drop, or less, of specimen has led to widespread POCT implementation. Handheld portable analysers can measure substances such as glucose, haemoglobin, cholesterol and electrolytes. Most require only a drop of blood, usually obtained by finger stick. Thus in the near future many of the routine clinical laboratory tests will be available in remote villages of developing countries although in the urban setting large automated system will continue to function. The clinical laboratories, however, will have to stay involved in making recommendations and compare the results with the classic procedures.

TIME-SAVING DEVICES AND KITS

The automated systems are undoubtedly fast, reliable and prove to be cheaper in the long run. However, the initial investment is high and a breakdown can be disastrous. As most of these automated systems are computerized, unfavourable weather, intermittent electrical supply and lack of technical repairmen make it hard for the laboratories to decide whether to invest in them or not. Modular systems are more advantageous as spare module can be used in case of breakdown, and the defective machine can be sent for repair. Automated system is profitable only when the work load is high.

For smaller laboratories, **kits** have proved to be profitable. Kits must be purchased from reliable companies (Appendix at the end of this volume). The laboratory should purchase such time-saving gadgets as automatic pipetter, diluter, mixer and others to expedite the work. Excessive mechanical work will cause fatigue in the technician and this will result in erroneous results. All technicians must, however, master the manual procedures before they plan for any kind of automation. This reduces the sense of helplessness when the automated system fails.

CONCLUSION

Automation is a buzzword among clinical laboratories, but it is not a pie in the sky. The future for automation in clinical labs is here. Many clinical laboratory tasks have already been automated, and several manufacturers are offering automated laboratory systems in the marketplace. Automation continues to be the province primarily of hospital laboratories, however, although most large commercial laboratories continue to eschew its usage. Unreliable energy source and instrument breakdown are the main problems of automation in developing countries, other than the high purchase cost and repair. If the laboratory totally relies on automation and neglects the written manual procedures about what to do, when there's a system interruption, which inevitably occurs, it will make a big folly. The laboratory must have a backup plan. The patient has no time to wait until the machine gets fixed.

Technology enables automation, which in turn, drives efficient laboratories. As automation has become an indispensable part of modern laboratories, its adoption varies by geography. Developing countries tend to take a longer time to adapt the oncoming changes and should select from the large array of automated systems to suit their own conditions. The ideal automated system for the laboratories of developing countries ought to be simple, trouble-free, low-maintenance and reliable. It ought to be able to function with basic chemicals and versatile enough to switch to manual system, if necessary, without sacrificing the accuracy of the results. In developing countries, a good backup system must be thought well before the laboratory adopts an automated system. Use of the computers and computer-assisted process control is encouraged and should be leveraged to drive productivity and achieve efficiency.

REVIEW QUESTIONS

1. What is the difference between continuous flow analysis and discrete analysis? Name one instrument in each of these automated systems.
2. Why is it that the centrifugal fast analyser is chosen for enzyme assays?
3. How is the protein interference minimized by the automated systems?
4. Why is it that,the discrete system is preferred over the continuous flow system in the laboratories of developing countries?
5. What are the difficulties you anticipate in computerizing a laboratory located in a developing country?
6. Define: CPU, Byte, Hardware, Software and Algorithm.
7. What are the main components of a computer and what are their functions?
8. If you are asked to install a computer in the laboratory where will you place it?
9. List the most important components of an AutoAnalyzer and state their functions.
10. Why does the AutoAnalyzer introduce air bubbles into the flowing stream of the sample and reagent and how are the air bubbles removed before the detector reads the colour of the solution?
11. What are the functions of the microcomputer system in the Clinical Corona?

Chapter

33

Routine Biochemical Test Procedures

Kanai L Mukherjee, Chhotelaal Pande and Rohini Chakravarthy

Chapter Outline

- Introduction
- Routine Diagnostic Tests in Clinical Chemistry
- Blood Glucose
  - Glucose Assay by o-toluidine Method
  - Glucose Oxidase (enzymatic) Method
  - Glycated or Glycosylated Haemoglobin (HbA1c)
- Serum Protein
  - Total Protein in Serum
  - Serum Albumin
  - Myoglobin
  - Troponin
- Blood Urea Nitrogen (BUN)
- Uric Acid
- Creatinine
- Bilirubin
  - Total Bilirubin
  - Total and Conjugated Bilirubin
- Diagnostic Enzymology
  - Measurement of Enzyme Activity
  - Routine Analysis of Diagnostic Enzymes
- Brain Natriuretic Peptide (BNP)
- Lipid Profile
  - Cholesterol
  - Triglycerides
  - Subgroups of Cholesterol

INTRODUCTION

Clinical biochemistry or clinical chemistry is the area of clinical pathology that is generally concerned with the **chemical analysis of bodily fluids**. Laboratory findings of the biochemical tests may be of use in **diagnosis and in the monitoring of treatment.** The biochemical findings may also be of value in screening for disease or in assessing the prognosis, once a diagnosis has been made.

The discipline of clinical biochemistry developed since 19th century and went through revolutionary changes with the use and measurement of enzyme activities and advent of instrumental analyses like spectrophotometry, electrophoresis, and immunoassay. In recent years, clinical biochemistry tests comprise over one-third of all laboratory investigations. Although these tests are occasionally performed with other body fluids like **urine and CSF** but serum or plasma are most common. Materials other than body fluids such as **faecal specimen and forensic samples** are submitted under special circumstances. It is estimated that the biochemistry laboratory receives the majority of specimens for diagnosis and treatment.

There are four basic types of biochemical investigations:

- routine
- special (includes electrophoresis and clinical endocrinology)
- toxicology
- therapeutic drug monitoring

In this chapter, we will focus on the **routine biochemical tests,** keeping in view the needs of the developing countries. One should keep in mind that most current biochemical laboratories in urban settings are highly automated in order to accommodate the high workload, which is typical of an urban hospital. This chapter, however, suggests manual procedures to suit the rural settings of developing countries.

Chemical constituents in a healthy body are in a delicate balance, or equilibrium, that is influenced by both internal and external factors. This equilibrium is known as **homeostasis.** Diseases alter the levels of biochemical factors and disrupt the body's natural equilibrium. By analysing the composition of various body fluids such as blood, urine and cerebrospinal fluid, one can determine the underlying cause of disease (infection, metabolic disturbance, toxicity or other pathologic mechanisms). Physicians regularly use the results of clinical chemistry tests to aid in the diagnosis, treatment, and prevention of disease. Interpretation of test results is based on understanding the **physiological and biochemical processes occurring in health and in disease** (Chapter 29).

ROUTINE DIAGNOSTIC TESTS IN CLINICAL CHEMISTRY

This chapter will provide descriptions and protocols for routinely ordered clinical laboratory tests. A list of these frequently requested tests, along with the expected values and sample type, is given in Table 33.1.

TABLE 33.1 *Routine diagnostic tests in clinical and their normal range*

S.No.	Test Name	Normal Range (Adults)	Types of Sample
1	Acid phosphatase (ACP)	0.0–4.3 U/L	Serum
2	Alanine aminotransferase (ALT)	10–35 U/L	Serum
3	Albumin (Alb)	3.5–5 g/dL	Serum and urine
4	Alkaline phosphatase (ALP)	35–100 U/L	Serum
5	Aspartate aminotransferase (AST)	0–35 U/L	Serum
6	Amylase	< 160 U/L	Serum
7	Bilirubin (total) Direct bilirubin (conjugated) Indirect bilirubin (total-conjugated)	0.3–1 mg/dL 0–0.5 mg/dL < 1.0 mg/dL	Serum
8	Blood urea nitrogen (BUN)	7–22 mg/dL	Serum
9	Brain natriuretic peptide (BNP)	0–100 ng/L	Serum
10	Calcium (Ca)	8.2–10.5 mg/dL	Serum and urine
11	Carbon dioxide (CO_2)	23–29 mEq/L	Whole blood
12	Chloride (Cl)	96–106 mEq/L	Serum
13	Cholesterol (total)	<200 mg/dL	Serum
14	Creatinine (Creat)	0.6–1.2 mg/dL	Serum and urine
15	Creatine kinase (CK) or Creatine phosphokinase (CPK)	55–170 U/L	Serum
16	Glucose	70–110 mg/dL	Serum
17	High-density lipoprotein (HDL)	>50 mg/dL	Serum
18	Lactate dehydrogenase (LD, LDH)	100–190 U/L	Serum
19	Low-density lipoprotein (LDL)	< 100 mg/dL	Serum
20	Myoglobin	<85 ng/mL	Serum
21	Potassium (K)	3.6 5.2 mEq/L	Serum
22	Protein (total)	6.0–8.0 g/dL	Serum
23	Sodium (Na)	135–145 mEq/L	Serum
24	Thyroid stimulating hormone (TSH)	0.4–4.2 μU/mL	Serum
25	Thyroxine (T4)	4.5–11.2 μg/dL	Serum
26	Triglyceride (Trig)	<I 50 mg/dL	Serum
27	Triiodothyronine (T3)	75–220 ng/dL	SerumTSH
28	Uric acid	3–7 mg/dL	Serum

Clinical laboratory tests can be performed by traditional "wet laboratory" techniques, newer "dry chemistry" kits, or by automated equipment. However, complete automation of clinical laboratory testing is unlikely in areas with modest financial resources, unreliable power supplies and limited healthcare facilities (Chapter 32). This book primarily outlines **manual techniques** which are performed regularly in developing countries. Furthermore, the "dry chemistry"techniques provide an affordable alternative to many traditional techniques. As science continues to advance, more of these **routine chemical tests are becoming available at home** (i.e., pregnancy testing, blood sugar tests).

A patient's diagnosis and treatment depends on **reliable test results.** So, it is important to calculate and report results with precision. Specimen collection, handling, storage and testing all impact the overall reliability of the results. Even, if results are calculated automatically by machine, this does not necessarily mean they are all reliable.

BLOOD GLUCOSE

Glucose, the major and simplest carbohydrate in blood, is used as the primary **energy source** by the body cells. Glucose level of blood is controlled by hormonal mechanism. This is why patients with abnormal glucose level of blood are referred to the **endocrinology department** for evaluation and treatment.

Clinical significance

Due to its importance in metabolism, glucose level is a key diagnostic parameter for many metabolic disorders. Increased glucose levels have been associated with ***diabetes mellitus***, hyperactivity of thyroid, pituitary and adrenal glands. Decreased levels are found in insulin secretion tumours, myxoedema, hypopituitarism and hypoadrenalism. A fasting blood sugar rarely exceeds 120 mg/dL. Increased levels may be observed in infectious diseases, and intracranial diseases such as meningitis, encephalitis, tumours, and haemorrhage. Glucose values as high as 500 mg/dL or higher is found in **diabetes mellitus (Type II).** High glucose values are also encountered (>200 mg/dL) during anaesthesia, depending on the duration and degree.

Glucose and diabetes

Two disorders of glucose metabolism are common.

- *Diabetes mellitus,* commonly called diabetes, is a chronic disease in which the body either produces insufficient insulin or is unable to use insulin properly. As a result, there is increased blood glucose **(hyperglycaemia).**
- *Deficiency of growth hormone (ACTH),* in which there is decreased level of blood glucose **(hypoglycaemia).**

Note There is another kind of diabetes, called **diabetes insipidus.** This is unrelated to carbohydrate metabolism. It is caused by the deficiency of antidiuretic hormone (ADH which is made in hypothalamus and secreted by pituitary at the base of the brain) that affects water metabolism of the body. Symptoms of diabetes insipidus include polyuria (increased urination) and increased thirst.

Diabetes management

Diabetes is a serious disease and uncontrolled diabetes may lead to a number of ailments including heart attack, coma, high blood pressure, blindness, kidney failure and increased infection risk. Therefore, early diagnosis is very important because proper management of diabetes can lessen, postpone, or even prevent many of these complications.

Diabetes cannot be cured but can be managed. **Diabetes management** is the process of keeping a person's blood glucose levels within normal range by adjusting diet, exercise, and medications. A patient's normal range depends on a number of factors (age, comorbidities, etc.) and should be discussed with his or her doctor. In general, if the blood glucose level is too low (<40 mg/dL), the person may experience weakness, visual disturbance, hunger, faintness, diaphoresis, palsy and confusion. When the level is too high (>400 mg/dL), the person may experience confusion, nausea, intense thirst, dry skin, weak pulses, or coma. The signs and symptoms vary with the individual. Therefore, measuring blood glucose is the first step in diabetes management. Glucometers are machines that allow patients to measure their blood glucose levels at home. Measuring **haemoglobin A1c** (also called **glycated haemoglobin** or **glycosylated haemoglobin)** is a useful technique to see how well a patient is managing his or her diabetes. Haemoglobin A1c is a measure of the percent of haemoglobin that is bound to sugar. This measurement gives an indication of the average blood glucose over several months. If diabetes is not managed properly, the A1c will be high.

There are two common types of diabetes—**type I** where blood glucose is controlled by oral medication without insulin injection. Here the insulin production is insufficient. In **type II,** the person has to take insulin injection as their body does not produce sufficient insulin.

Diagnosis of diabetes involves three major tests: fasting blood glucose, oral glucose tolerance, and haemoglobin A1c. Table 33.2 gives the diagnostic indicators of laboratory findings for various stages of diabetes.

TABLE 33.2 *Diagnostic indicators for diabetes and prediabetes*

	Normal	Prediabetes	Diabetes
Fasting blood glucose	<100 mg/dL (5.6 mmol/L)	100–125 mg/dL (5.6–6.9 mmol/L)	>126 mg/dL (7 mmol/L)
Oral glucose tolerance (Blood glucose tested 2 h after drinking glucose solution)	Blood glucose level <140 mg/dL (7.8 mmol/L)	Blood glucose level 140–199 mg/dL (7.8–11 mmol/L)	>200 mg/dL (>12 mmol/L)
Haemoglobin A1c (HbA1c)	4.5–5.7%	5.7–6.4%	>6.5%

Specimen

The first step to accurately measure blood glucose is to **collect a proper sample.** When a specimen is drawn for serum or plasma, glucose testing in a laboratory must be processed before it is analyzed. Hence, there exists opportunities for error in the **pre-analytical phase.** The following are some considerations that must be addressed to avoid potential problems.

- If **fasting blood glucose** is ordered, it is imperative that the patient is informed and the preparation is verified before the collection occurs.
- **Glucose is constantly metabolized by the cells** present in the blood specimen after it is added to the collection tube. If the specimen is not processed by centrifugation and tested within an hour of collection, the results may not reflect the accurate glucose level of blood as the cells might have metabolized the glucose. If a delay is expected in testing after collection, a tube with sodium fluoride or potassium oxalate additives should be used for the specimen collection. This slows down the utilization of glucose by the cells, and allows the plasma glucose to remain stable for approximately 24 h at room temperature.

Normal Range

80-110 mg glucose/dL

Glucose assay by o-toluidine method

Simple, direct and automation-ready procedures for measuring glucose concentrations find wide applications in clinical laboratories. Most bioassay is designed to **measure glucose directly in serum or plasma without any pre-treatment (removal protein).** The improved *o*-toluidine method utilizes a specific colour reaction with glucose. Absorbance (A) of the colour at 630 nm wavelength is directly proportional to the amount of glucose present in the specimen/solution. The method is sensitive and accurate that involves addition of a single working reagent and incubates for 8 min in a boiling water bath. Kits from reputable companies have improved stability of the reagents. For big laboratories, the kit can be used for 96-well plates if plate readers are available.

Principle of assay

In the presence of heat and acetic acid, ortho-toluidine (*o*-toluidine) reacts rapidly with aldohexoses (mostly glucose) to form a **green-coloured complex** that can be measured at 630 nm wavelength. Optical density or absorbance of the sample at 630 nm is directly proportional to the concentration of glucose.

$$o\text{-toluidine} + \underset{\text{(aldehyhe)}}{\text{Glucose}} \xrightarrow{\text{Acid}} \underset{\text{(intermediate)}}{\text{Glycosylamine}} \xrightarrow{\text{Heat}} \underset{\text{(Schiff base)}}{\text{Green complex}}$$

Special *note* and caution

- This test is not specific for glucose.
- Do not run this test on patients who have **hypergalactosemia.** Galactose is the only aldohexose other than glucose, small enough that it does not significantly interfere with the test.
- You should avoid contacting the skin with the reagent. The reagent should be dispensed from an all glass automatic pipettor.

Reagents

All reagents are available in the form of kit. Kits from reputable companies have improved reagent stability. For big laboratories, the kit can be used for 96-well plates if plate readers are available. One can, however, prepare *o*-toluidine reagent in the laboratory as described below.

1. *o-toluidine reagent:* Work inside a fume hood with fan on. Weigh and transfer thiourea into a flask of 500-mL capacity and then add glacial acetic acid (470 mL) into the flask.

Thiourea	0.75 g
Glacial acetic acid (CH_3COOH)	470 mL

   Dissolve thiourea in glacial acetic acid. Avoid the fumes of acetic acid. If the solute (thiourea) is difficult to dissolve, stand the flask in a bowl of hot water. Add to this:

o-toluidine	25 mL

   Mix well. Transfer the reagent to a **brown bottle** and store at room temperature. Prepare the reagent at least 24 h before use. The reagent is stable for several months but should be discarded if it becomes darkened in colour or gives an increased reading in the reagent blank, *o*-toluidine reagent should be dispensed only with an automatic dispenser

   Warning: Glacial acetic acid is highly corrosive. Figure 33.1 gives an idea of how to prepare a stock solution from the scratch after weighing the requisite amount of material on a balance.

2. *Benzoic acid solution (1 g/L):* In a 1000-mL flask, take approximately 800 mL of distilled water. Bring it almost to boiling and add 1 g benzoic acid. Mix until it is dissolved, cool the flask and then make it to 1000 mL volume (q.s.).

Benzoic acid	1 g
Distilled water (q.s.)	1000 mL

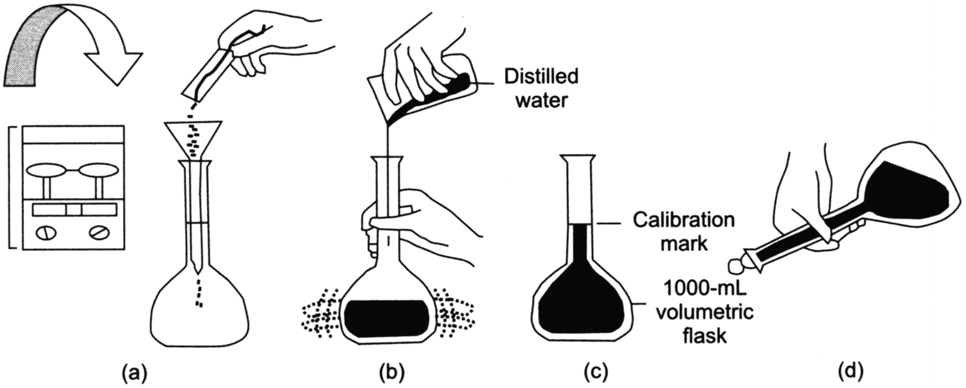


Figure 33.1 *Preparation of stock solution: (a) Weigh the standard on an analytical balance on a pre-weighed paper and transfer the standard into a volumetric flask, (b) Pour distilled water to dissolve by swirling, (c) Bring to the mark (q.s.) of the volumetric flask, (d) Finally, mix by inversion*

3. *Trichloroacetic acid (TCA),* 3% (w/v, 30 g/L)

TCA(CCl_3COOH)	15 g
Distilled water (q.s.)	500 mL

Weigh the acid quickly, since it is highly deliquescent. Transfer it to a beaker (500-mL). Add a portion (300 mL, by guess) of the total volume of distilled water (500 mL) to dissolve the acid. Then, dilute to 500 mL in a volumetric flask.

Storage: Store the reagent in a refrigerator. Reagent is stable for one month.

Warning: **TCA is highly corrosive.**

4. *0.2% Benzoic acid (200 mg, 100 mL):* In preparing glucose standards, 0.2% benzoic acid will be used as a diluent. Benzoic acid is a good preservative for glucose. 2 g benzoic acid is dissolved in a 500-mL beaker and then made to 1000 mL in a volumetric flask (q.s.).
5. *Glucose standards*
   (a) Accurately weigh 1.000 g of glucose standard and carefully transfer it to a 100-mL volumetric flask. Use a funnel and wash bottle during transfer in order to prevent any loss of pre-weighed glucose. Add to this the requisite amount of 2% benzoic acid. Dilute to the mark of 100-mL, put the stopper and thoroughly mix by repeated inversion of the flask. This standard solution contains 1000 mg glucose in 100 mL of solvent or benzoic acid. It is a 1% solution or 1000 mg/100 mL.
   (b) A second standard solution may be prepared by dissolving 200 mg of glucose (0.200 g) in a 100-mL volumetric flask with 2% benzoic acid. This is equivalent to 200 mg/ l00mL (0.2%). Working standards can be made by diluting 20 mL of the stock to 100 mL(= 5 ×) with 0.2% benzoic acid solution. This will be 40 mg/dL.

These standard solutions can be diluted to get series of standard. In each case, use 2% benzoic acid as diluent.

Materials required, but not provided in the kit

- Pipetting devices
- Centrifuge tubes with screw cap
- Boiling water bath
- Tube holder
- Spectrophotometer and cuvettes for measuring optical density (A) at 620–650 nm

Procedure

1. Add 0.1 mL of serum/plasma/urine/CSF/glucose standard to a test tube (19 × 150 mm) and dilute with 0.90 mL of water.
2. Add 7.0 mL of *o*-toluidine reagent and mix.
3. Put the tubes on a tube rack and place in a boiling water bath.
4. Heat for 10 min.
5. Put in ice water for 2–3 min and then cool down to room temperature. Remix.
6. Measure absorbance of the coloured solution at 630 nm against a water blank within 30 min.

Modifications

- Add 0.05 mL (50 μL) of undiluted serum directly to 3 mL of reagent (total volume 3.5 mL).
- ***Note*** This is same as in Step 1 and 2.
- If the sample has marked haemolysis, lipaemia, hyperbilirubinaemia, or if analysis of whole blood is desired, a TCA filtrate must be used. A 1 : 10 protein-free filtrate is prepared by mixing 0.2 mL of specimen with 1.8 mL of 3% TCA. Allow it to stand for 5 min, filter or centrifuge, and pipette 1 mL for analysis.

Calculation

In the calculation below, A represents the absorbance at 630 nm.

$$\frac{A_{\text{Specimen}}}{A_{\text{Standard}}} \times 200^{*} = \text{mg glucose/dL specimen}$$

*Refers to the concentration of the glucose standard (200 mg/100 mL or dL)

Glucose Oxidase (enzymatic) Method

Glucose oxidase method reports **"True glucose'** value. It is simple, quick, and specific for glucose and has been adapted for use in many types of glucose analyzers, both large and small. In general, tests that use hexokinase or glucose dehydrogenase are more specific and have less interference than that using glucose oxidase.

Specimen

A serum specimen is recommended for this procedure.

Note Serum must be separated from the clot within 30 min after collection. **Delay** in separation of serum from the clot would result in **glycolysis and lower glucose values.** This is true for all glucose analyses irrespective of the method.

For plasma, collect venous blood in tubes or bulbs containing **heparin.** Mix by repeated inversions. Centrifuge the blood to separate the plasma and then transfer the plasma into a clean, dry test tube for the test. 20 μL of either serum or heparin plasma is used. Samples not assayed within one hour of collection should be frozen. **Do not use whole blood.** Haemolyzed specimens must be discarded. The colour of the haemoglobin imitates that of the chromogen.

Colorimetric method

Principle

The glucose oxidase method of analysis is a three-step reaction.

$$\text{Glucose} + H_2O + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{Peroxidase}} H_2O + O \text{ (active oxygen)}$$

$$\text{Phenol} + O + \text{Chromogen} \xrightarrow{\text{Chemical reaction}} \text{Colour formation}$$

Conversion of glucose occurs in three steps, as shown above. In the first step, glucose is converted to **gluconic acid** in the presence of glucose oxidase and oxygen, resulting in the formation of hydrogen peroxide. The amount of hydrogen peroxide (H_2O_2) produced is proportional to the amount of glucose (substrate) present in the specimen. In the second step, **hydrogen peroxide** reacts with the enzyme peroxidase releasing water and active oxygen. Finally, in the third step, the active oxygen is accepted by an oxygen acceptor (phenol) and then transferred to a chromogenic substance, **aminoantipyrine**, a compound that yields a strong red coloured solution. The colour is proportional to the amount of available hydrogen peroxide or the amount of glucose (substrate) in the specimen. The intensity of colour complex is measured calorimetrically at 515 nm in the spectrophotometer (or, 500–530 nm, if a filter is used).

Interfering substances: The method is specific for glucose. High concentrations of other reducing substances (e.g., ascorbic acid) may interfere by competing with glucose and thus resulting **false low values** of glucose. Haemoglobin can also interfere by causing a premature decomposition of hydrogen peroxide (see urinalysis for dip-stick screening of haemoglobin) which may give low results. Haemoglobin concentration of less than 600 mg/dL does not interfere with the test. Bilirubin concentration up to 18 mg/dL does not interfere with the test. If the specimen is haemolyzed, icteric (jaundiced with yellow-green colour) or lipaemic, use protein-free filtrate. Creatinine, uric acid, and antidiabetic drugs are not known to interfere with the test. Patients with vitamin C therapy (ascorbic acid) may yield low values of glucose.

Reagents

Various commercial kits are now available in the market. They provide prepared reagents and thus eliminate the tedious process of preparing them in the laboratory. The following account is based on one such commercial kit. Since you may be using another kit to perform your procedure, you should carefully read and follow the manufacturer's directions which are provided inside the kit as inserts. Store the unopened kit at 4–8°C during its useful life.

Note Some manufacturers sell the final glucose working reagent and thus the intermediate steps may be avoided.

1. Enzyme in buffer
   100 mM phosphate buffer, pH 7.0
   0 1.2 U/mL horseradish peroxidase
   12 U/mL aspergillus glucose oxidase
2. Phenol
   1.25 M phenol in 57% ethanol
3. 4-aminophenazone
   2.95 mM (4-aminophenazone)
4. Glucose standard
   - Stock standard (1 g/dL, 10 mg/mL): Dissolve 1 g (1000 mg) of reagent grade glucose in 0.2% benzoic acid solution and dilute to 100 mL in a volumetric flask. The solution contains 10 mg of glucose/mL of solution.
   - Working standard (200 mg/dL): Dilute the above working standard 5×. Dilute 20 mL of the stock to 100 mL (5×) with 0.2% benzoic acid solution.

The working reagent whether commercially available or made in house, **must be stored in the refrigerator** when not in use. When properly stored, the reagent is stable and reactive for approximately 4–6 weeks. Any indication of pink colour, turbidity, or crystallization is the cause for discarding the reagent. For laboratories located in remote areas, prepare the working reagent once a month, as needed. Change the volume of the working reagent proportionately.

Procedure

- Take three test tubes (13 × 100 mm) and mark them as reagent blank (BL), standard (ST) and specimen (SP; increase the number of test tubes according to number of specimens SP_1 SP_2 etc.)
- Add 2.0 mL of glucose working reagent to all the tubes.
- Add 20 µL of deionized water in the tube marked BL.
- Add 20 µL of working standard solution in tube marked ST.
- Add 20 µL of specimen into tube marked SP.
- Mix all tubes by hand or Vortex mixer and let them stand at room temperature for 20 min (incubation).
- After 20 min, transfer the contents of tubes into matched cuvettes. (The colour is stable for 30 min).
- Measure absorbance in a colorimeter/spectrophotometer at 500 nm after adjusting the zero absorbance with reagent blank (BL).

Calculation

$$\text{mg glucose/dL specimen} = \frac{\text{Absorbance (A) of Specimen}}{\text{Absorbance (A) of standard}} \times 200^*$$

*200 = Concentration of standard

Ultraviolet (UV) Method

Principle

In the conversion of glucose to 6-phosphogluconate by hexokinase and glucose-6-phosphate dehydrogenase (G6PD), NADP which does not absorb light at 340 nm is converted to NADPH which does absorb light at 340 nm. **The amount of NADPH formed is proportional to the glucose concentration.** The test is specific for glucose but you need to have a spectrophotometer with UV capabilities (340 nm) to perform the test.

$$\begin{array}{lcl}
\text{Glucose + ATP} & \xrightarrow{\text{Hexokinase}} & \text{glucose-6-phosphate + ADP (1)} \\
\text{Glucose-6-phosphate} & \xrightarrow{\text{G6PD}} & \text{6-phosphogluconate (2)} \\
\text{NADP} & \longrightarrow & \text{NADPH} \\
\text{(No absorption at 340 nm)} & & \text{(Absorbs UV light at 340 nm)}
\end{array}$$

Potential errors in laboratory glucose testing

There exists opportunities for error in the pre-analytical phase. The following are some considerations that must be addressed to avoid potential problems.

- The **timing of the blood drawn** is critical. For instance, if fasting blood glucose is ordered, it is imperative that the patient is informed and the preparation is verified before the collection occurs.
- **Glucose is constantly metabolized** by the cells present in the blood specimen after it is added to the collection tube. If the specimen is not processed by centrifugation and tested within an hour of collection, the results may not reflect the accurate glucose level of blood as the cells might have metabolized the glucose. If a delay is expected in testing after collection, a tube with sodium fluoride or potassium oxalate additives should be used for the specimen collection. This slows down the utilization of glucose by the cells, and allows the plasma glucose to remain stable for approximately 24 h at room temperature.

Quick screen of blood glucose

Using test strip

For quick screening of blood glucose in case of diabetic patients, plastic reagent strips with glucose testing spot is convenient (Dextrostix, Ames, Miles of India Ltd.). **Hyperglycaemia can be easily recognized with this.** The procedure is described here:

1. Perform skin puncture and squeeze out a large drop of blood.
2. Touch the test spot on the drop of blood and see that the entire surface of the spot is covered.
3. Begin timing immediately by exactly 60 s (or according to the manufacturer's instructions). Simultaneously hold dry surgical gauze over the puncture site.
4. After 60 s, rinse the spot on the strip, blot off the excess water and read the glucose concentration by comparing the colour of the spot with the colour chart. Use the spot reader for glucose, if available.

Using electronic devices

Several hand-held glucose meters (Figure 33.2) are available in the market, which are based on electrochemical technology. This allows the **diabetic patients to check their blood sugar at home** several times a day without any pain. Patient samples are applied to disposable **biosensors,** strips that look similar to other reagents strips. These biosensors, in addition to containing reagents for the chemical reactions, also contain electrodes called **electrochemical sensors.** When the sample interacts with the reagents in the biosensor strip, the current (electrons) generated is detected by the meter and converted into glucose unit.

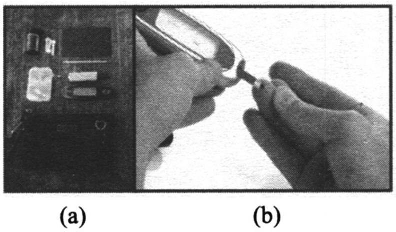


FIGURE 33.2 *(a) Glucometer kit, and (b) Glucometer testing*

Use of glucometer kit

1. Clean and warm the testing site, usually a fingertip.
2. Obtain a drop of whole blood using a lancet or lancet pen.
3. Follow glucometer directions. The amount and method of blood sample placement vary with manufacturer.
4. Allow the correct amount of time for determination of glucose level in the specimen. Most glucometers have automatic timer and will display the results on completion.
5. Record the results in a logbook and, if prescribed by the physician, administer insulin if the levels are elevated.
6. Clean and store back the equipment in the kit according to manufacturer's instructions.

Common errors

Quality control and care of glucometer are important considerations for getting accurate results from this simple equipment.

1. Periodically check the equipment using pretested solutions of glucose.
2. Reagent strips must be stored as instructed by the manufacturer.
3. Always keep a clear record of glucose measurements for the doctor to analyse.
4. Never use the lancet more than once.
5. Always use A1cohol before puncturing the skin.

Glycated or Glycosylated Haemoglobin (HbA1c)

Haemoglobin (Hb) is present in all red blood cells and is the molecule that **transports oxygen** from the lungs to the tissues. **The major haemoglobin is Hb A**. After glucose is absorbed from the gastrointestinal tract and enters circulation, it is taken up by cells for energy. Glucose, the major and simplest carbohydrate in blood, is used as the primary energy source by all cells and especially by the red cells which cannot consume any other form of carbohydrate, the energy package of the biological world.

During period of high blood glucose levels, glucose molecules enter red blood cells and bind to haemoglobin, forming **HbA1c (glycated haemoglobin).** The amount of HbA1c is proportional to the amount of glucose in circulation. Since average life span of red blood cells is approximately 120 days, the level of HbA1c is related to the average amount of glucose it is exposed to in the blood for that period of time. In reality the HbA1c more closely represents levels over the previous 2–4 weeks.

As the **fasting glucose level** gives the patient an idea of the lowest level of glucose on the daily basis, the physician reads the HbA1c value forjudging how tightly the patient has controlled the blood glucose level over a period of about a month. Recent studies have shown that **HbA1c is the single best test for evaluating the risk of damage to nerves** and the small blood vessels of the eyes and kidneys. This damage leads to the complications of diabetes, such as blindness and kidney failure. Clinical trials have shown that reducing the HbA1c level in diabetics and maintaining it below 7% will prevent the development of, or further progress of, complications from diabetes. It is true for both type I and type II diabetes.

The traditional methods of HbA1c determination are HPLC, TLC and electrophoresis. These are available only in reference laboratories. Point of care analysis HbA1c is a recent innovation and has proved to be very helpful to diabetic patients. Results are obtained within few minutes. Additionally, some home glucose meters **calculate the HbA1c from the average of the glucose values in memory.**

Manual Procedure

Laboratory determination of HbA1c may use a latex agglutination reaction in which a **monoclonal antibody** is used as an epitope which recognizes glucose bound to HbA1c. The measurement, however, will require an **immunophotometer.**

Preliminaries

1. Take necessary precautions for self-protection (gloves, laboratory coat, hand sanitization etc.).
2. Arrange the following:
   - Sample dilution kit pouch
   - Test cartridge pouch
   - Specimen–capillary puncture or well-mixed heparinized blood sample (used in haematology)
   - Gauze pad, biohazard waste container
   - Quality control materials

Procedure

1. Verify the test ordered and specimen received.
2. Bring samples, reagents, and monitors, at room temperature.
3. Wash hands and apply gloves.
4. Assemble the necessary equipment before starting the test.
5. Verify the kit has not expired.
6. Prior to the performance of the test, verify whether a quality control (QC) test needs to be run with the control specimen (provided by the manufacturer). If so, complete that test before the patient's test is performed. The results must be in conformity with the manufacturer's directions.
7. Verify that all lot numbers match on the monitor, the sample dilution kit pouch, and the test cartridge pouch.
8. Open the sample dilution pouch and remove the dilution device.

9. Perform the capillary puncture if you are working in a physician's office or mix the whole blood (heparinized) sample to prepare for the next step.
10. Add a 5-μL blood sample to blood collection device.
11. Plug blood collection device into the sampler body. Push firmly so that there is no gap on insertion.

    (*Note* If a gap is present, the sample will not combine with the dilution solution inside the sampler body, and the results will be invalid).
12. Thoroughly mix/shake the sample (6-8 times) with the dilution solution.
13. Set sample assembly on the table top as the test cartridge is prepared.
14. Tear open the test cartridge pouch and ensure that the code number on the cartridge matches the code number printed on the instrument.
15. Insert the test cartridge into the monitor until it is seated firmly and an audible click is heard.
16. Verify that the instrument screen display reads WAIT.
17. When the instrument screen displays SAMPLE (or SMPL), pick up the sampler device and remove the base piece, exposing the plunger.
18. Deliver the sample by pressing the plunger gently and firmly into the corresponding sample application area on the cartridge. Remove after 1 s.
19. The instrument screen will display a countdown from 5 min to 0. Do not move the monitor during this time.
20. The results will display as percentage on the screen. Record results after comparing with the chart provided by the manufacturer.
21. Dispose off the sample dilution, cartridge, and test cartridge, as bio-hazardous trash and disinfect work area.
22. Remove gloves and sanitize hands.
23. Document your results immediately in record book and in patients' request slip.

Automated procedure

The automated system commonly available for analysing HbA1c is Afinion Analyzer and others. This instrument uses **boronate affinity assay** for the determination of percentage of HbA1c in human whole blood. All reagents are contained in the disposable test cartridge which also has an integrated sampling device taking only 1.5 μL of whole blood from a finger prick. The blood sample is automatically diluted and mixed with a liquid that releases haemoglobin from the erythrocytes. The **haemoglobin precipitates.** The sample mixture is transferred to a blue boronic acid conjugate, which binds to glycated haemoglobin. The reaction mixture is soaked through a filter membrane and all **precipitated haemoglobin remains on the membrane.** All measurements and Calculations are performed by the analyzer and percentage HbA1c is displayed on the screen. The analyzer system is easy to use, gives reliable and accurate results. The analysis time is about 3 min and measuring range is 4–15% HbA1c. The system is therefore particularly suitable for use in diabetic clinics.

Diabetic Association has provided guidance regarding the frequency of this test and the interpretation of the result. **A non-diabetic patient will have HbA1c levels below** 6%. For diabetic patients, the goal is to remain below 7%, as it is understood that there may be spikes in the blood glucose that are unavoidable. As a part of the management of diabetes, those who have unstable blood sugars should have heir HbA1c tested quarterly; those who appear to have good glycaemic control should be checked at least two times per year. In addition, the recommendation includes performance of an annual lipid profile and urine micro-albumin testing to screen for renal damage. Micro-albumin testing will be discussed later in this chapter.

SERUM PROTEIN

Serum protein consists of **albumin** and **globulin.** Serum protein analysis can help diagnose liver disorders, nutritional deficiency of protein, renal failure and lymphoproliferative disorders. **Decrease in total protein** values is associated with liver disorders (cirrhosis, nephrotic syndrome), malnutrition, and neoplastic disease. **Increased total protein** values may be diagnostic for multiple myeloma, and conditions associated with high globulin concentration (autoimmune and lymphoproliferative disorders).

Total Protein in Serum

The total protein test measures the total amount of two classes of proteins found in the fluid portion of your blood. These are **albumin and globulin.** Proteins are important parts of all cells and tissues. Albumin helps prevent fluid from leaking out of blood vessels. **Globulins** are an important part of the immune system.

Clinical significance

This test is often done to diagnose nutritional problems, kidney disease, or liver disease. If total protein is abnormal, more tests will need to be done to look for the exact cause of the problem.

Normal range (adult)

Total protein: 6.0–8.0 g/dL
Albumin fraction: 3.5–5.0 g/dL
Globulin fraction: 2.3–3.5 g/dL
Normal value ranges may vary slightly among different laboratories.

Specimen

Serum or plasma; specimen is collected by routine procedure.

Biuret method

Principle

Biuret method, modified by Lowry, for determining **total proteins in serum,** has been used for decades. The procedure is simple and sensitive. It is based on two chemical reactions. The first is the **biuret reaction,** in which the alkaline cupric tartrate reagent complexes with the peptide bonds of the protein. In the second reaction, the **phenol** reagent (Folin and Ciocalteu's phenol reagent) is reduced to a purple colour compound. Absorbance of the coloured solution is read at 540 nm (range 530–560 nm). The protein concentration is determined from a calibration curve or compared with a standard. The following description is based on the technical bulletin of Sigma, manufacturer of laboratory kits.

Interfering substances: Haemolyzed serum or plasma samples should not be used in this testing procedure. Lipaemia has no effect on the total protein values but **grossly turbid serum** should not be used. If necessary, treat the serum with isopropanol, discard the solvent and use the serum for protein assay.

The following account is taken from the brochure of Sigma kit in order to illustrate the procedure. There are many other manufacturers who offer similar kits for the local market.

Caution Read and comprehend the procedure given in the insert before performing the test.

Reagents (provided with the kit)

1. Lowry reagent, powder 5 × 2 g
2. 0.15% Deoxycholate (DOC) solution 20 mL
3. Folin and Ciocalteu's Phenol reagent, 1 bottle
4. Protein standard, 5 × 1 vial—2 mg of BSA (Bovine serum albumin) per vial

Handle all chemicals carefully. Consider all chemicals poisonous. Specimens should be considered as potential biohazards.

Reconstitute the reagents according to manufacturer's direction.

- The Lowry reagent solution is prepared by adding 40 mL of water to a bottle of Lowry reagent powder. Mix well by inverting to completely dissolve the contents. Do not shake so as to minimize foaming.
- The Folin and Ciocalteu's phenol reagent working solution is prepared by transferring the Folin and Ciocalteu's phenol reagent (18 mL) to the amber glass bottle provided for the working solution. Rinse the Folin and Ciocalteu's phenol reagent bottle with 10 mL of water and add the rinse solution to the working solution bottle. Add an additional 80 mL of water to the working solution bottle and mix well. Store the Folin and Ciocalteu's phenol reagent working solution at room temperature.
- A protein standard solution (400 μg/mL) is prepared by adding an appropriate volume of water (5 mL) to the vial. Read the instructions carefully. The exact protein content of the protein standard vial may be found on a lot-specific Certificate of Analysis. Swirl gently to completely dissolve the contents.

Storage and Stability

- Store the Lowry reagent solution and the Folin and Ciocalteu's phenol reagent at room temperature. **Do not refrigerate.**
- Store the protein standard solution in a **refrigerator** (2–8°C) or freezer (below 0°C). The solution is stable for at least 3 months when stored refrigerated. Discard the protein standard solution if turbidity develops.

Procedure

A. Protein determination without protein precipitation

1. Set up three test tubes (19 × 150 mm), marked blank (B), standard (S) and test (T). If there are more specimens, mark them as T1, T2, etc.
2. Add 1.0 mL of water in tube marked B, 1 mL of standard (400 ug/mL or 40 mg/dL) in tube marked S and 1 mL of test specimen in the tube marked T.
3. Add 1.0 mL of the Lowry reagent solution to all the tubes.
4. Mix well by hand or Vortex.
5. Allow solutions to stand at room temperature for 20 min.
6. With rapid and immediate mixing, add 0.5 mL of the Folin and Ciocalteu's phenol reagent working solution to each tube.
7. Allow colour to develop for 30 min.

   Note If your cuvette requires more than 2.5 mL volume, add an appropriate volume of water to each tube before reading. Treat standard, blank, and sample/test tubes identically. Then transfer solutions to cuvettes.
8. Zero the instrument (colorimeter/spectrophotometer) with the blank in order to make all measurements of absorbance against the blank.
9. Measure the absorbance of the standards (A_S) and test specimen (A_T) at a wavelength 540 nm (range 530–560 nm).
10. Absorbance reading is good for 30 min.

    Note The absorbance reading is proportional to the amount of protein present.

Calculation

Concentration of protein in specimen (mg/dL) = $A_T/A_S \times 40$
Concentration of protein in specimen (μg/mL) = $A_T/A_S \times 400$

Serum Albumin

Albumin is the major protein in serum (about 60%). It is synthesized in liver. The other protein is **globulin.** Antibodies are all globulins. Albumin plays important physiological roles, including maintenance of colloid osmotic pressure and binding of key substances such as long-chain fatty acids, bile acids, bilirubin, haematin, calcium, and magnesium. It has antioxidant and anticoagulant effects, acts as a carrier for nutritional factors and drugs, and is an effective plasma pH buffer.

Clinical significance

Decreased levels of albumin in serum may be found in certain conditions such as cirrhosis of the liver and other liver disorders. Loss of albumin due to kidney disorder such as nephritic syndrome will also lead to a decline of serum albumin level as also with malnutrition, malignancy, and chronic protracted conditions like ulcerative colitis. Serum albumin is a reliable prognostic indicator for morbidity and mortality, and protein-losing enteropathies. High level of albumin may be associated with dehydration.

Normal range

3.5–5.0 g/dL

Specimen

Serum or plasma; use fresh samples and store in refrigerator until use.

Bromocresol purple (BCP) method

Principle

Albumin selectively binds with **bromocresol purple (BCP)** dye or **bromocresol green (BCG)** in a suitable buffer to form respective coloured complexes with bromocresol. The intensity of the colour is measured as absorbance at 610 nm. The absorbance is directly proportional to the albumin concentration in the sample. We will focus on BCP although the method is same as in BCG.

The use of **bromocresol purple (BCP) albumin assay kit,** provided by various manufacturers, has simplified the assay in diagnostic laboratories. The following account is taken from the technical bulletin published by Sigma-Aldrich. It is designed to measure albumin directly without any pre-treatment of samples, such as serum, plasma or urine. The optimized formulation substantially reduces interference by other substances (lipids/other proteins) in the raw samples. It may also be used to measure effects of drugs and other compounds on albumin metabolism.

The kit may be used for cuvette or multi-well plate assays. The multi-well plate assay uses samples as small as 5 mL and can be readily automated.

Reagents

These reagents are supplied in the kits:

BCP Reagent	50 mL
Albumin standard (5 g/dL)	1 mL

Instructions for handling the reagents

- Use double distilled water for dilutions.
- Bring reagent to room temperature and shake before use.
- Do not use outdated kit.
- The kit is stored at room temperature or in a refrigerator (2–8°C).
- Store the albumin standard at –20°C. Frozen standards must be thawed completely before use.
- All reagents must be considered as biohazards.

Supplies needed other than kit

- 96-well plate assay: 96-well flat-bottom plate—It is recommended to use clear bottom plates for colorimetric assays.
- Spectrophotometric multi-well plate reader.
- For cuvette assay: Cuvettes suitable for reading absorbance at 610 nm.
- Spectrophotometer suitable for reading absorbance at 610 nm.
- Automatic micropipettor.

Procedure

The procedure involves addition of a single working reagent (BCP) and 5-minute incubation. The optimized formulation has greatly enhanced reagent and signal stability.

96-well plate assay

Follow instructions given by the manufacturer.

1. Transfer 20 µL of blank (B), standards (S), and test specimen (T) to appropriate wells.
2. Add 200 µL of BCP reagent and tap lightly to mix. Avoid bubbles.
3. Let stand for 5 min at room temperature (incubation).
4. Measure absorbance at 610 nm (range 590–630 nm).

Calculations

Concentration of albumin (g/dL) = $\Delta A_T/\Delta A_S \times 5$
Where,

ΔA_S = Difference of absorbance between standard and blank
ΔA_T = Difference of absorbance between test and blank
5 = Concentration of albumin in standard (5 g/dL)

Note If the absorbance of a specimen is higher than the absorbance of standard, dilute the specimen with double-distilled water (ultrapure) and repeat the assay.

Cuvette Assay

1. Transfer 100 µL of water in tube marked B (blank), 100 µL of albumin standard (5 g/dL) in tube marked S, and 100 µL test specimen in rube marked T.
2. Add 1000 µL (1 mL) of BCP reagent in all and tap lightly to mix.
3. Wait for 5 min (incubation at room temperature).
4. Transfer incubated mixtures to different cuvettes B, S and T. (***Note*** Matched cuvettes recommended).
5. Zero the instrument (colorimeter/spectrophotometer) with blank (cuvette B).
6. Read absorbance of the standard (cuvette S) at 610 nm.
7. Read absorbance of the test specimen (cuvette T) at 610 nm.

Calculation

Concentration of albumin in serum specimen (g/dL) = $A_T/A_S \times 5$
Where,

A_T = Absorbance of test specimen
A_S = Absorbance of standard
5 = Albumin concentration in standard in g/dL

Conversion factors for albumin: 0.1 g/dL= 15 mM = 0.1% = 1000 ppm

Note If absorbance (at 610 nm) of a test specimen (T) is higher than the standard (S), dilute the sample with double distilled water (ultrapure) and repeat the assay.

Preparation of Standard Curve

Bring the reagent to room temperature and shake before use. Dilute Bovine Serum Albumin Standard or BSA (5 g/dL) in double distilled water.

Standard #	Amount of Albumin standard μL (5 g/dL)	Amount of water added (in μL) to make up a total volume of 100 μL	Final concentration of Standard (g/dL)
1	100	0	5.0 (1 × undiluted)
2	80	20	4.0 (1.2 × diluted)
3	60	40	3.0 (1.7 × diluted)
4	40	60	2.0 (2.5 × diluted)
5	30	70	1.5 (3.3 × diluted)
6	20	80	1.0 (5 × diluted)
7	10	90	0.5 (10 diluted)
8 (blank)	0	100	0 (No standard)

Note

- Diluted standard may be stored at –20°C for future use.
- The calibration curve must be drawn with every new batch of reagent and must be made for each individual colorimeter used.
- Calibration curve must be linear (straight line).
- If the specimen does not fall within the linear scale, the specimen should be diluted with saline and in final calculation multiply the result by dilution factor.

Additional information

- Bilirubin does not seriously interfere with the test.
- If a slight precipitate is formed in the colour reagent during storage, filter it before use. The reagent does not lose its sensitivity.
- Saline is not used in diluting the specimen. The addition of electrolytes decreases the absorbance. Neither bilirubin nor succinate buffer seems to interfere.
- In case of low serum albumin levels, the results tend to be high owing to the attachment of dye to other proteins. The technician should be aware of this problem and the physician should be informed.

Calculation of Globulin Concentration of Serum

Globulin concentration in serum is indirectly determined by **subtracting the albumin concentration from the total protein** concentration. The normal globulin concentration in serum is in the range of 2.3–3.5 g/dL.

Myoglobin

Myoglobin is a protein that is commonly found in the **heart and skeletal muscles.** The only time that myoglobin is found in the bloodstream is when injury to a muscle has occurred. In particular, **injury to the heart muscle results in the release of myoglobin.** Thus, when detected through a blood test, the presence of myoglobin is clinically significant. Currently developed serum myoglobin blood test is relatively easy to follow that measures the levels of myoglobin in the blood stream.

Clinical significance

This test is typically ordered when it is suspected that a patient is having a heart attack. Most of the time, heart attacks are obvious. However, in some cases, a heart attack is not outwardly clear. Serum myoglobin can be elevated in cases of inflammatory and degenerative muscle diseases and following muscle trauma.

Serum myoglobin test has, for the most part, been replaced by the **troponin level test** to provide a positive diagnosis of a heart attack. This is because troponin levels are more specific to heart damage than myoglobin levels. Additionally, troponin levels will stay higher than myoglobin levels for longer when there is a heart attack. However, serum myoglobin is still used in some cases. The test is commonly ordered in conjunction with other tests for cardiac problems. This is why it is called one of the biomarkers. Other tests associated with the "biomarker" include troponin, creatine kinase (CK), and creatine kinase-MB (CK-MB). These will be discussed at appropriate places. Negative results obtained from a myoglobin test can be used to rule out a heart attack. However, positive results do not confirm that a heart attack has indeed occurred. In order to diagnose a heart attack, troponin levels must also be measured.

The serum myoglobin test may also be ordered following the diagnosis of a heart attack. Once damage to the heart muscle has been confirmed, values obtained from the serum myoglobin test can help physicians estimate the amount of muscle damage that has occurred. Serum myoglobin may also be ordered if the patient has symptoms of kidney disease or kidney failure.

Normal range

0–85 ng/mL

Specimen

Serum, plasma (EDTA or heparin anticoagulated)

ELISA Kit for determination of Myoglobin

The ELISA kit for myoglobin contains 96-well strip plate coated with anti-myoglobin. The minimum detectable value is 0.30 ng/mL. The kit has high sensitivity and excellent specificity for the detection of myoglobin. No significant cross-reactivity or interference between myoglobin and analogues is noticed. The kit has high linearity and stability.

Principle of assay

The test principle applied in this kit is **sandwich enzyme immunoassay** which is shown in Figure 33.3. The microtitre plates (provided in the kit) are pre-coated with an **antibody specific to myoglobin.** This is the solid phase. Standards or test samples are then added for capturing the analyte or antigen (myoglobin). Following incubation, myoglobin (antigen) sticks to the antibody and is not washed away in the following step. After wash, a second antibody to myoglobin and conjugated to Horseradish Peroxidase (HRP) is added to each microplate well (detection reagent A). This is then allowed to re-incubate at 37°C. After incubation, TMB substrate solution (detection reagent B) is added and re-incubated. Only those wells that contain myoglobin, biotin-conjugated antibody and enzyme-conjugated-antibody will exhibit a change in colour. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the colour change is measured spectrophotometrically at a wavelength of 450 nm ± 10 nm. The concentration of myoglobin in the samples is then determined by comparing the absorbance of the samples to the standard.

Reagents

The kit provides all necessary reagents for the test and instruction manual. Strictly follow the instruction manual while performing the test.

- Pre-coated, ready to use 96-well strip plate
- Plate sealer
- Standard
- Detection reagents A and B
- Substrate
- Wash buffer (30 × concentrate)

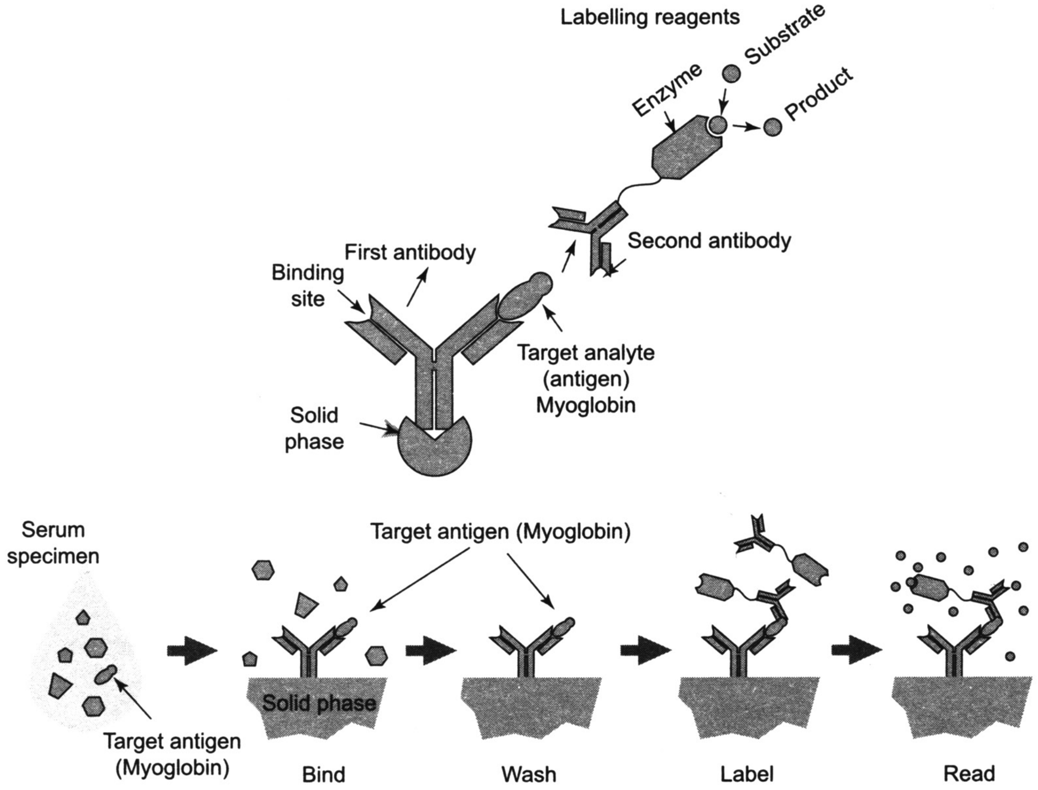


FIGURE 33.3 *Sandwich enzyme immunoassay*

- Standard diluent
- Assay diluents
- Stop solution

Other supplies

- Automatic micropipettors
- Well reader

Procedure

1. Prepare all reagents according to manufacturer's instructions.
2. Label test samples (T_1 T_2, etc.) and standards (S) on the plate.
3. Bring everything to room temperature.
4. Add 100 µL standard (S) and test sample (T) to respective well. For blank, use 100 µL of double distilled water.
5. Incubate for 2 h at 37°C.
6. Aspirate and add 100 µL prepared Detection Reagent A.
7. Incubate for 1 h at 37°C.
8. Aspirate and wash 3 times.

9. Add 100 μL prepared Detection Reagent B.
10. Incubate 30 min at 37°C.
11. Wash and aspirate 5 times.
12. Add 90 μL substrate solution (colour developer).
13. Incubate 15–25 min at 37°C.
14. Add 50 μL Stop Solution (sulphuric acid).
15. Read absorbance at 450 nm immediately.

Troponin

Troponin is another protein present in serum. The normal value of serum troponin is less than 0.01 ng/mL. It consists of a complex of three regulatory proteins (troponin C, troponin I, and troponin T) that is integral to muscle contraction in skeletal muscle and cardiac muscle, but not smooth muscle. An increased level of the cardiac protein isoform of troponin circulating in the blood has been shown to be a biomarker of heart disorders.

Clinical significance

Physicians request for troponin assay in the diagnosis of acute myocardial infarction (AMI). Raised troponin levels indicate cardiac muscle cell death as the enzyme is released into the blood upon injury to the heart.Its release pattern is similar to CK-MB (4–6 h after the onset of AMI). However, CK-MB levels return to normal after 36–48 h, while levels of troponin I remain elevated for upto 6–10 days. The level of troponin I is very low in normal healthy people, and is not detected in patients with skeletal muscle injury. Therefore, troponin I is a specific marker for the diagnosis of AMI.

Normal values

<0.01 ng/mL
Values > or = 0.01 ng/mL have been shown to have prognostic value.

Specimen collection and preparation

Freshly **collected serum** is recommended. If delay is expected, store the specimen in refrigerator. Allow the sample to reach room temperature before proceeding. **Sodium azide** (0.1%) may be used as a preservative without affecting the test results. If it is used, dilution factor of the serum should be noted.

Laboratory assay

The Rapid Troponin I test is described here which is based on **immune-chromatography** (sandwich immunoassay). It is a one-step test designed for a qualitative determination of cardiac troponin I in human serum specimens. It is very helpful in the diagnosis of myocardian infarction under emergency situation in remote areas of developing countries. It is available from rapid diagnostics as a test strip.

Principle

Rapid Troponin I test is based on **sandwich immunoassay.** The manufacturer provides a solid pad on which there is a spot with colour-producing antibody. When the serum moves through the solid pad through capillary action, *c*-troponin, if present, reacts with the **anti-c troponin conjugate** (antibody) present on the pad and appears as a coloured band on the test region of the pad. This happens only when the level of *c*-troponin in the serum is more than 1.5 ng/mL (diagnostic level). Thus, a positive reaction is the formation of a pink to purple colour, otherwise the spot remains colourless (normal serum). The pad also provides a control area. If there is no *c*-troponin I in the sample, the area will remain colourless. The sample, however, continues to move to the control area by capillary action and forms a pink to purple colour on the "control" spot. This confirms the test and the results are considered valid.

Reagents

Rapid Troponin I Test device (test strips) provided in a kit by the manufacturer.
Carefully follow instructions given in the insert.

Supplies not provided in the kit

- Serum collection containers
- Timer or clock

Storage: Store the test device at 2–30°C. Do not freeze.

Precautions

Do not use commercial kits with expired date.

Quality control

- The control band is an internal reagent and procedural control. It will give colour reaction if the test has been performed correctly and the reagents are reactive.
- Good laboratory practice recommends the daily use of control materials to validate the reliability of the device.

BLOOD UREA NITROGEN (BUN)

Urea is the final degradation product of protein and amino acid metabolism. In **protein catabolism** the proteins are broken down to **amino acids and deaminated.** The ammonia formed in this process is synthesized to urea in the liver. The urea passes into the blood and filtered out of the kidneys and excreted in the urine. This is the most important catabolic pathway for eliminating excess nitrogen in the human body. If the kidneys do not remove urea, the concentration in the blood is increased which is the sign of renal dysfunction.

Clinical significance

The determination of serum blood urea nitrogen currently is the most widely used screening test for the **evaluation of kidney function.** The test is frequently requested along with the serum creatinine test since simultaneous determination of these two compounds appears to aid in the differential diagnosis of pre-renal, renal and post-renal hyperuremia. There could be **pre-renal clinical problems** that results in an increase protein breakdown like glomerulonephritis, chronic nephritis, nephrotic syndrome, etc. Hence the results must be carefully evaluated. Acute upper gastrointestinal bleeding can cause increase in protein into the gut which after absorption may cause high level of BUN. Looking to the other side of the kidney, **post-renal problems** like obstruction of urinary tract may also cause increase in urea. The value must, however, be compared with creatinine level before concluding the diagnosis. Post-renal problems lead to increase of both urea and creatinine proportionately. In other words, the ratio does not change significantly.

Normal range

Urea: 15.0–38.5 mg/dL (values vary with diet)

Specimen

Serum specimen is commonly submitted for reporting blood urea concentration. Since **urea may be lost through bacterial action,** the specimen should be analyzed within 2 h after blood collection or should be preserved by refrigeration.

Interfering substances: Avoid using haemolyzed and icteric serum to prevent loss of urea to bacterial contamination. Moderate haemolysis (0.15 g dL Hgb), bilirubin levels up to 20 mg/dL, and moderate lipaemia do not cause significant interference with this method.

Diacetyl monoxime method (colorimetric)

The diacetyl monoxime methodology for BUN determination is direct and measures a chromogen formed from the **condensation of urea with diacetyl.** This condensation methodology does not suffer from ammonia interference and utilizes less caustic reagents than other methods. Diacetyl monoxime is hydrolyzed under acidic conditions to produce diacetyl which then condenses with urea to form a pink chromogen that is measured at 520 nm. **Thiosemicarbazide and ferric ions** are employed to enhance the colour development.

Reagents

- BUN colour reagent—16.6 mmol/L diacetyl monoxime (Reagent #1): BUN colour reagent should be a clear to pale yellow solution. Darkening or formation of a precipitate in the reagent would indicate contamination and the reagent should be discarded.
- BUN acid reagent—0.21 mmol/L ferric chloride, 0.01 mol/L phosphoric acid, and 1.9 mol/L sulphuric acid (Reagent #2): The BUN acid reagent should be a clear colourless solution. Failure to achieve assay values on freshly prepared control sera may indicate reagent deterioration.
- Urea nitrogen standard solution—Concentration is provided by the manufacturer; follow direction for reconstitute. Store the solution in refrigerator at 0–5°C. The final concentration of the standard is usually 100 mg/dL which widely varies.

Warning: All chemicals should be considered poisonous and harmful. Avoid contact to all chemicals with skin, eyes, or clothing. Flush affected area with water and seek medical attention. All specimens are potential biohazard.

Materials required but not provided in the kit

- Spectrophotometer or suitable instrument to read well, calibrated to read absorbance at 520 nm.
- Test tubes and cuvettes.
- Automatic pipettor capable of dispensing 0.02 mL (20 μL), 1.5 mL, and 3 mL.
- Timer or stopwatch.
- Boiling water bath (100°C) or use a heat block capable of maintaining that temperature.

Procedure

1. Label three or more test tubes or cuvettes as blank, standard, Test 1, Test 2, etc.
2. To each, add 1.5 mL BUN colour reagent.
3. To blank (B), add 20 μL water. To standard (S), add 20 μL of urea nitrogen standard solution, and to tube marked T add 20 μL serum or plasma.
4. Mix contents by gentle swirling.
5. Add 3.0 mL BUN acid reagent into all tubes and mix well.
6. Incubate for 10 min at 100°C in a heat block or 8 min in a boiling water bath.
7. Remove all tubes and cool to room temperature using cold tap water.
8. Mix all tubes with hand or Vortex.
9. Set the wavelength of the spectrophotometer at 520 nm (or use appropriate filter in case of colorimeter). Set the "Zero" with the reagent blank (use water in place of serum).
10. Read the absorbance of standard (or control) and that of test specimen (patient's serum). ***Note*** Final colour is stable for 6 h at room temperature. Calculate the concentration of urea in serum specimen.

Calculations

Conc. of urea in serum (mg/dL) = A_T/A_S × Conc. of standard × Serum dilution factor
Where,

A_T = Absorbance reading of the test specimen against blank
A_S = Absorbance reading of the standard against blank

Note If the concentration of urea in the specimen is more than 150 mg/dL, the specimen must be diluted with saline and test repeated. Multiply results by the dilution factor (total volume/amount of specimen) when calculating the unknown.

Example 1 mL of serum mixed with 2 mL of saline, the dilution factor = 3/1 = 3 ×

Quality Control

Linearity extends to 150 mg/dL. Check the linearity for every batch of chemicals used. The two levels of assayed control, i.e., Monitrol I and Monitrol II, can be diluted to get various concentrations of urea to make the standard curve.

Enzymatic method

There are many kits available in the market for the assay of urea by enzymatic method. Of these, the microplate method is more popular. This allows smaller size of the sample and large number of assays. The depressions on the plate are used as cuvettes. Hence, in the long-run, microplates may be cost effective as it requires smaller amount of reagents. We have chosen one of the microplate-based kit for the purpose of illustration. Instruction may vary with manufacturers. Hence, always follow the instructions given in the kit. In absence of microplate, cuvettes may be used after making a few changes.

Principle

Enzyme-based assay procedure utilizes urease to specifically detect urea in serum. In presence of urea, urease produces ammonia as follows:

$$\underset{\text{Urea}}{CO(NH_2)_2} + H_2O \xrightarrow{\text{Urease}} CO_2 + \underset{\text{Ammonia}}{2NH_3}$$

The ammonia produced from the urea is then directly detected by a colorimetric chemical reaction.

The method is rapid, accurate, and proven to be reliable. The linearity range of urea concentration in this assay is 1–20 mg/dL. If the concentration is beyond this range, the specimen is diluted with saline and the dilution factor is used during calculation. The kit contains urea standards to construct a linear calibration curve and verify assay performance. The kit is designed to be used with a microplate reader.

Specimen and its preparation

Always try to get fresh specimen and run the test immediately. Standing specimen may lose urea.

1. Allow blood sample to coagulate in a microfuge tube for 20 min at room temperature.
2. Centrifuge for 5 min at 9,000 rpm.
3. Transfer the supernatant (serum) to a clean tube.
4. If the samples cannot be tested within 6 h of collection, store them at 4°C and test no later than 3 days after collection.

Note Immediately before testing, dilute the serum 1 : 4 (dilution factor 5×) with normal saline or PBS (phosphate buffered saline).

Kit Contents

- Store the kit in a refrigerator. The shelf life is 6 months when the kit is properly stored.
- Microtitre Plate (1 × 96-well plate that contains 8 wells in 12 strips).
- Urea Standard: 0–20 mg/dL (higher standards may be included).
- Urease Mix: 1 vial (keep refrigerated)
- Alkaline hypochlorite solution: 20 mL

Storage and Shelf Life

- Do not intermix reagents from different kits or different lots.
- Try to maintain a laboratory temperature of 20–25°C (68°–77°F).
- Use only distilled-deionized water since water quality is very important.

Reconstituting the reagents

- ***Urease mix***: It comes in lyophilized form in the kit and need to be reconstituted. Follow manufacturer's instruction. *Here is a general outline:* Add exactly 20 mL of deionized-distilled water to the urease mix powder (in kit). Mix by swirling or inverting several times until the powder is dissolved. Wait for 10 min at room temperature. The urease mix is stable for 4 months in the refrigerator after reconstitution with water. The reconstituted urease mix, however, can be left at room temperature for short periods prior to use. Between uses, the reconstituted urease mix should be stored in the refrigerator. Discard the urease mix 4 months after reconstitution.
- ***Urease standard***: Reconstitute by direction provided by the manufacturer. The final concentration of the standard is 20 mg/dL.

Materials/Equipment required but not provided with the kit

- Microtitre plate with 96-wells
- Microtitre plate reader (with 620 nm absorbance filter)
- Microcentrifuge
- Microcentrifuge tubes
- Automatic pipettors
- Normal saline or PBS (Phosphate-buffered saline, pH 7.4)

Note You can use narrow cuvettes after making necessary alteration in the procedure.

Warnings and Precautions

- Read the instructions thoroughly as provided by the manufacturer.
- Standard curve should be made with each kit and check the linearity.
- Do not use the kit past the expiration date.
- When pipetting samples or reagents into an empty microtitre plate, place the pipette tips in the lower corner of the well, making contact with the plastic.
- A standard curve must be constructed for each new kit. Here the absorbance of the standard (blank corrected) is plotted (y-axis) against the concentration (x-axis) and check the linearity. The curve should be linear up to 100 mg/dL.

Procedure

1. Set-up
   - Warm up kit reagents to room temperature and turn on the plate reader. This will allow the light source to warm up.
   - Set absorbance wavelength to 620 nm (other wavelengths in the range of 590–630 nm can also be used for the assay, but the sensitivity will be slightly lower).
   - Before testing, dilute serum samples 1 : 4 in either normal saline or PBS. Here the dilution factor = 5 × (1 mL + 4 mL = 5 mL, so the dilution is five times; 1 made to 5).
2. Add 5 μL of diluted serum (see Step #1) to the microplate wells. Duplicate sample is recommended.
3. Then add 150 μL of reconstituted urease mix solution to the wells.
4. Tap the plate gently several times to mix the sample and enzyme.
5. Allow to incubate for 15 min at room temperature.

6. Add 150 μL of alkaline hypochlorite to each well. This stops the reaction in progress.
7. Incubate for another 10 min at room temperature.
8. Read the absorbance in duplicate at 620 nm.

Calculation

Calculation of urea concentration is made by standard formula:

$$\text{BUN concentration (mg/dL)} = A_T/A_S \times 5 \text{ (Dilution factor)}$$

Where,

A_T = Absorbance of Test (specimen)
A_S = Absorbance of Standard
5 = Dilution factor

URIC ACID

Uric acid is a heterocyclic compound of carbon, nitrogen, oxygen and hydrogen with formula $C_5H_4N_4O_3$. It forms ions and salts known as **urates** and **acid urates** such as ammonium acid urate. Uric acid is the end product of nucleoprotein metabolism with low threshold excretory product.

Clinical significance

High blood concentrations of uric acid can lead to **gout**. The chemical is associated with other medical conditions including diabetes and formation of ammonium acid urate kidney stones. The determination of serum uric acid has diagnostic value in differentiating gout from other causes of arthritis. Uric acid levels are also increased in renal failure, uraemia and leukaemia.

Normal range

Serum uric acid (Adult) – Male: 3–7 mg/dL; Female: 2-6 mg/dL
A serum uric acid >12 mg/dL calls for further investigation.

Principle

Determination of serum uric acid by enzymatic method is a two-step reaction. In the first step, the enzyme **uricase** (present in working reagent) acts on the uric acid present in the specimen to catalyse the splitting of **uric acid**. This reaction produces hydrogen peroxide (H_2O_2). In the second step, another enzyme, **peroxidase** (present in the working reagent) oxidizes the H_2O_2 releasing nascent oxygen that oxidizes the phenolic chromogen (present in the reaction mixture). The oxidized form of the chromogen is a coloured compound, whose intensity can be measured at 500 nm (500–530, green filter).The intensity (absorbance) of red colour is proportional to the amount of uric acid present in the serum sample.

Here are the steps of chemical reactions:

$$\text{Uric acid} + 2H_2O + O_2 \xrightarrow{\text{Uricase}} \text{Allantoin} + H_2O_2 + CO_2$$

$$H_2O_2 \xrightarrow{\text{Peroxidase}} H_2O + [O]$$

$$\text{Phenolic chromogen} + [O] \longrightarrow \text{Coloured chromogen}$$

Specimen

Serum, blood collected by routine procedure.

Reagents

A. Stock reagents: This comes in lyophilized form in the kit. Reconstitute according to manufacturer's direction.

• Buffer (pH 7.5)	100 mmol/L
• Uricase	100 IU/L
• Peroxidase	140 IU/L
• Chromogen	2.5 µmol/L
• Surfactants/stabilizer	

Stability of reagents: Lyophilized reagents are stable at 2–4°C. Reconstituted reagents must be stored in the refrigerator.

B. Uric acid standard: This comes as standard solution (5.0 mg/dL).

C. Working reagent solution or enzyme reaction mixture is made from the lyophilized stock reagent. The stock is diluted with distilled water according to manufacturer' direction. Working reagent is stable at 2–4°C for 60 days. 1 mL of working reagent is used in testing.

Procedure

1. Label three test tubes as blank (B), standard (S), and test (T). If there are more specimens, name the test tubes T_1, T_2, T_3, etc., according to the number of tests to be done.
2. Pipette 1.0 mL of working reagent solution in all test tubes.
3. Add:
   0.02 mL of water in the blank tube (B).
   0.02 mL of standard (5 mg/dL) in the tube marked S (standard).
   0.02 mL of serum specimen in tube marked as T (Test).
   Continue if more serum specimens are to be tested (T_1, T_2, T_3, etc.).
4. Keep the test tubes at room temperature 25 ± 5°C for 10 min.
5. Transfer the contents of the tubes to cuvettes to take absorbance readings at 500 nm (use green filter for colorimeters).
6. Set the zero of colorimeter (spectrophotometer) with the blank. Read the absorbance of test specimen (A_T) and that of the standard (A_S) against the blank which is used to set the zero.
7. Calculate the concentration of uric acid in test specimens.

Calculation

Serum uric acid (mg/dL) = $A_T/A_S \times 5$

Where,

A_T = Absorbance of test (specimen)
A_S = Absorbance of standard
5 = Concentration of standard in mg/dL

A_T/A_S ratio remains constant for the same kit.

Note

- Method is linear up to 25 mg/dL.
- Glucose, bilirubin, ascorbic acid, urea, proteins, and haemoglobin do not interfere with this test, if present in moderate concentrations.

Determination of Uric Acid in Urine

1. Dilute urine 1 : 10 in distilled water and perform the test as described above.
2. Modify Calculation by multiplying with 10 (dilution) to report the concentration of uric acid in urine.

$$\text{Urine uric acid (mg/dL)} = A_T/A_S \times 5 \times 10$$

CREATININE

Creatinine is a breakdown product of creatinine phosphate in muscle, and is usually produced at a fairly constant rate by the body (depending on muscle mass). It is a substance stored in muscles and used for energy. Creatinine is removed from plasma through glomerular filtration and is then excreted in the urine without being reabsorbed by the tubules to any significant extent. When **renal function** is impaired, blood creatinine levels rise, but more than 50% of kidney function is lost before this happens. So, **elevation** usually indicates significant **insufficiency.** Other reasons for the increase of serum creatinine level are shock, water imbalance, dehydration and ureter blockage. Simultaneous determination of urea and creatinine is desirable in order to trace the aetiology as both increases with decreased kidney function. Creatinine determinations have one advantage over urea determinations as they are not affected by a high protein diet as is the case for urea levels. Determination of creatinine clearance is a highly sensitive test for measuring the glomerular filtration rate.

Clinical significance

Creatinine is produced from creatine which provides energy to the function of muscles. Thus, it is a waste product of the body that comes from muscle metabolism. Creatinine leaves the body through the urine. Thus, creatinine levels in the blood are related to **excretion rate by the kidneys.** Increased concentrations of creatinine in serum indicate that the kidneys are not functioning properly (diseased or damaged). The test is simple but it is unsuitable for detecting early-stage of kidney disease. A better estimation of kidney function is given by calculating the estimated glomerular filtration rate (eGFR). eGFR can be accurately Calculated using serum creatinine concentration. Many laboratories will automatically Calculate eGFR when a creatinine test is requested. **Urine creatinine concentration** is also checked during standard drug tests. But one should keep in mind that the trend of serum creatinine levels over time is more important than absolute creatinine level.

Normal range

Creatinine is typically reported in mg/dL but also expressed as umol/L. For the conversion of creatinine concentration in mg/dL unit to the International unit of μmol/L, apply the following formula:

$$\text{mg/dL} \times 88.4 = \mu\text{mol/L}$$

Serum creatinine range:
Males: 0.7 to 1.3 mg/dL (60–110 μmol/L)
For women: 0.6 to 1.q mg/dL (45–90 μmol/L)
Urine: 90–150 mg/dL (100 times higher than serum)
Urinary discharge per day: 1.0–2.0 g/day

Laboratory assay

Two methods are available for the laboratory assay of creatinine—colorimetric method and enzymatic method. Only the colorimetric method will be described here as the enzymatic method is more expensive with no additional advantage.

Specimen and its preparation

Blood is collected in plain tube through venepuncture. Prepare the serum specimen by routine procedure:

- Clot the blood at room temperature
- Centrifuge
- Separate the serum

- Alternatively, use the serum separator tubes (not cost effective in developing countries).
- At least 20 μL of serum will be needed for each test.

Caution Avoid hemolysis as it may release erythrocyte creatinine into the serum. Creatinine in serum is reported stable for 2 days at 4°C (refrigerator) and 3 months when frozen and properly protected against evaporation.

Alkaline picrate method (colorimetric)

Colorimetric assay kits are available in the market which makes the test simple, direct, and automation compatible. Because of increasing popularity, we have described here the procedure for microplate instead of cuvette. With minor adjust of volume, the kit can be used for cuvette as well.

Principle

At high pH (alkaline), **creatinine reacts with picrate** reagent forming a red-coloured complex. This is called Jaffee reaction.

$$\text{Creatinine} + \text{Alkaline Picrate} \longrightarrow \text{Red Coloured Complex}$$

The intensity of red colour, as measured at 510 nm, is proportional to the concentration of creatinine in the sample.

Reagents

Most kits provide sufficient prepared reagents necessary for 40 duplicate specimens. Kits can be stored at room temperature, except the standard which is stored in the refrigerator. The shelf life is 6 months if properly stored.

• Microtitre plate	
• Microplate cover sheet	
• Creatinine colour reagent (Reagent #1)	20 mL (picric acid)
• Creatinine buffer reagent (Reagent #2)	20 mL
• Creatinine standard (20 mg/dL)	0.75 mL (reconstitute)

Note **Reagent mix refers to mixing of Reagent 1 and 2 and keep it ready for the test.**

Special instructions

- A new standard curve must be established for each new kit in order to check the linearity. The creatinine standard provided in the kit should be used to calibrate the assay.
- Strictly follow manufacturer's instructions.
- *While preparing standard curve using microplate, always remember:* Add standards to plate only in the order from low concentration to high concentration, as this will minimize the risk of compromising the standard curve.
- Treat all specimens as potential biohazard.
- Wipe up any spillage since dried picrate (picric acid) is explosive.
- Handle all reagents cautiously.

Additional materials required but not provided in the kit

- Microtitre plate reader (510 nm) that reads absorbance
- Water bath or incubator to maintain 37°C temperature
- Centrifuge (for preparing serum)
- Deionized-distilled water (superpure)
- PBS (phosphate buffer saline, pH 7.3)
- 1.5 mL microfuge tubes
- Multichannel pipette or repeating pipettor (recommended but not required)

Working reagent preparation

Mix equal amount of Reagent #1 (colour reagent) and Reagent #2 (buffer reagent) as needed for the number of tests to be performed. This will be referred as "Reagent Mix".

For example: You will need a total of 300 µL per testing. Hence if you have to perform 10 tests, combine 1.6 mL of Reagent # 1 with 1.6 mL of Reagent #2 in order to yield a total of 3.2 mL of reagent mix. This will allow you to perform 10 tests (each requiring 300 uL of total mixture), implying that you will need 3.0 mL of mixture leaving behind 0.2 mL (20 µL) excess reagent to avoid entry of any air bubble into the pipette.

Procedure

The general summary of the procedure is given below. After preparing the sera, the assay is performed by adding reagent mix into microplate wells containing 10 µL sera. After a brief incubation, the absorbance of each well at 510 nm is then measured using a plate reader. The concentration of creatinine in each sample is then directly determined from the 510 nm absorbance. The step-by-step-account of these steps is given below:

1. Use three microplate-wells as blank (B), standard (S) and test specimen (T). If more than one specimen is there, mark them as T_1 T_2, etc.
2. Add 10 µL of water in well-marked B, 10 µL of test specimen in well-marked T, and 10 µL of standard (20 mg/dL) in the well-marked S.
3. Add 300 µL of reagent mix in all the wells. (***Note*** Reagent mix is the mixture of Reagent 1 and 2 in equal amounts. This has been explained earlier).
4. Carefully cover the wells with adhesive cover sheet, mix in the shaker and incubate at 37°C for 15 min.
5. After incubation, carefully remove the adhesive cover sheet and zero the absorbance reading of the instrument with blank.
6. Treat the test (T) and standard (S) in the same way and measure the absorbance of each specimen (A_S) and that of the standard (A_T) at 510 nm. As the instrument is zeroed with blank, all absorbance readings are against the blank (0 mg/dL).
7. Samples with values above 20 mg/dL should be diluted with PBS or normal saline and re-tested. (Multiply results by dilution factor).

Calculation

Concentration of creatinine in specimen (mg/dL) = $A_T/A_S \times 20 \times DF$
Where,

A_T = Absorbance of test specimen
A_S = Absorbance of standard
20 = Concentration of standard (20 mg/dL)
DF = Dilution factor (in case the specimen is diluted)

Preparation of standard curve

Within limits there is a linear relationship between the concentration of creatinine in the specimen and the absorbance at 510 nm. Therefore, a standard curve provides a reference for the linear range of the assay. Once the linearity is established, the concentration can be calculated mathematically, as long the concentration falls within the linearity range: A_T/A_S, where A_T is the absorbance of the Specimen (Test) and A_s is the absorbance of the Standard.

How to make dilutions of the standard to be used in preparing the standard curve?

Label four centrifuge tubes to prepare various dilutions of the standard.

The four dilutions of the standard (plus one blank, #5) are made as follows:

Table showing the dilution process that gives various standards to plot the standard curve

Tube #	1	2	3	4	5
Creatinine standard (20 mg/dL)	100 μL	50 μL	25 μL	5 μL	0 μL
Water (deionized/distilled)	0 μL	50 μL	75 μL	95 μL	100 μL
Creatinine concentration	20 mg/dL	10 mg/dL	5 mg/dL	1 mg/dL	0 mg/dL (blank)

10 μL of each diluted standard is used for reaction.

Determination of creatinine clearance

The group of tests, generally referred to as renal clearance tests, are useful in assessing the capacity of kidneys to eliminate (or clear) certain substances present in plasma. Two of these substances are routinely considered—creatinine and urea. **Clearance study of creatinine has proved to be more reliable and reproducible** because the serum level of urea varies considerably depending on the type of food taken by the patient. The request for the clearance study is made by the physician when the patient shows an increase of non-protein nitrogenous constituents of blood—mainly urea, creatinine and uric acid. The correct assessment of the functional capacity of the kidney comes from the clearance study (Chapter 34 of this volume).

BILIRUBIN

Bilirubin is one of the degradation products of haemoglobin formed when red blood cells die in the spleen, liver, and bone marrow. Bilirubin is conjugated with glucuronic acid in the liver to form a soluble compound. This **conjugated bilirubin** passes down the bile duct and is excreted into the gastrointestinal tract. An unconjugated, albumin bound form is also present in the circulation. It is insoluble and does not normally pass through the kidneys into the urine. Thus, in serum, bilirubin exists as insoluble **unconjugated** form (also **indirect** bilirubin), or soluble glucuronide **conjugated** form (also called **direct bilirubin).** Conjugated bilirubin moves into the bile duct of the liver and then to the gall bladder. When stimulated by eating, bile (including the conjugated bilirubin) is excreted into the small intestine, where bilirubin is converted into **urobilinogen.**

Clinical significance

Jaundice is caused by the elevation of **bilirubin** in blood. It gives a yellowish discolouration of skin, mucous membranes, and whiteness in eyes. Bilirubin is a brownish yellow substance found in bile. It is produced in liver from old red blood cells after they cease functioning. Bilirubin is normally taken out of the body through stool and urine. This is why they have the normal yellowish colour. If the liver does not function properly, bilirubin is back-fired into the blood stream. This clinical condition is jaundice.

Bilirubin is a key diagnostic indicator. **High levels of bilirubin** result when too much haemoglobin is broken down or the removal of bilirubin does not function properly. The accumulation of bilirubin in the body causes **jaundice.** Jaundice occurs in case of toxic infectious diseases of the liver, e.g.,viral hepatitisor obstruction of the bile duct and also in case of incompatible blood transfusion. In newborn babies, excess bilirubin can lead to retardation or physical abnormalities; early detection is therefore extremely important.

Useful information may be obtained by determining which **form of bilirubin** is elevated. **Unconjugated** or indirect bilirubin can be determined by subtracting the direct bilirubin level from the total bilirubin result. High levels of **conjugated** or direct bilirubin indicate that bile is not being properly excreted; therefore an obstruction may be present in the bile duct or gall bladder. High levels of unconjugated bilirubin indicate that too much haemoglobin is being destroyed or that the liver is not actively treating the haemoglobin it is receiving.

Normal Range

Adult:	
Conjugated (direct)	0.0–0.2 mg/dL
Unconjugated (indirect)	0.2–0.8 mg/dL
Total (direct + indirect)	0.2–1.0 mg/dL
Infant:	
Premature	>10 mg/dL
Full term	>4 mg/dL

Conversion to international unit: mg/dL × 17.1 = μmol/L

Specimen

Adults should not eat or drink for 4 h before drawing blood. No special preparation is needed for children before having a bilirubin test. Serum is the specimen of choice for the quantitative assay of bilirubin in blood. Collect blood by venepuncture in a plain tube (without any anticoagulant). **Avoid haemolysis.** Specimens for bilirubin determination should be protected from light, since **bilirubin is light-sensitive** and will break down under exposure.

Here are the basic steps to get the serum:

1. Collect blood by venepuncture in a plain tube (red top).
2. Allow the blood to clot at room temperature for 10 min.
3. Centrifuge to separate the clot.
4. Remove serum layer to a clean tube avoiding the "buffy coat" layer.
5. Store the serum samples in a refrigerator; do not freeze.
6. Serum samples can be stored in a refrigerator for one week.
7. If turbidity is observed, centrifuge the sample and use clear supernatant for assay.
8. Always bring the specimen to room temperature before taking the aliquot for analysis.

Laboratory assay

Physician may ask the laboratory to report:

- Total bilirubin
- Total and Conjugated (direct) bilirubin
- Indirect bilirubin (it is calculated by the difference of total and conjugated (direct) bilirubin)

Here we have presented two different kits using same colorimetric method of (Jendrassik and Grof, 1938) with minor modifications.

Total Bilirubin

Clinical significance

Jaundice is the most common reason to check the bilirubin level of blood. An increased total bilirubin may reflect liver problem, incompatible blood transfusion in infants or a suspected case of gallbladder problem.

Principle

Aqueous diazotized sulphanilic acid reagent modifies bilirubin in the sample to an azobilirubin form (colour) which absorbs light at 550 nm. The absorption is proportional to the concentration of total bilirubin in the sample. A separate serum blank eliminates endogenous serum interferences.

The Total Bilirubin Assay Kits available in the market makes the test procedure simple and rapid to follow. Kits from reputed manufacturer are recommended as they are more sensitive and has low detection limit (1 mg/dL). The kit also contains a control solution containing a standard (equivalent to 20 mg/dL bilirubin) which can be used to calibrate the assay and verify kit performance.

We have used here the microplate method, using standard calorimetric procedure for bilirubin determination. It can be adopted to use small size cuvettes if microplates are not available. Appropriate adjustments in the procedure will then be necessary.

Always follow manufacturer's instructions. The following is a general outline:

1. Prepare serum by the procedure described before. (Always protect the serum from sunlight).
2. Label the microplates—blank (B), standard (S), and tests (T_1, T_2, etc.).
3. Add requisite amount of sulphinilic acid and sodium nitrite reagent in the microplate. This may be sold as bilirubin reagent mix.
4. Add the serum sample.

Sensitivity (Detection Limit)

Specimen type: Serum
Detection limit: 1 mg/dL

Reagents and requisites

Purchase the kit from a reliable vendor. Check the content of the kit against manufacturer's instructions available in the insert. Store the kit in a refrigerator; the shelf life is 6 months when the kit is properly stored.

- Bilirubin reagent mix
- Nitrite reagent
- Calibration standard (20 mg/dL)
- Microplate

Required Materials but Not Provided With the Kit

- Microplate reader (550 nm): Use of micro cuvette is recommended if microplate reader is not available. In that case, minor adjustments may be necessary for the amount of reagents, standards and specimens.
- Microcentrifuge: If not available, use table centrifuge.
- Deionized-distilled water (***Note*** Double distilled water may be used but deionized water is more efficient).
- PBS (phosphate buffer saline, pH 7.3). Use this in place of normal saline.
- 1.5 mL microfuge tubes.
- Multichannel pipette or repeating pipettor (recommended).

Warnings and Precautions

- Read and understand the instructions of the manufacturer thoroughly before performing the test.
- Do not use the kit past the expiration date.
- Try to maintain a laboratory temperature close to 20–25°C (68–77°F).
- Use a refrigerator with outside temperature of 4°C (above freezing).
- Avoid running assays under or near air vents, as this may cause excessive cooling, heating and/or evaporation.
- Do not run assays in direct sunlight, as this may cause excessive heat and evaporation.
- Cold bench tops should also be avoided.
- Use only distilled or deionized water since water quality is very important.
- When pipetting samples or reagents into an empty microtitre plate, place the pipette tips in the lower corner of the well, making contact with plastic.

Procedure (using microplate)

1. Turn on the microplate reader, allow light source to warm up, and set the absorbance wavelength to 550 nm.

2. Allow reagents to warm up to room temperature for 30 min.
3. Add 263 µL of bilirubin reagent mix in the microplate wells.
4. Then add 7 µL nitrite reagent to the microplate wells.
5. Add 30 (µL of serum or diluted standard to each well. Gently triturate (pipette up and down) each sample 4–5 times to mix (avoid causing bubbles in wells).
6. Incubate 5 min at room temperature.
7. Measure the absorbance of each sample at 550 nm to determine the total bilirubin levels.

Calculation

Concentration bilirubin (mg/dL) = $A_T/A_S \times 20 \times DF$
Where,

A_T = Absorbance of test specimen
A_S = Absorbance of standard
20 = Concentration of standard
DF = Dilution factor if the serum is diluted

Note Samples with values above 20 mg/dL should be diluted 1 : 1 with PBS and retested. Multiply results by 2 (DF).

Construction of standard curve

A standard curve should be constructed for each kit in order to check the linearity.

1. Label 6 microfuge tubes: 1, 2, 3, 4, 5 and 6 (blank, B).
2. Dilute the calibration standard using distilled or deionized water as described in the table below.
3. After dilution, briefly mix each tube before performing the next dilution. Thus, a series of 5 dilutions will be available to plot the curve.

Table showing the dilution process that gives various standards to plot the standard curve

Tube # and serial standards	Calibration Standard (20 mg/dL)	Water	Concentration
S_1	100 µL	0 µL	20 mg/dL
S_2	80 µL	20 µL	16 mg/dL
S_3	60 µL	40 µL	12 mg/dL
S_4	40 µL	60 µL	8 mg/dL
S_5	20 µL	80 µL	4 mg/dL
6 (blank)	0 µL	100 µL	0 mg/dL

4. Follow the procedure described earlier to determine the absorbance at 550 nm (A_{550}) for each dilution. If run in duplicate, take the average value of absorbance readings.
5. Construct a standard curve by plotting the absorbance for each standard (y-axis) against its concentration in mg/dL (x-axis).
6. The values must be linear.
7. Once the linearity is established, apply formula A_T/A_S (Absorption of Test over Absorption of Standard) × Concentration of standard

Direction for remote laboratories: If you do not have an arrangement for using microplate, purchase the "prepared chemicals" from vendors and use reagents and specimens according to the same proportion.

Total and Conjugated Bilirubin

Red blood cells are removed from circulation after it becomes non-functional (about 120 days). Haem of the haemoglobin then becomes bilirubin and bile. Bilirubin as such is insoluble in water. Liver gets the bilirubin conjugated and thereby it is thrown out of the body through urine or stool that gives them the yellow colour. Thus, in the blood two kinds of bilirubin circulate—soluble (conjugated) and insoluble (unconjugated). Physician wants to know the levels of both these kinds of bilirubin in order to diagnose the cause of jaundice. The laboratory, however, cannot determine the unconjugated bilirubin as it does not dissolve or react with the specific reagent for bilirubin determination. So the laboratory treats the specimen with a substance (caffeine) that conjugates the insoluble bilirubin and behaves like conjugated bilirubin. Thus, when the serum is directly subjected to bilirubin determination (without caffeine treatment), the result is that of conjugated bilirubin, also called **"Direct bilirubin".** When the same serum is first treated with caffeine before subjected to bilirubin assay, the result is that of **"Total bilirubin".** The difference between the two is the value of **"Indirect bilirubin"** (or unconjugated bilirubin).

Principle

Direct (conjugated) bilirubin reacts with **diazotized sulphanilic acid in alkaline medium** to form a blue coloured complex. Total bilirubin is determined in the presence of caffeine, which releases albumin bound bilirubin, by the reaction with diazotized sulphanilic acid. The improved Jendrassik-Grof method utilizes the reaction of bilirubin with diazotized sulphanilic acid, in which a red coloured product is formed. The intensity of the colour, measured at 510–550 nm, is an accurate measure of the bilirubin level in the sample. Total bilirubin is assessed using caffeine benzoate to split bilirubin from the unconjugated bilirubin protein complex.

$$\text{Sulphanilic acid} + NaNO_2 \longrightarrow \text{Diazotized sulphanilic acid}$$

$$\text{Bilirubin diazotized} + \text{Sulphanilic acid} \longrightarrow \underset{\text{(Blue colour complex)}}{\text{Azobilirubin}}$$

The procedure involves addition of a single working reagent and incubation for 10 min.

With easy availability of commercial kits, preparation of reagent in the laboratory has been discontinued. In addition, use of disposable plastic multi-well plates is proving to be more convenient and cost effective. It eliminated the use of cuvette and test tubes and their consequence cleaning. But this will require special plate reader for taking the absorbance reading of the reagent medium. Bilirubin assay kit that we used here measures bilirubin in blood specimen in 96-well or cuvette formats.

Caution The procedure described here may be different than the kit available through your local vendor. Always follow the instructions given by the manufacturer in the insert. It is important that the technician must understand the procedure thoroughly before performing the test. Adapt to the procedure with minor modifications.

Reagents

1. Reagent A: 30 mL Diazo I (sulphanilic) reagent
2. Reagent B: 10 mL Diazo II – sodium nitrite solution
3. Reagent C: 30 mL Caffeine-benzoate reagent (for total)
4. Saline (0.85% NaCl): 50 mL
5. Standard (5 mg/dL Bilirubin): 2 mL

Storage conditions: The kit is shipped at room temperature. Store all reagents in a refrigerator. Shelf life is 12 months after receipt. Protect specimen from light.

A. Micro-well Plate Method

Materials required but not provided in the kit.

- Pipetting devices and accessories
- 96-well plates
- Plate reader

Precautions Normal precautions for laboratory reagents should be exercised while using the reagents. Always treat the reagents as poison and specimen as potential biohazard.

Procedure

1. Freshly prepare at least 200 µL working reagents (total, direct and blank) for each well as indicated in the following table:

Assay	Reagent A (µL)	Reagent B (µL)	Reagent C (µL)	Saline (µL)	H_2O(µL)
For total	50	20	130	0	0
For direct	50	20	0	130	0
For blank	50	0	0	130	20

Note

- The above table gives the proportion of the reagents to mix. Calculate the total volume to be made after you see the number of specimens to be analyzed. Multiply all values by the number of specimens and one extra to avoid the air bubbles that may come with the last drop.
- "Total Bilirubin" is determined with working reagent that contains Reagent C (caffeine), and "Direct Bilirubin" with working reagent that does not contain Reagent C but saline instead. Caffeine benzoate (Reagent C) splits bilirubin from the bilirubin glucuronide conjugate.

2. Transfer 50 µL standard solution (5 mg/dL) and 50 µL H_2O into two separate wells of clear-bottom 96-well plate.
2. Add 200 µL H_2O into each well making a total volume of 250 µL.
3. The former is standard and the latter is blank.
4. Transfer 50 µL of specimen into separate wells of the plate for the total, direct and blank measurement.
5. Add 200 µL of the appropriate working reagents (total and direct) to each of the sample wells.
6. Incubate for 10 min.
8. Read absorbance at 530 nm (510 to 550 nm, A_{530}).

B. Cuvette Method

1. Prepare at least 800 µL of working reagents (total, direct, and blank) for assay as indicated in the table below. Prepare enough of the total, direct, and blank working reagent for each sample tested.

Assay	Reagent A (µL)	Reagent B (µL)	Reagent C (µL)	Saline (µL)	H_2O(µL)
For total	200	80	520	0	0
For direct	200	80	0	520	0
For blank	200	0	0	520	80

2. Transfer 200 µL of standard solution and 200 µL of water into two cuvettes. Add 800 µL of water into each cuvette for a final volume of 1,000 µL (=1 mL).
3. Transfer 200 (µL of sample into cuvettes (one each for total, direct, and blank measurement).
4. Transfer 800 µL of the appropriate working reagent to each of the cuvettes to develop colour.
5. Mix by inversion (use pieces if parchment papers as lid).
6. Incubate at room temperature for 10 min.

7. Zero the instrument with blank. This will eliminate the step to subtract the absorbance of blank from each of the subsequent readings.
8. Take all absorbance reading at 530 nm (A_{530}).

Calculation

Bilirubin concentration = $A_{SP}/A_{ST} \times (5\ \text{mg/dL})$

Where,

A_{SP} = Absorbance value of the specimen (total or direct)
A_{ST} = Absorbance value of the standard or calibrator
5 mg/dL= Equivalent bilirubin concentration of the standard when assay is performed as indicated

Note If the instrument is zeroed with blank, the above formula will read as follows:

$$\text{Bilirubin concentration} = \frac{(A_{530})_{\text{specimen}}}{(A_{530})_{\text{standard}}} \times (5\ \text{mg/dL})$$

DIAGNOSTIC ENZYMOLOGY

Diagnostic enzymology deals with the quantitative measurement of those enzymes, which have clinical significance. In most modern clinical laboratories, enzyme analysis comprises nearly 30% of the workload. This is because the diagnostic enzyme assay is becoming more dependable and the information that it provides regarding functional disorders of various organs of the body is not available by other laboratory procedures. The reason behind enzyme analysis is that various organs of the body contain specific enzymes as their cellular components to carry on their specific biochemical functions. When these organs are in distress, a leakage of the organ-specific enzymes into the blood stream occurs especially with inflammation and cell death. Quantitative biochemical analyses of the blood and other body fluids can thus assist the physician to evaluate the severity of the diseased organ. Because of its growing significance, some of the basic facts of enzyme assay need to be discussed here.

Role of enzymes

Enzymes are organic catalysts that accelerate various biochemical reactions. They are needed in small quantities and apparently, they are not used up in the reaction. The whole thing can be explained with a comparable story, meant for beginners.

Once upon a time there was an Arab Sheikh who owned 17 camels. He left a will for his three sons to share the camels after him in such a way that the distribution would be in the ratio of 1/2 (first son), 1/3 (second son) and 1/9 (third son). After the Sheikh's death, the sons began to fight as they found that it is not possible to split the 17 camels without killing some of them. So, they went to the Kaji, the wise man. The Kaji added his own camel to the 17 camels of the Arab; that resulted in 18 camels, and then he could distribute the camels to the three sons as desired by the Sheikh: 9 (1/2) + 6 (1/3) + 2 (1/9) and then he walks away with his own camel: (***Note*** Kaji's camel is compared with the enzyme.)

17 camels of Sheikh* + 1 camel of Kaji** = 18 camels§
Distribution to the sons by the wish of the Sheikh
9 (1/2) + 6 (1/3) + 2 (1/9) = 17†
Kaji (enzyme) walks away with his own camel.

* = Substrate
** = Enzyme
§ = Substrate-enzyme complex
† = Products (same as substrate)

Chemical nature of enzymes

Enzymes are protein molecules that can effectively convert **substrates** (on which it works) into **products** at normal body temperature. Enzyme actions are also **reversible** which means that the product can also give the substrate if the conditions of the enzyme reaction are changed. A good example is amylase. It can convert starch into sugar in your mouth and sugar into starch in a potato plant. Then what determines the direction of enzyme action? It is the **reaction condition,** especially, the concentrations of substrate or product. If the substrate is plenty (more than the product) the reaction will be forward until all of it is exhausted. It will reverse if the concentration of glucose is higher than starch. There are many other conditions to determine the direction of enzyme action like pH.

Concentration of enzymes in serum sample is measured by the **rate of enzyme action,** using unit volume of serum. The rate is measured by the rate of depletion of the substrate or increase in the amount of product or any kind of change that can be optically measured. Thus, the rate reflects enzyme concentration per, unit volume of the serum.

Many of these reactions involve the **coenzyme nicotinamide adenine dinucleotide** or **NAD.** Coenzymes are small organic **non-protein molecules** that carry chemical groups associated to the specific enzymes. They do not form a permanent part of the enzyme but are required by the enzyme for its activity. Therefore, they are sometimes referred as cofactors or co-substrates. The best example is NAD. During the course of enzymatic reaction, NAD (oxidized state, with no absorptivity at 340 nm) will be reduced to NADH (reduced state, high absorptivity at 340 nm). As a result, optical density of the reaction medium increases: which can be measured at 340 nm wavelength (in the ultraviolet range). From the change of optical density (Δ/A), one can measure the rate of enzyme activity per unit time. It is expressed in the International Unit (IU) of enzyme activity, which is defined as the amount of substrate consumed in micromoles (μmole) or amount of product formed in micromoles (μmole) in 1 min. The results are expressed in IU/L (International unit per litre of specimen) at 37°C or any other specified temperature, which may be 25 or 30°C.

This is exemplified in the following reaction in which lactic dehydrogenase (LDH) converts lactic acid into pyruvic acid whereby NAD forms NADH:

$$\text{Lactic acid} + \text{NAD} \xrightleftharpoons{\text{LDH}} \text{Pyruvic acid} + \text{NADH}$$

Enzyme reactions are clinically relevant because certain organs contain enzymes that are unique to that organ. When these organs are in distress, these organ-specific enzymes leak into the bloodstream and can be measured by laboratory tests. Thus, quantitative biochemical analyses of the blood and other body fluids can aid the physician in diagnosing the affected organ and evaluating the severity of disease.

Measurement of Enzyme Activity

Quantitative assay of enzymes requires the measurement of rate of activity of the enzyme, rather than its weight or volume. The rate of enzyme activity, however, depends on many variable factors. Therefore, test conditions are chosen in a way that the reaction rate will depend only on the amount of enzyme present in the sample to be measured.

Two methods are currently followed in the measurement of enzyme activity—**two point method and continuous method.** In the two-point method, the reaction is terminated after a specified time, and the **amount of substrate used or product formed** is measured by a suitable analytical procedure. The enzyme activity is computed from the change in the concentration of the substrate (used) or the product (formed) in micromoles during a 1-min period, taking into consideration the unit volume of serum specimen. On the other hand, in continuous assay, the enzymatic reaction is not terminated, and the change in substrate or product concentration is constantly monitored while the reaction is in progress. The former can be compared with the measurement of distance *(d)* travelled between the starting and finishing time *(t)* and expressed as *d/t;* while the latter can be compared with the radar system for tracking the speed of a moving object. In order to accomplish the continuous assay of the enzyme, the reaction mixture must be tested without disturbing the enzyme activity. Two coenzyme pairs in their oxidized and reduced states—**NAD/NADH and NADP/NADPH**—are commonly used in the **continuous assay.** This has been mentioned earlier for lactic dehydrogenase.

Occasionally these coenzymes are not directly used in the principal enzyme reaction; and they are **'coupled'** with a secondary reaction to accomplish the continuous analysis of the principle enzyme under study (e.g., ALT or GPT).

Routine Analysis of Diagnostic Enzymes

Following routinely requested enzyme assays would be discussed in the following pages.

- *Acid phosphatase (ACP):* Prostate, semen detection (rape case)
- *Alkaline phosphatase* (ALP): Liver
- *Transaminases* (AST and ALT): Liver
- *Creatine kinase* (CK): Heart
- *Lactic dehydrogenase* (LD): Heart and liver
- *Amylase:* Pancreatic function

Acid Phosphatase (ACP)

Prostatic acid phosphatase (PAP), a glycoprotein synthesized by the prostate gland, is a member of a diverse group of isoenzymes, the acid phosphatases. This group of enzymes is capable of hydrolysing phosphate esters in an acidic medium. They are classified on the basis of their **electrophoretic mobility.**

Clinical significance

Although PAP was a major tumour marker for **prostate cancer** for more than 50 years but now is no longer used to diagnose prostate cancer. In most instances, serum **prostate specific antigen** (PSA) is used instead. PAP measurement is now limited to niche applications. Pre-treatment PAP measurement may add unique, clinically useful prognostic information for predicting recurrence of prostate cancer in men. PAP also may be useful for following the progression of disease response to therapy in men treated by androgen ablation. However, for both of these applications, **PSA provides more information and also should be utilized.** There is, however, considerable controversy in considering PSA test to be as useful as claimed earlier.

Normal range

Serum total ACP: 0–9 IU/L
Serum prostatic ACP: 0–4 IU/L

Specimen

Serum should be collected within 30 min of blood collection. Hydrolyzed serum is not preferred for the test. Add 0.02 mL (20 μL) of acetate buffer per 1 mL of serum (to stabilize ACP). The dilution is insignificant.

Laboratory assay

Principle

$$\text{Alpha-naphthyl phosphate} + H_2O \xrightarrow{\text{ACP}} \text{Alpa-naphthol} + \text{Inorganic phosphate}$$

$$\text{Alpha-naphthol} + \text{Fast Red TR} \longrightarrow \text{Diazo Dye}$$

Alpha-naphthyl phosphate is hydrolyzed by serum acid phosphatase to alpha-naphthol and inorganic phosphate. The rate of hydrolysis is proportional to ACP activity in serum. The alpha-naphthol produced is coupled with Fast Red TR to produce a coloured complex. The rate of formation of the coloured complex is monitored at fixed intervals (visible kinetics up to 3 min) at 405 nm. This chemical reaction gives the value of total acid phosphatase (TAP). After determining the total, tartrate is used to inhibit prostatic acid phosphatase (PAP).

For the determination of prostatic acid phosphatase (PAP), L-Tartrate is used which inhibits prostatic acid phosphatase (PAP). If testing is performed in the presence and in the absence of L-Tartrate, the difference between the results of the two assays gives the concentration of Prostatic Acid Phosphatase (PAP) in serum.

Reagents

These reagents are provided in the kit. Strictly follow manufacturer's instructions given in the insert.

- Buffered Substrate:
  - Alpha-naphthyl phosphate (3 mM)
  - Fast Red TR(1 mM)
  - Sodium citrate (6 mM) pH 5.3 ± 0.1
- L-Tartrate Reagent
  - Sodium L-Tartrate (2 M)
  - Citric acid (10 mM) pH 5.3 ± 0.1
- Acetate Buffer (5 M) pH 5.0

The reconstituted reagents are stable at 2–8°C (in refrigerator) for 2 weeks.

Supplies required not provided in the kit

- Test tubes 15 × 125 mm (or matched cuvettes)
- 1.0 mL automatic dispenser
- Constant temperature water bath (37°C)
- Special type of photometer (or spectrophotometer) equipped with thermos-cuvettes.

Procedure

A. Total Acid Phosphatase

1. Reconstitute buffered substrate.
2. Pipette 1 mL of reconstituted buffered substrate in a test tube or cuvette and place at 37°C for 5 min.
3. Add 0.1 mL (100 µL) of serum and mix well.
4. Read absorbance exactly after 5 min.
5. Determine difference of absorbance (initial and final).
6. Divide the difference of absorbance (ΔA) by five to get ΔA/min.

B. Non-Prostatic ACP (acid phosphatase)

1. Pipette 1 mL buffered substrate in a test tube or cuvette.
2. Add 0.01 mL of L-Tartrate reagent with a micropipette (10 µL) and mix well.
3. Add 0.01 mL serum with a micropipette and mix well.
4. Read initial absorbance and absorbance exactly after 5 min.
5. Divide the difference ΔA by 5 to determine ΔA/min.

Calculation

$$\text{Serum total ACP (IU)} = \frac{\Delta A/\text{min} \times 10^{6} \times 1.11}{12.9 \times 10^{3} \times 1.0 \times 0.1} = \Delta A/\text{min} \times 853$$

$$\text{Serum non-prostatic ACP} = \frac{\Delta A/\text{min} \times 10^6 \times 1.11}{12.9 \times 10^3 \times 1.0 \times 0.1} = \Delta A/\text{min} \times 860$$

Prostatic ACP (IU) = Total ACP (IU) – Non-Prostatic ACP (IU)

Where,

10^6 = Conversion of moles to millimoles
1.1 = Total reaction volume (total ACP)
1.11 = Total reaction volume (non-prostatic ACP)
12.9×10^3 = molar absorptivity of alpha-naphthol Fast Red TR at 405 nm
1.0 = Light path in cm
0.1 = Sample volume in mL

Limitations: The method is linear up to 35 IU/L. Samples with values of ACP above 35 IU/L should be diluted 1 : 5 with normal saline and determination is performed. Final result should be multiplied by 5.

Interference: High level of bilirubin (icteric samples) inhibit ACP activity.

Note Run quality control sample first before running patient's sample.

Alkaline phosphatase

Alkaline phosphatases are a group of enzymes that split off a terminal phosphate group from an organic ester in alkaline solution. Their optimum pH is usually around 10, but this varies with particular substrate and isoenzymes. ALP is made mostly in the liver and in bone with some made in the intestines, kidneys, and placentas of pregnant women.

Clinical significance

Alkaline phosphatase (ALP) is mostly made in liver and in bones. Hence an increased level of ALP in blood is associated with liver or bone disorder. In order to pinpoint the source, ALP isoenzymes are called for. The normal level of ALP is age-dependent. During active bone growth (growing age), it is high and goes down in old age. Serum level of ALP activity is raised in all bone disorders accompanied by increased osteoblastic activity. This includes Paget's disease (osteitis deformans), osteoblastic tumours with metastases, hyperparathyroidism when there is mobilization of calcium and phosphate from the bone, rickets, and osteomalacia.

A moderate elevation of ALP may be attributed to several disorders including Hodgkin's disease, congestive heart failure, abdominal bacterial infections, and pregnancy. Low levels, on the other hand, are found in hypophosphatemia, dwarfism, hypothyroidism, and pernicious anaemia. Isoenzymes of ALP have been identified in the liver, bone, intestinal mucosa, placenta, and bile.

Normal Range

20–130 U/L (p-nitrophenyl phosphate method)
Children: Less than 350 U/L

Specimen

Serum is the specimen of choice, although heparinized plasma can be used. Chelating anticoagulants, which remove the activator magnesium, are unacceptable. Since haemoglobin may interfere spectrophotometrically, *in vitro* haemolysis should be avoided. Assay within 4 h of specimen collection is recommended; frozen specimens should be kept at room temperature for 12 h prior to assay to assure complete enzyme reactivation. Separate the serum promptly and store in a refrigerator if immediate analysis is not possible. ALP activity increases with storage, hence, as a general rule, it is best to analyse ALP specimens the same day they are drawn.

Principle

Most commonly followed method for the assay of ALP in clinical laboratories is the **p-nitrophenylphosphate** method. The p-nitrophenylphosphate is not a natural substrate for the phosphatases. It is used because the enzyme uses this synthetic compound and gives a reasonably rapid rate of reaction. In addition, it is analytically convenient to measure the product formed (p-nitrophenol). The liberated phenol is yellow in colour in alkaline medium and is colourless in acidic medium. Continuous assay can be done for ALP by measuring the rate of formation of p-nitrophenol at pH 10. The two point method described here is suitable for the routine laboratories of developing countries and the reagents are easily available. In this method, the enzyme reaction is terminated by alkali treatment. The assay can be performed using cuvettes or plates. The procedure provided is for the detection using cuvettes with a 1-mL reaction volume.

In this method, ALP catalyzes the hydrolysis of p-nitrophenylphosphate (pNPP) to p-nitrophenol. pNPP is colourless but p-nitrophenol is yellow in alkaline medium, having strong absorbance at 405 nm. Some divalent ions like Mg^{++} are added to the system, which act as activators. pNPP is colourless in acid and alkaline medium, while pNP is yellow in alkaline medium and colourless in acidic medium.

$$\underset{\text{(colourless)}}{\text{p-Nitrophenyl phosphate}} + H_2O \xrightleftharpoons{\text{ALP/ACP}} \underset{\text{(yellow)}}{\text{2p-Nitrophenol}} + H_3PO_4$$

The rate of increased absorbance at 405 nm is proportional to the enzyme activity. The procedure is standardized by means of the millimolar absorptivity of p-nitrophenol 405 nm under the specified conditions. The results are based on the change in absorbance per unit time. One International Unit (IU/L) is defined as the amount of enzyme that catalyzes the transformation of one micromole of substrate per minute under specified conditions.

Reagents included in the kit

Kits for alkaline phosphatase measurement are available in the market. Follow manufacturer's instructions for reconstituting the chemicals and running the test. The chemicals may be lyophilized (solid). Hence, while reconstituting, make sure that all particles are dissolved.

We are following one such kit in describing the procedure.

1. ***pNPP substrate reagent:*** pNPP solution is reconstituted with 15 mL of distilled water. Swirl gently to dissolve. When reconstituted as described, the reagent contains p-nitrophenylphosphate (10.0 mM), magnesium ions (1.0 mM), buffer at pH 10.0, along with activator and binder. Un-reconstituted reagent of the kit is stored in a refrigerator at 2-8°C. It is stable for at least 2 years. Once reconstituted, the reagent is stable for 48 h at 25°C and for 30 days at 2-8°C.
2. ***Alkaline phosphatase standard solution:*** This is prepared immediately before use. Mix briefly to ensure the alkaline phosphatase is dissolved completely. Consult manufacturer's instructions for concentration of alkaline phosphatase in standard. We are using a standard of 200 IU/L.

Supplies not provided in the kit

- Spectrophotometer (or colorimeter). Spectrophotometer with arrangement of keeping the cuvette at 37°C is convenient.
- Cuvettes (or 96-well plates)
- Distilled or deionized water
- Automatic pipettors and tips
- Physiological saline (0.9% NaCl)
- Paraffin square (for closing the cuvette-top during mixing)
- Heating block or water bath 3 7°C
- Timer

All reagents and specimens must be considered as potential biohazard.

Procedure

1. Turn on the spectrophotometer and let it warm up for at least 15 min.
2. Set the wavelength to 405 nm.
3. Label 3 (or more) cuvettes as blank (B), standard (S) and test specimen (T) for the patient's serum. If there are more specimens, continue labelling T_1 T_2, etc.
4. Add 1.0 mL of distilled or deionized water to cuvette B (blank).
5. Add 1.0 mL of pNPP reagent to others, i.e., standard (S) and test (T) cuvettes.
6. Incubate all cuvettes at 37°C for 5 min.
7. Add 25 µL of activator to each of the standard (S) and test, or serum, specimen (T) cuvettes.
8. Mix each by inversion using the paraffin squares to prevent spillage.
9. Incubate the cuvettes at 37°C for 1 min.
10. After one minute, place blank cuvette in the spectrophotometer and set the absorbance to read 0.000.
11. Read and record the absorbance for each of the standard and test cuvettes following the zero setting.
12. Return these cuvettes (T and S) to 37°C and repeat readings for each cuvette every minute for the next two minutes.
13. Calculate the average absorbance difference per minute (ΔA/min) for Standard and Test.
14. Calculate the activity of ALP in Test (or serum) specimen.

$$\frac{\Delta A/\text{min Test}}{\Delta A/\text{min Standard}} \times \text{ALP activity of sandard or Control}$$

$$\frac{\Delta A/\text{min Test}}{\Delta A/\text{min Standard}} \times 200$$

Where, 200 IU/L is the standard that we are using.

The following calculation will help you to find out the activity of ALP of standard, if not known:

$$\text{ALP (IU/L)} = \frac{\Delta A/\text{min} \times \text{TV} \times 1000}{18.75 \times \text{LP} \times \text{SV}}$$

Where,

ΔA/min = Average absorbance change per minute
1000 = Conversion of IU/mL to IU/L
1.025 = (TV) or total reaction volume in mL
18.75 = Millimolar absorptivity of p-nitrophenol
0.025 = (SV) Sample/Standard volume in mL
1 = (LP) Light path in cm

$$\text{ALP(IU/L)} = \Delta A/\text{min (S)} \times \frac{1.025 \times 1000}{18.75 \times 1 \times 0.025}$$

$$\text{ALP (IU/L)} = \text{AA/min (S)} \times 2187$$

Transaminases

Transamination is an important step in the metabolism of amino acids. During this process the amino group of the amino acid is transferred to analpha-ketoacid. The enzymes responsible for transamination are called transaminases (now called, **aminotransferases).** Two most useful enzyme markers of acute hepatocellular injury are ALT (alanine amino transferase) and AST (aspartate amino transferase). In the past, these were called GOT (glutamate oxaloacetate transaminase) and GPT (glutamate pyruvate transaminase), respectively.

Alanine amino transferase (ALT) and Aspartate amino transferase (AST)

Clinical significance

Increased serum transaminase activity is seen in **liver dysfunction.** Greater activity of AST (GOT) over ALT (GPT) is typical of myocardial infarction and muscle injury as in case of intramuscular injection and rhabdomyolysis.

Normal range

The adult reference range for both AST and ALT is roughly 10–40 U/L when measured at 37°C. Although men have slightly higher values than women do, most laboratories use a single range for both genders.
AST 10–40 units per liter
ALT 7–56 units per liter

Mild elevations are generally considered to be 2–3 times higher than the normal range. In some conditions, these enzymes can be severely elevated (1000 times higher).

Specimen and its preparation

Serum is the specimen of choice. The serum specimen submitted for the enzyme assay of ALT and AST should be free from haemolysis. Collect the blood by venepuncture without anticoagulant and separate the serum promptly. If analysis cannot be done within an hour, refrigerate the specimen.

Chemical reactions of ALT and AST

The enzymatic chemical reactions of ALT and AST are shown here:

$$\text{L-alanine} + \alpha\text{-oxoglutarate} \xrightarrow{\text{ALT}} \text{Pyruvate} + \text{L-glutamate}$$

$$\text{L-aspartate} + \alpha\text{-oxoglutarate} \xrightarrow{\text{AST}} \text{Oxaloacetate} + \text{L-glutamate}$$

For the quantitative assay of these enzymes, **two methods are available:**

- Spectrophotometric method
- Colorimetric method

Spectrophotometric method is now widely used and is ideal for the automated continuous assay. It is also more accurate. Unfortunately, it requires a spectrophotometer that will read absorbance at 340 nm. Some of the newer colorimeters are able to read at this range, which is not available to many peripheral laboratories. Hence, a colorimetric method is also presented here. The **colorimetric method** has proved to be cheaper.

The basic principle of the spectrophotometric method is to measure the **changing optical density of the reaction medium** as the enzyme reacts on the substrate. The indicator system used is the coenzymes NAD–NADH (or NADP–NADPH). In the colorimetric method, which is a two-point method, the reaction is stopped and the amount of substrate formed (ketoacid) is chemically reacted to yield colour. The intensity of the colour developed per unit time is a measure of the enzyme activity.

Spectrophotometric method

This method is more sensitive and accurate than the colorimetric method but is expensive to perform and also requires spectrophotometer that will work in the ultraviolet range (340 nm).

Principle

When the enzymatic reaction of ALT is coupled with LD activity, it utilizes one of the products (pyruvate) as a substrate. This is in collaboration with its coenzyme NADH. As a result the optical density or absorbance of the reaction medium changes due to the oxidation of NADH. ***(Note*** NAD has minimal absorbance at 340 nm wavelength). By following the change (ΔA), the rate of ALT is determined.

$$\text{L-alanine} + \alpha\text{-oxoglutarate} \xrightarrow{\text{ALT}} \text{Pyruvate} + \text{L-glutamate}$$

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LD}} \text{Lactate} + \text{NAD}$$

Similarly, the enzyme assay for AST involves the coupled reactions of AST and MDH (malate dehydrogenase), using the substrates L-aspartate and α-oxoglutarate. Here again, the coenzyme **NADH converts to NAD.**

$$\text{L-aspartate} + \alpha\text{-oxoglutarate} \xrightarrow{\text{AST}} \text{Oxaloacetate} + \text{L-glutamate}$$

$$\text{Oxaloacetate} + \text{NADH} \xrightarrow{\text{MDH}} \text{L-malate} + \text{NAD}$$

Note

- Because none of the reactants or products of these reactions absorbs in the ultraviolet or visible region of the spectrum, they must be coupled to indicator reactions for analysis. ALT requires LD, whereas AST requires MDH.
- The reactions are monitored by following **a decrease in the absorbance** at 340 nm. This because NADH is consumed in the reaction. (***Note*** NADH has absorbance at 340 nm whereas NAD has no absorbance).
- Pyridoxal phosphate, an additional coenzyme, is needed to run the above reaction, which is abundantly present in serum. It may have to be added in patients with vitamin B deficiency.

Reagent and supplies

The commercial kit supplies all the required reagents, except

- Spectrophotometer
- Laboratory supplies required for chemical analyses

Colorimetric Method

The colorimetric method is commonly followed in the peripheral laboratories of developing countries. The reference laboratory, however, uses the spectrophotometric method.

Note

- Colorimetric assay of ALT requires 20 µL of specimen for each reaction (well).
- Serum specimens can be directly added to wells.
- If the specimen is diluted, note the dilution factor. It is important that the reading of the specimen is within the standard curve range.
- For the positive control (optional), add 5 µL of the ALT Positive Control to appropriate well. Adjust the volume.
- Adjust well volume to 20 µL with ALT Assay Buffer.

Principle

The activity of transaminases (aminotransferases) is determined by measuring the colour of the hydrazone (brown) which is formed by the reaction between 2,4-dinitrophenyl hydrazine (DNPH) and the ketoacid which is one of the products of transaminase reaction. The DNPH reacts with all oxoacids. These include oxoglutarate and oxaloacetate, as well as pyruvate, which are on the two sides of the above equations. DNPH gives more colours with oxaloacetate and pyruvate than with oxoglutarate, thus making the method feasible with an acceptable limit of error. In both estimations—ALT and AST, the substrates are suboptimal, to reduce the background colour given by the alpha-ketoglutarate (or oxoglutarate) in the reaction with DNPH.

$$\text{L-alanine} + \text{2-oxoglutarate} \xrightleftharpoons{\text{ALT/GPT}} \text{Pyruvate} + \text{L-glutamate}$$

$$\text{L-aspartate} + \text{2-oxoglutarate} \xrightleftharpoons{\text{AST/GOT}} \text{Oxaloacetate} + \text{L-glutamate}$$

Though not as accurate as the spectrophotometric method (UV), the colorimetric method is easily adaptable to the local conditions of developing countries and is much faster to perform than spectrophotometric method.

Alanine Aminotransferase (ALT): Colorimetric assay

The ALT enzyme is also known as **serum glutamic-pyruvic transaminase** or **SGPT.** It is a pyridoxal phosphate dependent enzyme that catalyses the reversible transfer of an amino group from alanine to α-ketoglutarate, generating pyruvate and glutamate. ALT is found primarily in liver and serum, but occurs in other tissues as well. Hepatocellular injury often results in an increase of serum ALT levels and these levels can be used as a marker for liver injury. The ALT Activity Assay Kit provides a simple and direct procedure for measuring ALT activity in a variety of biological specimens. The following procedure was adopted from Sigma-Aldrich kit. Kits must be stored in the refrigerator, preferably at –20°C.

Principle

ALT activity is determined by a coupled enzyme assay, which results in a colorimetric (570 nm) product, proportional to the pyruvate generated. One unit of ALT is defined as the amount of enzyme that generates 1.0 µmole of pyruvate per minute at 37°C.

$$\text{L-alanine} + \alpha\text{-ketoglutarate} \xrightleftharpoons{\text{ALT/GPT}} \text{Pyruvate} + \text{L-glutamate}$$

$$\text{Pyruvate} + \text{2, 4-DNPH-ine} \xrightarrow{H^+} \text{Pyruvate-2, 4-DNPH-one (colour)}$$

In this procedure, ALT (SGPT) catalyzes L-alanine and α-ketoglutarate to form pyruvate and glutamate. The pyruvate is then reacted with **2,4-dinitrophenylhydrazine** (2,4-DNPH-ine) to form 2,4-DNPH-one (colour). The addition of sodium hydroxide dissolves this complex, allows coloured complex 2,4-DNPH-one to be measured at 570 nm. The product glutamate is measured by the generation of a blue colour product through an enzyme coupled reaction cycle. The signal can be read by an absorbance microplate reader at ~570 nm.

Reagents

The kit is sufficient for 100 assays in 96-well plates.

• ALT Assay Buffer	25 mL
• Fluorescent Peroxidase Substrate, in DMSO	0.2 mL
• ALT Enzyme Mix	1 vial
• ALT Substrate	1 vial
• Pyruvate Standard	100 nmole/µL 0.1 mL
• ALT Positive Control	1 vl

Reagents and equipment required but not provided in the kit

- 96-well flat-bottom plate. It is recommended to use black plates with clear bottoms for fluorescence assays and clear plates for colorimetric assays.
- Spectrophotometric multi-well plate reader.

Precautions

Consider all chemicals to be poisonous and specimen as potential biohazard.

Reagent Preparation

Briefly centrifuge vials before opening. Use ultrapure water for the preparation of reagents. To maintain reagent integrity, avoid repeated freeze/thaw cycles. ***Note*** Allow the reagents in the kit (kept in the refrigerator) to come to room temperature before use.

- ALT Assay Buffer
- Fluorescent Peroxidase Substrate: Mix well by pipetting. Aliquot and store, protected from light and moisture, at –20°C.
- ALT Enzyme Mix: Reconstitute in 220 µL of water. Mix well by pipetting and store at –20°C. Use within two months of reconstitution.
- ALT Substrate: Reconstitute in 1.1 mL of ALT Assay Buffer. Mix well by pipetting. Aliquot and store at –20°C. Keep cold while in use. Use within two months of reconstitution.
- ALT Positive Control: Reconstitute in 100 µL of water. Mix well by pipetting. Store at –20°C. Use within two months of reconstitution.

Storage/Stability: The kit is shipped on wet ice. Storage at –20°C, protected from light, is recommended.

Preparation for Assay

Pyruvate Standards for Colorimetric Detection:

- Dilute 10 µL of 100 nmole/µL Pyruvate Standard with 990 µL of ALT Assay Buffer to prepare a 1 nmole/µL standard solution. (Stock standard diluted 100 × or 100 nmol/100 = 1 nmole/µL)
- Add 0, 2, 4, 6, 8, and 10 µL of the working standard solution (1 nmole/µL) 96-well plate, generating 0 (blank), 2, 4, 6, 8, and 10 nmole/well as series of standards.
- Add ALT Assay Buffer solution to each well to bring the total volume to 20 µL. For example, in the well marked “standard 8 nmole”, you have 8 µL of diluted standard and 12 µL of buffer.

Procedure

Note All specimens and standards should be run in duplicate.

1. Set up the Master Reaction. Mix according to the scheme given in the table below.
   - 100 µL of the Master Reaction Mix is required for each reaction (well).
   - Prepare enough Master Reaction Mix for the number of specimens, positive controls, and standards to be performed.
   - Master Reaction Mix.

     (***Note*** Volumes will proportionately increase if cuvette is used. Always make slightly more than what you need in order to avoid air bubble of the last drop).

Reagent	**Volume**
ALT assay buffer	86 µL
Fluorescent peroxidase substrate	2 µL
ALT enzyme mix	2 µL
ALT substrate	10 µL

2. Add 100 µL of the Master Reaction Mix to each of the standard, positive control, and test wells; mix well.
3. After 2–3 min, take the initial absorbance reading at 570 nm (A_{570}).
4. Incubate the plate at 37°C taking measurements every 5 min. (Protect the plate from light during incubation).

5. Continue taking measurements until value of the most active specimen is greater than value of the highest standard. At this time the most active specimen is near or exceeds the end of linear range of the standard curve.
6. The final measurement for calculating the enzyme activity would be the penultimate reading or the value before the most active specimen is near or exceeds the end of linear range of the standard curve. The time of the penultimate reading is T_{final}.
7. Calculate the change in measurement from $T_{initial}$ to T_{final}.

$$\Delta A_{570} = (A_{570})_{final} - (A_{570})_{initial}$$

Note It is essential that the initial and final measurements fall within the linear range of the reaction.

Calculations

- Correct for the background by subtracting the value obtained for 0 (blank) standard from all readings. Plot the pyruvate standard curve.
- Compare the difference in the absorbance (ΔA_{570}) for each specimen and compare it with the standard curve to determine the amount of pyruvate generated between $T_{initial}$ and T_{final} (B).

  Note A new standard curve must be set up each time the assay is run.
- The ALT activity of a specimen may now be determined by the following equation:

$$\text{ALT Activity} = \frac{\text{B} \times \text{Sample Dilution Factor}}{(T_{final} - T_{initial}\ \text{minutes}) \times \text{V(mL)}}$$

Where,

B = Amount (nmole) of pyruvate generated between $T_{initial}$ and T_{final}
$T_{initial}$ = Time of first reading in minutes
T_{final} = Time of penultimate reading in minutes
V = Specimen volume (mL) added to well

Reporting of result

ALT activity reported as nmole/min/mL = milliunit/mL, where one milliunit (mU) of ALT is defined as the amount of enzyme.

Aspartate Aminotransferase (AST): Colorimetric assay

Aspartate Aminotransferase (AST), formerly called serum glutamate-oxaloacetate transaminase (SGOT), is a pyridoxal phosphate (PLP) dependent enzyme that catalyses the conversion of aspartate and α-ketoglutarate to oxaloacetate and glutamate.

$$\text{L-aspartate} + \alpha\text{-oxoglutarate} \xrightarrow{\text{AST/GOT}} \text{Oxaloacetate} + \text{L-glutamate}$$

Clinical significance

Similar to Alanine Aminotransferase (ALT), AST levels in blood are commonly used as **a marker for liver function**. However, AST has a broader tissue distribution than ALT and perturbations in AST levels can occur in response to diseases or injuries in multiple tissues including skeletal and heart.

The Aspartate Aminotransferase (AST) Activity Assay kit of Sigma-Aldrich (supplier) provides a simple and direct procedure for measuring AST activity in a variety of specimens. In the chemical reaction, an amino group is transferred from aspartate to α-ketoglutarate and results in the generation of oxaloacetate and glutamate. Both the end products have the ability to produce colour when reacted with DNPH (hydrazine). The absorbance is measured at 450 nm which is proportional to the AST enzymatic activity present. One unit of AST is the amount of enzyme that will generate 1.0 µmole of glutamate per minute at pH 8.0 at 37°C.

Principle

$$\text{L-aspartate} + \text{2-oxoglutarate} \xrightleftharpoons{\text{ALT/GOT}} \text{Oxaloacetate} + \text{L-glutamate}$$

$$\text{Oxaloacetate} + \text{2,4-DNPH-ine} \xrightarrow{H^+} \text{Pyruvate} + \text{2,4-DNPH-one}$$

Reagents (supplied in the kit)

AST Assay Buffer	25 mL
Enzyme Mix	1 vl
AST Developer	1 vl
AST Substrate	1 vl
Glutamate Standard, 0.1 M	0.1 mL
AST Positive Control	1 vl

Keep the kit in a refrigerator. Storage temperature of –20°C is recommended.

Reagents and equipment required but not provided in the kit

- 96-well flat-bottom plate. It is recommended to use clear plates for colorimetric assays.
- Spectrophotometric multi-well plate reader
- Automatic pipettor

All reagents must be considered poisonous and specimens as potential hazards.

Preparation of Reagents

Briefly centrifuge vials before opening. Use ultrapure water for the preparation of reagents. To maintain reagent integrity, avoid repeated freeze/thaw cycles.

- AST Assay Buffer: Allow buffer to come to room temperature before use.
- AST Enzyme Mix: Reconstitute with 220 µL of water. Mix well by pipetting (don't vortex). Store at –20°C protected from light and use within 2 months of reconstitution.
- AST Developer: Reconstitute with 820 µL of water. Mix well by pipetting (don't vortex), and store, protected from light, at –20°C. Use within 2 months of reconstitution.
- AST Substrate: Reconstitute with 1.1 mL of AST Assay Buffer. Mix well by pipetting (don't vortex). Store, protected from light, at –20°C. Use within 2 months of reconstitution.
- AST Positive Control: Reconstitute with 100 µL of water. Mix well by pipetting, and store, protected from light, at –20°C. Use within 2 months of reconstitution and keep cold while in use.

Storage/Stability: The kit is shipped on wet ice and storage at –20°C, protect from light.

Procedure to prepare standard

Run all specimens and standards in duplicate.

Glutamate Standards for Colorimetric Detection

- Dilute 10 µL of 0.1 M Glutamate Standard solution with 990 µL of the AST Assay Buffer to prepare a 1 mM standard solution. (100 × diluted 1M solution becomes 1 mM solution). Standard curve is made from the diluted standard.
- Add 0, 2µL, 4µL, 6µL, 8µL, and 10 µL, of 1 mM standard solution into a 96-well plate, generating 0 (blank), 2, 4, 6, 8, and 10 nmole standards in different wells. (Here, 1 µL = 1 nmole glutamate standard).
- Add AST Assay Buffer to each well to bring the volume to 50 µL. (This means 50, 48, 46, 42, and 40 mL in the above series of well).

Preparation of serum specimen for assay

- Serum specimens can be directly added to wells.
- For unknown specimens, it is suggested to test several specimen dilutions to ensure the readings are within the linear range of the standard curve.
- Bring specimens to a final volume of 50 µL with AST Assay Buffer. (Volume of specimen + Volume of buffer = 50 µL)
- For the positive control (optional), add 5 µL of the AST positive control solution to wells and adjust to 50 µL with the AST Assay Buffer. (5 µL positive control + 45 µL volume of buffer = 50 µL)

Procedure

1. Set up the Master Reaction Mix according to the scheme given in the following table. 100 µL of the Reaction Mix is required for each reaction (well).

Reagent	Volume
ALT Assay buffer	80 µL
ALT Enzyme mix	2 µL
AST Developer	8 µL
ALT Substrate	10 µL

2. Add 100 µL of the Reaction Mix to each of the wells. Mix well by automatic shaker or by pipetting. Protect the plate from light during incubation.
3. Incubate the plate at 37°C. After 2–3 min, take the initial absorbance at 450 nm $(A_{450})_{\text{initial}}$ and click the stopwatch simultaneously. ***Note*** It is essential that $(A_{450})_{\text{initial}}$ is in linear range of the standard curve.
4. Continue to incubate the plate (37°C) taking measurements (A_{450}) every 5 min. Protect the plate from light during incubation.
5. Continue taking measurements until value of the most active specimen is greater than value of the highest standard (10 nmole/well). At this time the most active specimen is near or exceeds the end of linear range of the standard curve.
6. The final measurement $[(A_{450})_{\text{final}}]$ for calculating the enzyme activity would be penultimate reading or the value before the most active specimen is near or exceeds the end of linear range of the standard curve (see Step 5). The time of the penultimate reading is T_{final}. ***Note*** It is essential that the final measurement falls within linear range of the standard curve.

Calculations

Correct for the background by subtracting the final measurement $[(A_{450})_{\text{final}}]$ obtained for 0 (blank) from Glutamate standard and specimens. Background values can be significant and must be subtracted from all readings. Plot the Glutamate standard curve. ***Note*** A new standard curve must be setup each time the assay is run.

Calculate the change in measurement from T_{initial} to T_{final} for the specimens.

$\Delta A_{450} = (A_{450})_{\text{final}} - (A_{450})_{\text{initial}}$ = Difference in absorbance at 450 nm between initial and final

Compare the ΔA_{450} of each specimen to the standard curve to determine the amount of glutamate generated by the aminotransferase assay between T_{initial} and T_{final} (B).

The AST activity of a specimen may be determined by the following equation:

$$\text{AST Activity} = \frac{\text{B} \times \text{Specimen Dilution Factor}}{(\text{Reaction Time}) \times \text{V}}$$

Where,

B = Amount (nmole) of glutamate generated between $T_{initial}$ and T_{final}

Reaction Time = $T_{final} - T_{initial}$ (minutes)

V = Specimen volume (mL) added to well

AST activity is reported as nmole/min/mL = milliunit/mL

One unit of AST is the amount of enzyme that will generate 1.0 µmole of glutamate per minute at pH 8.0 at 37°C.

Example Glutamate amount (B) = 5.84 nmole
First reading ($T_{initial}$) = 3 min
Second reading (T_{final}) = 32 min
Specimen volume (V) = 0.01 mL
Specimen dilution is 1
AST activity is:

$$\frac{5.84 \times 1}{(32 - 3) \times 0.01} = 20.14 \text{ milliunits/mL}$$

Creatinine kinase

Creatine kinase (CK), also known as creatine phosphokinase, is an enzyme chiefly found in brain, skeletal muscles, and heart. An elevated level of CK is seen in case of muscle damage, muscle disorders or heart attack. It is an enzyme that catalyses the transfer of one phosphate group from ATP to creatine generating phosphocreatine, an important energy reservoir in muscle and brain tissue. It exists as three isoenzymes: CK-MM, CK-MB, and CK-BB. The isoenzyme locates the source of the enzyme and thus gives the diagnosis.

Clinical significance

CK levels are elevated in various pathological conditions including myocardial infarction, rhabdomyolysis (muscle disorder), muscular dystrophy, and renal failure. Laboratory assay of creatinine kinase in serum and other specimens is done routinely. The procedure is simple and associated chemicals are available from various manufacturers in kits.

Normal range

Adult males: 52–336 U/L
Adult females: 38–176 U/L

Specimen

Blood sample should not be haemolyzed. Separate the serum as soon as possible after clotting. Assay the specimen within 4 h after collection if stored at room temperature. The specimen, if not analyzed, can be stored in refrigerator (2–8°C) for 12 h.

Principle

The basic reaction comprised of the action of CK enzyme on the substrate—creatine phosphate and ADP in presence of Mg ions. The products of the enzymatic reaction are creatine and ATP.

$$\text{Creatine phosphate} + \text{ADP} \underset{\text{Mg++}}{\overset{\text{CK}}{\rightleftarrows}} \text{Creatine} + \text{ATP}$$

ATP is then taken through two coupled reactions involving hexokinase and glucose-6-phosphate dehydrogenase (G6PD). In the second enzymatic reaction, NAD is reduced to NADH that allows the measurement of absorbance of the reaction medium at 340 nm (UV range).

$$\text{ATP} + \text{Glucose} \xrightarrow{\text{Hexokinase}} \text{Glucose-6-phosphate}$$

$$\text{Glucose-6-phosphate} + \text{NADP} \xrightarrow{\text{G6PD}} \text{6-phospho-D-gluconate} + \text{NADPH}$$

One unit of CK is the amount of enzyme that will transfer 1.0 µmole of phosphate from phosphocreatine to ADP per minute at pH 6.0. The kit provided by Sigma has a linear range of 30–1,800 units/L activity.

Reagents

Assay buffer	12 µL
Enzyme mix	120 µL
Substrate solution	1 µL
Calibrator (standard)	150 µL

Storage of reagents: Store all chemicals at –20°C. Protect the reagents from light.

Equipment and supplies not provided with the kit

- Ultraviolet spectrophotometric multi-well plate reader.
- Clear 96-well flat-bottom plate suitable for use in UV absorbance assay. These are special clear plates that are UV transparent.
- Automatic micro pipettor.
- Laboratory supplies.

Procedure

Although the following procedure is given for convenience but the manufacturer's direction must be followed while using their kit.

1. Bring all reagents to room temperature.
2. Prepare Reconstituted Reagent before use. It should be sufficient enough to cover all samples. Each specimen will require 100 µL of reconstituted reagent, assay buffer (100 µL), substrate solution (10 µL), and enzyme mix (1 µL).
3. Transfer 110 µL of water into one well (blank) and 100 µL of water plus 10 µL of the calibrator (standard) into a separate well of 96-well plate.
4. Transfer 10 µL of specimens into separate wells and 100 µL of reconstituted reagent to each specimen well. Tap the plate to mix.
5. Incubate at room temperature for 20 min. CK is fully activated during that period.
6. After 20 min take the **initial absorbance** reading at 340 nm (A_{in}).
7. Continue to incubate (room temperature) for 20 additional minutes.
8. Take the **final absorbance** reading at 340 nm (A_{fn}). ***Note*** If the activity in specimen is expected to be higher than 300 U/L, dilute the specimen.

Calculation

$$\text{CK activity (U/L)} = \frac{\Delta A_{SP}}{\Delta A_{ST}} \times 150$$

Where,

ΔA_{SP} = Difference of Absorbance between final and initial ($A_{fn} - A_{in}$) of specimen.

ΔA_{ST} = Difference of Absorbance between final and initial ($A_{fn} - A_{in}$) of standard.

150 = Equivalent activity (U/L) of the calibrator (or standard) when assay is read at the same interval (20 min) as the specimen.

Note If the specimen is diluted, multiply with dilution factor.

Linearity: Above procedure gives a linearity of 30–1800 U/L.

Definition of unit (V): One unit of CK is the amount of enzyme that will transfer 1.0 µmole of phosphate from phosphocreatine to ADP per minute at pH 6.0.

Lactic dehydrogenase

Lactate dehydrogenase (LD or LDH) is an oxidoreductase enzyme that catalyses the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD+. It is an enzyme that helps produce energy. It is present in almost all of the tissues in the body and its levels rise in response to cell damage. An increase in the level of LD in serum is nonspecific. LD is found in all of the body's cells and released from cells into the blood circulation when cells are damaged or destroyed.

Clinical significance

When any disease or injury or toxic material damages body tissues, cells release LD into the bloodstream. Since LD is a fairly stable enzyme, it has been widely used to evaluate the presence of damage and toxicity of tissue and cells. Elevations in serum lactate dehydrogenase (LD) occur from myocardial infarction, liver disease, pernicious and megaloblastic anemias, pulmonary emboli, malignancies, and muscular dystrophy. A combined analysis of LD and CK (creatine kinase) isoenzymes provides a definite diagnosis of **acute myocardial infarction.**

Normal range

140–280 U/L or 2.34–4.68 mkat/L

Specimen

Fresh serum specimen is always preferred. Even minimal haemolysis causes significant increase in LD due to high levels of this enzyme in red cells. Hence, avoid haemolysis and reject specimens showing haemolysis.

Laboratory assay

Assay kits are available in the market manufactured by different commercial companies. We chose Sigma-Aldrich. In most colorimetric assay kits, LD reduces **NAD to NADH,** which then interacts with a specific probe to produce a colour with peak absorption at 450 nm. The kit quantifies LDH activity in variety of biological samples including serum or plasma. The assay is quick, convenient, and sensitive. The kit can detect 1–100 mU/mL of LDH directly in samples.

Principle

The LD procedure described here utilizes the forward reaction of **lactate to pyruvate which simultaneously reduces NAD to NADH.**

$$\text{Lactate} + \text{NAD+} \xrightarrow{\text{LD}} \text{Pyruvate} + \text{NADH} + \text{H}^{+}$$

This changes the absorbance of the reaction medium which can be measured at 450 nm (colorimeter) or 340 nm (UV spectrophotometry). The rate of change of absorbance (ΔA) is directly proportional to the LD activity in the sample.

Reagents

The reagents that come with the kit are sufficient for 500 assays in 96-well plates.

LDH Assay Buffer	50 mL
LDH Substrate Mix	1 vial
NADH Standard, 0.5 mmole	1 vial
LDH Positive Control, 20 mL	1 vial

Most conventionally, the final concentration of reactive ingredients in the reaction mixture consists of Buffer (pH 8.9 at 37°C) 230 mmol/L, Lactate 70 mmol/L, and NAD+ 10 mmol/L. Preservatives and stabilizing reagents are further added over the basic ingredients.

Reagents and equipment required but not provided in the kit

- 96-well flat-bottom plate: It is recommended to use clear plates for colorimetric assays.
- Spectrophotometric multi-well plate reader.

All reagents and specimen must be handled safely as they may be potential biohazard.

Reagent preparation

- ***Vials:*** Briefly centrifuge vials before opening. Use deionized distilled water for the preparation of reagents. To maintain reagent integrity, avoid repeated freeze/thaw cycles.
- ***LDH Assay Buffer:*** Allow buffer to come to room temperature before use.
- ***LDH Substrate Mix:*** Reconstitute in 1 mL of water. Mix well by pipetting and keep cold while in use. This Substrate Mix is stable for one week at 4°C and 1 month at –20°C.
- ***1.25 mM NADH Standard:*** Reconstitute in 400 mL of water to generate 1.25 mM standard solution. Mix well by pipetting and keep cold while in use. The NADH standard solution is stable for one week in a refrigerator (4°C) and 1 month in a freezer (–20°C).
- ***LDH Positive Control:*** Dilute the LDH Positive Control 10-fold (1 : 9) with LDH Assay Buffer before use and use 2–5 mL of the diluted LDH Control as positive control.

Storage/Stability: The kit is shipped on wet ice. Storage at –20°C, protected from light, is recommended. Unopened reagents are stored in a refrigerator (2–8°C) until the expiration date. Opened reagents are stable for 30 days when stored in the refrigerated compartment of an analyzer.

Indications of Deterioration: Visible signs of microbial growth, turbidity or precipitate, or any change in reagent colour may indicate degradation and warrant discontinuance. Never use expired reagents.

Interference: No significant interference is reported for the presence of bilirubin up to 40 mg/dL. Lipaemia does not significantly interfere with the result.

Procedure

1. Set up the Master Reaction Mixture with the proportion of 48 µL of assay buffer and 2 µL of substrate. (***Note*** This means a total of 50 µL of the Master Reaction Mixture is required for each reaction well. If there are 8 wells, you need to make for 10 wells so that you do not run out of mixture to draw through the pipette. In other words, you need 500 µL of reaction mixture. This comes from combining 480 µL of buffer and 20 µL of substrate).
2. Mark three wells as blank (B), standard (S) and test (T).
3. Add 50 µL of the master reaction mixture to each well. Mix well using a horizontal shaker or by pipetting. Protect the plate from light during incubation.
4. Add 50 µL of water in cuvette marked "B", 50 µL of standard (original standard of 1.25 mM diluted 10 × yielding 12.5 nmole NADH) in cuvette marked "S" and 50 µL of specimen in tube marked "T".
5. Start the stopwatch.
6. After 5 min, take the initial absorbance reading at 450 nm ($A_{initial}$). Leave first 5 min for temperature equilibrium.
7. Incubate the plate at 37°C taking measurements (A_{450}) after 5 min and after 10 min. Protect the plate from light during incubation.
8. Based on the observations after ten minutes and fifteen minutes, determine ΔA/min for both Standard and Test as compared to the blank (you can zero the instrument against blank).

Calculations

The LDH activity of a sample may be determined by the following equation:

$$\text{LDH Activity (mU/mL)} = \frac{\Delta A_T \times 12.5\ \text{nmole} \times \text{Dilution Factor}}{\Delta A_s \times V}$$

ΔA_T = ΔA/min for test (specimen)
ΔA_S = ΔA/min for standard (12.5 nmoles)
NADH Standard = 12.5 nmole (amount of NADH present in the system under identical conditions)
V = Volume of test specimen or standard = 50 µL or 0.050 mL
D = Dilution factor (in case the specimen is diluted)

$$\text{LDH Activity (mU/mL)} = \frac{\Delta A_T \times 250 \times D}{\Delta A_S}$$

B = Amount (nmole) of NADH generated between $T_{initial}$ and T_{final}
LDH activity is reported as nmole/min/mL = milliunit/mL

One unit of LDH activity is defined as the amount of enzyme that catalyzes the conversion of lactate into pyruvate to generate 1.0 mmole of NADH per minute at 37°C.

Special note

- Prepare standard curve with diluted standards at regular interval in order to provide reliable results.
- If spectrophotometer in ultraviolet range is available, NADH measurements are done at 340 nm.

Amylase

Amylase, Lipase and Trypsin are enzymes present in normal pancreatic glands which are secreted in pancreatic juice for digestion of starch, fat and protein respectively. Amylase is also present in saliva to facilitate digestion of complex carbohydrates taken as food to simple sugar.

$$\text{Starch} + \text{Water} \overset{\text{Amylase}}{\rightleftarrows} \text{Sugar(s)}$$

Amylase enzyme is found in **three forms** α, β **and** γ, depending on the way they react on the substrate. Alpha amylase (α-Amylase) is present in the saliva of humans and some other mammals, where it begins the chemical process of digestion. Foods that contain large amounts of starch but little sugar, such as rice and potatoes, may acquire a slightly sweet taste as they are chewed because amylase degrades some of their starch into sugar. The pancreas and salivary gland make amylase (α-Amylase). This helps to **hydrolyse dietary starch into disaccharides** and trisaccharides, simpler organic compounds. These ultimately convert to glucose which supplies energy to the body.

Clinical Significance

The clinical significance of the estimation of amylase lies almost entirely in the diagnosis of acute **pancreatitis,** in which the enzyme level frequently exceeds more than 10-times the normal range. Levels higher than 550 units/dL (SU) strongly support a diagnosis of acute pancreatitis. The rise is rapid and transient, reaching a peak within the first 12–24 h after onset and returning to normal usually in 2–3 days. Some of the other causes of high amylase activity in serum include salivary gland disorders, abdominal disturbances affecting the pancreas, and intake of some drugs (morphine). In these cases, the rise of the amylase activity is not as high. Chronic pancreatitis and carcinoma of the pancreas are rarely associated with a raised serum amylase.

Normal range

Reference range: <160 U/L (0–93 IU)

Specimen and specimen preparation

Amylase assay calls for whole blood (for plasma) or clotted blood (for serum). For getting plasma, whole blood is collected in tubes containing **heparin** which does not bind calcium which is needed for running amylase activity. Hence plasma with any other anticoagulant that binds calcium (e.g., EDTA) should not be used. Haemolysis and lipaemia will increase the amylase values. Amylase in serum or urine is reported stable for seven days at room temperature (18–30°C) and three months when stored at 4°C and properly protected against evaporation. Cover the tube with cellophane tape.

Laboratory Assay

Use of commercial kit has proved to be convenient, reliable and cost effective under some conditions. The following description is that of a typical kit (Sigma-Aldrich). Store the kit in a refrigerator. The shelf life is 6 months after receipt. Plate-based kinetic assays are becoming increasingly popular which is described here but it can be adapted to cuvette with minor modifications. The method is highly sensitive (detection limit = 15 U/L) and can handle urine and other body fluids and it does not require expensive instrumentation.

Principle

The chemical reaction involves three interlinked enzymatic assay scheme using a colour producing starch analogue called p-nitrophenyl-D-maltoheptaoside (PNPG7). Amylase works on the substrate (PNPG7) in the first reaction in which amylase selectively degrades the **PNPG7** substrate to form maltotriose and **p-nitrophenyl maltotetraoside (PNPG4).** In the next reaction, PNPG4 is cleaved by glucoamylase and glucosidase to form a yellow-coloured p-nitrophenol. The rate of increase in yellow colour is measured at 405 nm which is proportional to alpha-amylase activity in the sample. Amylase activity is measured as **"kinetic colorimetric assay"** as it progresses. A p-nitrophenol standard is used to verify assay performance.

$$\text{PNPG7} \xrightarrow{\text{alpha-amylase (in serum)}} \text{PNPG4 + maltotetrose}$$

$$\text{PNPG4} \xrightarrow{\text{glucoamylase}} \text{PNPG1 + glucose}$$

$$\text{PNPG1} \xrightarrow{\text{alpha-glucosidase}} \text{p-nitrophenol + glucose}$$

After preparing the sera, the assay is performed by adding Amylase Reagent into microplate wells containing 5 µL sera. Using a plate reader, the absorbance of each well at 405 nm is measured. The concentration of amylase in each sample is directly determined from calculating the change in absorbance over 5 min (ΔA).

Reagents

Following reagents are provided in the kit:

- Amylase reagent
- pNP standard
- pNP dilution buffer

Reconstitute the reagents as per manufacturer's instructions:

- Add exactly 27 mL of deionized or distilled water to the amylase reagent mix powder.
- Mix by swirling or inverting the bottle 10 times.
- Allow contents to dissolve for 10 min at room temperature. Do not shake.

Formerly made solutions	Amount	Storage condition
Microtitre plate	One	Room temperature
Amylase reagent	1 vial	4°C
pNP standard	0.8 mL	4°C
pNP dilution buffer	2×1.8 mL	4°C

Materials Required but Not Provided With the Kit

- Microtitre plate reader (405 nm)
- Centrifuge to prepare serum samples
- Deionized or distilled water (dH_2O)
- PBS (phosphate buffered saline, pH 7.3)
- Automatic pipettor

Warnings and Precautions

Read the inset carefully after opening the kit. All kits are not identical.

Special note

Add standards to plate only in the order from low concentration to high concentration, as this will minimize the risk of compromising the standard curve. Increased activity of pancreas may also increase the amylase level in serum.

Procedure

1. In the microplate well, mark three wells as blank (B), standard (S) and test specimen (T). If there are more specimens, mark them as T_1, T_2, etc.
2. Add 5 µL of water in the well-marked B; 5 µL of standard in the well-marked S, and 5 µL of specimen in the well-marked T. A duplicate run is always recommended.

   Note Concentration of undiluted standard is 800 U/L.
3. Add 245 µL of reconstituted reagent. Mix in the wells.
4. Add the reagent at one minute interval to facilitate exact timing.
5. Set the colorimeter (spectrophotometer) of the plate reader to zero with the blank.
6. Immediately measure the absorbance readings of standard (A_S) and sample (A_T) at 405 nm. This is absorbance at "0 min"–A_{S0} and A_{T0}

   Note The absorbance reading will decrease with time.
7. Exactly after 5 min, measure the absorbance again for both Standard (A_{S5}) and Test (A_{T5}).
8. Determine ΔA (difference in absorbance readings) for both standard (ΔA_S) and test specimen (ΔA_T) during 5 min. For Standard, it will be the difference between $A_{S5} - A_{S0}$ and for Test, $A_{T5} - A_{T0}$. These are the values of ΔA_S and ΔA_T respectively.

Calculation

Determine amylase activity in serum samples by the following equation:

$$\text{Amylase activity (U/L)} = \Delta A_S/\Delta A_T \times 1569$$

Note

- If the amylase activity of the specimen is too high (> 800U/L), dilute the specimen with phosphate buffered saline (PBS) and retest the serum. Multiply results by dilution factor.
- Grossly lipaemic sera may require the following adjustment to eliminate any error:

  Run a sample blank. Add 5 µL of sample to 250 µL PBS and mix thoroughly. Then read the absorbance against water. Subtract this reading value from the absorbance of each serum sample to obtain the corrected reading.

Preparation of Standard Curve

Each batch of new kit must be checked for linearity by constructing the standard curve.

1. Label 6 microfuge tubes: 1, 2, 3, 4, 5, 6.
2. Dilute the pNP standard according to following table using pNP dilution buffer. After dilution, briefly mix each tube by inversion before performing the next dilution. (The standard is equivalent to 800 U/L amylase.)

Tube#	pNP control	pNP Dilution Buffer	Dilution	Amylase Diluted standard (U/L)
1	150 µL	0	0×	800
2	100 µL of Tube #1	100 µL	2×	400
3	100 µL of Tube #2	100 µL	4×	200
4	100 µL of Tube #3	100 µL	8×	100
5	100 µL of Tube #4	100 µL	16×	50
6		100 µL	0	0

3.5 µL of each diluted standard is used for the assay as described with sample.

Gamma-Glutamyl Transferase (GGT)

Gamma-glutamyl transferase (also known as γ-glutamyl transferase, GGT, GGTP, gamma-GT) is an enzyme that transfers gamma-glutamyl functional group. This transfer can occur even on an artificial substrate (not found in the body) and that liberates a coloured compound **p-nitroanilide.** This makes the assay simple and accurate.

Clinical significance

γ-glutamyl transferase (GGT) is found in high level in liver, kidney, pancreas, heart, and in brain. Hence the test is requested to detect diseases of the liver or bile ducts. Results must be compared with other tests like ALP, AST and bilirubin in order to differentiate probable source. Serum GGT can also be elevated in many other pathophysiological conditions as well, such as cardiovascular diseases, pancreatic disorders, and metabolic syndrome. Some physicians use this test for monitoring alcohol consumption that may lead to cirrhosis.

Normal value

0-51 IU/L

Specimen

Fresh serum specimen is preferred. Reject specimens showing haemolysis.

Laboratory Assay

Colorimetric method

GGT colorimetric assay kits are now available in the market. The kit provides a convenient tool for detection of the GGT in a variety of samples. Kit from reputable company is easy to use, convenient, cost-effective and reliable. One should read and understand the instructions provided by the manufacturer before running the test.

Principle

The colorimetric assay to measure the activity of GGT in serum provides a simple and direct procedure that can be adapted easily to any laboratory. It is a coupled enzyme reaction in which the GGT transfers the γ-glutamyl group from the substrate **L-γ-glutamyl-p-nitroanilide,** liberating the **chromogen p-nitroanilide** (pNA at 418 nm) **proportional to the GGT present.** The activity of GGT can be easily quantified calorimetrically. This assay detects GGT activity as low as 0.5 mIU. ***Note*** One unit of GGT is the amount of enzyme that will generate 10 µmole of pNA per minute at 37**°C.**

$$\underset{\text{(Substrate)}}{\text{L-}\gamma\text{-glutamyl-p-nitroanilide}} \xrightarrow{\text{GGT}} \underset{\text{(Chromogen)}}{\text{pNA}}$$

Reagents

All reagents are supplied by the manufacturer in the kit.

GGT assay buffer	25 mL
GGT substrate	1 bottle
GGT positive control	1 vial
pNA standard	400 µL

Storage and handling: Store the kit at –20°C protected from light. Allow assay buffer to come to room temperature before use. Briefly centrifuge vials before opening. Read the entire protocol before performing the assay.

Reconstitution and general consideration

- GGT substrate solution: Add 10 mL assay buffer into substrate bottle and mix well. Take out enough substrate solution (90 µL per assay) for the number of assays to be performed in experiment. Store the rest of the GGT substrate solution at –20°C quickly. ***Note*** The GGT substrate solution is unstable at room temperature (can be hydrolyzed by itself) which increases the assay background.
- GGT positive control: Reconstitute with 100 µL deionized water. Pipette up and down several times to completely dissolve the pellet into solution (do not vortex). Aliquot enough GGT positive control (10 µL per assay) for the number of assays to be performed in each experiment and aliquot and freeze the rest immediately at –20°C for future use. The GGT positive control is stable up to 1 month at –20°C after reconstitution of freeze-thaw cycles (<5 times). Keep the GGT positive control on ice during the preparation.

Procedure

1. Warm up the standard for 1–2 min at 37°C until completely melt. When 20 µL of the 2 mM pNA standard solution is mixed with 80 µL of GGT assay buffer (total 100 µL), it yields 40 nmol standard.
2. Set up three matched cuvettes marked blank (B), standard (S) and test (T); if more than one mark them as T_1; T_2, etc.
3. Add 10 µL water in the cuvette marked B; 10 µL of standard (40 nmol) in cuvette marked S (40 nmol) and 10 µL serum specimen in the cuvette marked T.
4. Add 90 µL GGT substrate in all three cuvettes—B, S and T. (A positive control is occasionally included for quality control check).
5. Mix by inversion.
6. Incubate the mixture for 30 min at 37°C.
7. Measure absorbance (or optical density, OD) at 418 nm after 30 min, using blank to zero the photometer.

Calculation

$$\text{GGT activity (in pNA unit)} = \frac{A_T}{A_S} \times 40 \text{ nmol}$$

Where,

A_T = Absorbance of the test
A_S = Absorbance of the standard
40 = nmol of standard (pNA)

Note 1 pNA unit = 1.5 IU

Laboratory Assay

Enzymatic method

The enzymatic assay kit of γ-glutamyl transferase (GGT), as described here, is also a colorimetric procedure and all measurements are done at the lower end of visible range (405 nm). Some laboratories may have a spectrophotometer that records kinetic assay to detect changes in γ-glutamyl transferase level directly from serum samples. The instrument is highly sensitive and has low detection limit (<10 U/L). It requires only 10 µL of serum sample. The method is rapid (10 min) and can be adapted to laboratories with minimal facilities. Make minor modifications to suit your own requirements.

Specimen

- Carefully collect the blood specimen in serum collection tube making sure to avoid haemolysis.
- Allow to clot for 10 min at room temperature.
- Centrifuge the clotted blood (10,000/10 min).
- Separate the serum to a clean tube avoiding the "buffy coat" layer.
- Store the serum on ice or in refrigerator (at 4°C) prior to testing.
- Do not freeze.
- Serum sample can be stored at 4°C for up to one week.

Principle

The assay measures the cleavage of a specific **GGT substrate (γ-glutamyl-p-nitroanilide)** by the enzyme present in the specimen. The cleavage results in the transfer of an amino group (thus the name transferase). This cleavage results in the production of the **p-nitroanaline (pNA) product,** a coloured substance whose absorption is measured at 405 nm. Intensity of the colour is proportional to the level of GGT enzyme in the sample. The absorbance reading from each cuvette (or sample well) is measured at 405 nm in the spectrophotometer or colorimeter. (***Note*** Plate reader will be needed if plates with wells, holding the reaction mixture, are used). The concentration of GGT in each sample is then directly determined from the change in absorbance at 405 nm within 10 min. Check linearity as part of quality control. Dilute the specimen, if required.

$$\underset{\text{(Substrate)}}{\text{L-}\gamma\text{-glutamyl-p-nitroanilide}} \xrightarrow{\text{GGT}} \underset{\text{(Chromogen)}}{\text{pNA}}$$

Concentration of the chromogen p-nitroanilide (pNA) is proportional to the amount of GGT present in the serum when measured at 418 nm.

Stock reagents and supplies

GGT reagent mix powder	1 bottle
pNA control	0.4 mL
pNA dilution buffer	2 × 1.8 mL

Keep all reagents at 4°C.

Materials needed but not available in the kit

- Serum collection tubes (without any anticoagulant)
- Matched cuvettes of 0.5 mL capacity
- Microtitre plate
- Spectrophotometer (405 nm)
- Multichannel pipette or repeating pipettor (optional)

Working reagent

Kits are popularly used by laboratories. Follow manufacturer's instructions. Following instructions are taken from one such kit which may be different from the one in hand.

GGT reagent mix

- Constitute the GGT mixture according to manufacturer's direction. In this kit we have used exactly 27 mL of deionized or distilled water to be added to the GGT powder in the bottle provided by the manufacturer.
- Mix by swirling or inverting the bottle 10–12 times.
- Wait for 10 min at room temperature to dissolve the contents.
- After reconstituting the GGT reagent mix, the entire kit should be stored at –20°C between uses.
- The frozen liquid component should be thawed and brought to room temperature immediately before each use.

Important note: The reconstituted GGT reagent mix can be left at room temperature for short periods (30–60 min) while in use. Otherwise it must be stored in the freezer (–20°C) when not in use. The reconstituted GGT reagent mix can be stored for six months. Discard after 6 months of storage.

pNA standard

Follow manufacturer's instructions to reconstitute the pNA control (100 µL of control is equivalent to 125 IU/L). ***Note*** The value of the standard may vary.

Procedure

1. Bring all reagents and samples to room temperature.
2. Set up three matched cuvettes and labelled them as blank (BL), standard (ST) and test (TS).
1. Add 10 µL of water in the cuvette marked BL, 10 µL of standard in the cuvette marked ST, and 10 µL of specimen in the cuvette marked TS.
3. Add 240 µL of GGT reagent mix in each cuvette marked BL, ST and TS.
4. Mix gently.
5. Measure absorbance at 405 nm for all the cuvettes, zeroing the instrument with water blank.
6. Exactly after 10 min measure the absorbance again.
2. Determine the increase of absorbance during 10 min time interval by subtracting the absorbance at the initial time point from the absorbance at the 10 min time point.

Calculation

Concentration of GGT (U/L) = Difference in absorbance in 10 min × 353

The above equation can also be written in the following way:

$$\text{GGT (U/L)} = \Delta A_{10\ \text{min}} \times 353$$

For example: If the specimen shows a difference of absorbance during 10 min period as 0.2, the GGT activity will be 0.2 × 353 = 70.6 U/L

Note

- If the laboratory has arrangement for the use of microtitre plate with wells and a plate reader, it will be more convenient.
- If the GGT of the specimen is higher than 400 U/L (off linearity) dilute the specimen 1 : 1 with buffered saline and repeat the assay.

BRAIN NATRIURETIC PEPTIDE (BNP)

B-type natriuretic peptide (BNP) is a natriuretic peptide produced in the heart and released when the heart is stretched and working hard to pump blood. It is structurally similar but genetically distinct from peptide hormones.

Clinical significance

BNP is a substance secreted from the ventricles or lower chambers of the heart in response to changes in pressure that occur when heart failure develops and worsens. The level of BNP in the blood increases when heart failure symptoms worsen, and decreases when the heart failure condition is stable. The BNP level in a person with heart failure—even someone whose condition is stable—is higher than in a person with normal heart function.

Normal range

100–400 pg/mL

Specimen

Fresh serum

Laboratory assay

The Brain Natriuretic Peptide (BNP) concentration in serum is commonly determined by Enzyme Immunoassay (EIA). All reagents and laboratory supplies are available in the kit which needs to be reconstituted according to manufacturer's direction. We have discussed here a procedure based on the principle of **competitive enzyme immunoassay.** The BNP assay, like all laboratory tests, does not provide a definitive diagnosis. The test result should be interpreted by the physician in conjunction with clinical findings and other diagnostic testing.

Principle

The microplate in the kit is pre-coated with anti-rabbit secondary antibody. After a blocking step and incubation of the plate with anti-BNP antibody, both biotinylated BNP peptide and peptide standard, or targeted peptide, in samples interacts competitively with the BNP antibody. Bound biotinylated BNP peptide then interacts with **streptavidin-horseradish peroxidase** (SA-HRP), which catalyzes a colour development reaction. **The intensity of colorimetric signal is directly proportional to the amount of biotinylated peptide**–SA-HRP complex and inversely proportional to the amount of BNP peptide in the standard or samples. This is due to the competitive binding to BNP antibody between biotinylated BNP peptide and peptides in standard or samples.

A standard curve of known concentration of BNP peptide can be established and the concentration of BNP peptide in the samples can be calculated accordingly.

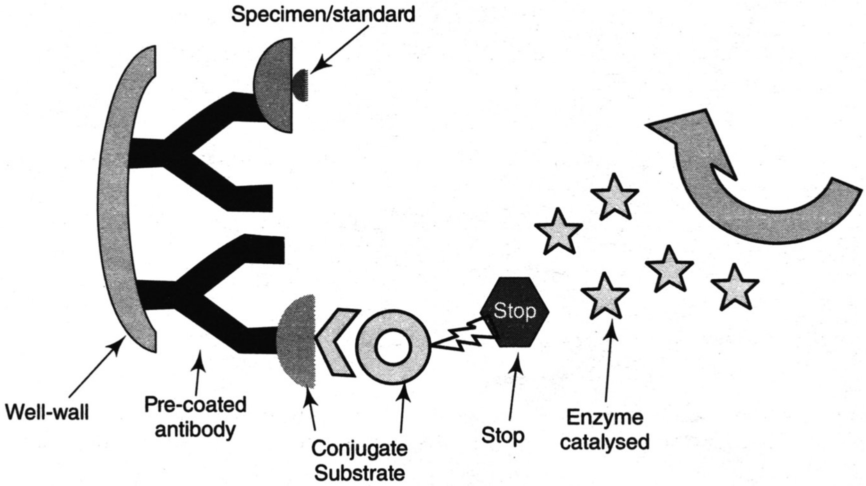


FIGURE 33.4

Reagents

The kit provides all reagents along with pre-coated microtubes or plates with wells. Reconstitute the chemicals according to manufacturer's instructions.

Procedure

Strictly follow manufacturer's instructions during the test and in preparing standard curve.

Preparation of standards

Label 6 wells or microtubes with the following concentrations:
1,000 pg/mL, 100 pg/mL, 10 pg/mL, 1 pg/mL, 0.1 pg/mL, and 0 pg/mL

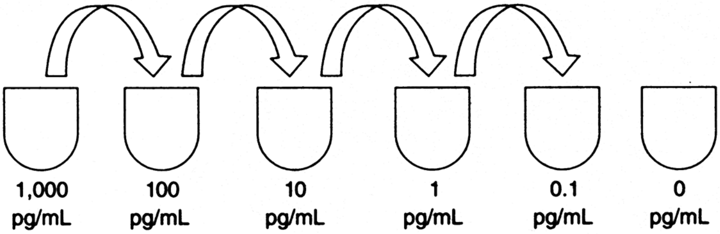


FIGURE 33.5 *Preparation of standards*

Each subsequent tube is diluted 10×.

1,000 pg/mL	100 pg/mL	10 pg/mL	1 pg/mL	0.1 pg/mL	0 pg/mL

BNP pmol/L	OD (A)
0	3.0
200	2.5
500	2.0
800	1.6
1500	1.3
3400	0.8
6200	0.3

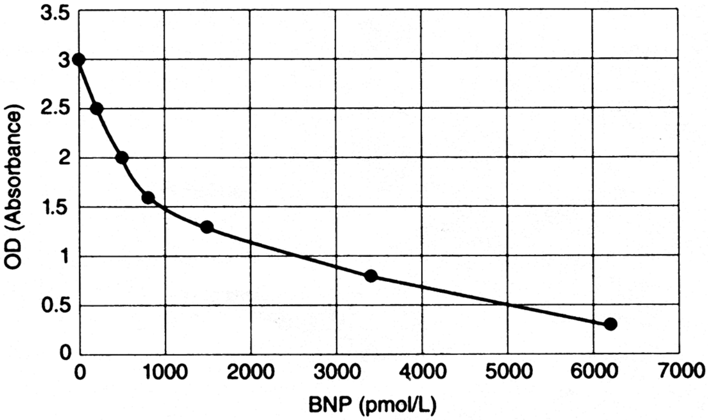


FIGURE 33.6 *Standard Curve*

LIPID PROFILE

Lipids are substances which are insoluble in water but soluble in organic solvents. Oils and fats are lipids. **Cholesterol** is another example of lipid found in our body along with several others. Our digestive system breaks down ingested fats into fatty acids, which are transported through the blood stream to the liver, where they are used to manufacture lipids. Because lipids do not dissolve in water, the body attaches lipids to a protein molecule for transport to the cells of the body to be used. These complexes are called **lipoproteins.** Lipids are used as an alternative source of energy when glucose is not readily available, and they are also necessary for hormone production, cell integrity, utilization of fat-soluble vitamins, hunger satiation, maintenance of appropriate body temperature, and skin health.

Clinical significance

In the laboratory, the two types of lipids most commonly analyzed in blood:

- Cholesterol
- Triglyceride

These provide valuable information to the physician about cardiovascular health and potential risk factors for the future. However, more information can be gained by breaking down the relative amounts of lipids according to subtype. These categories are:

- HDL (high-density lipoprotein)
- LDL (low-density lipoprotein)
- VLDL (very low-density lipoproteins)

This is the reason why the cardiologists order the test known as "lipid profile", routinely for patients with coronary artery disease and other atherosclerotic conditions. Elevated total cholesterol, triglyceride, and LDL levels may be considered to be the risk factors for coronary heart disease. This will be further discussed under each of the following analytes.

Some of the screening tests are now available in the physician's office but the reference laboratories of developing countries are better equipped for complete analysis.

Specimen

Cholesterol levels ordered individually do not require fasting samples. However, cholesterol levels are often ordered as part of a lipid panel with the triglyceride, HDL, LDL, and VLDL levels. These additional tests do require a fasting sample, so patient should be advised to abstain from eating for 12 h prior to the blood collection.

Although serum specimen is preferred but plasma can also be used for cholesterol assay. Cholesterol assay should be performed on the same day. The specimen is, however, stable for a week if refrigerated (2–8°C) and for one month if frozen (–10°C). The specimens should be brought to room temperature before analysis. Capillary whole blood is also used with some kits but the blood must be used instantly.

Serum or plasma appearance with high lipid content

An inexpensive and simple way to recognize **hyperlipidaemia** is to store the collected serum specimen in refrigerator (2–8°C) in clear tube with very little of the label obstructing the cross-sectional view through the tube, especially at the meniscus of the specimen. A **creamy layer** forming at the surface of the serum contains chylomicrons, which is an indication of either a non-fasting specimen or a serious defect in lipoprotein lipase production or function. Varying degrees of cloudiness are usually associated with **elevated triglyceride** levels. Consequently, the appearance provides observable data that aid in the correlations and validation of laboratory results. However, the appearance reveals little about the HDL and LDL cholesterol levels.

Cholesterol

Cholesterol is a **lipid,** classified as a **sterol.** Cholesterol is important in our metabolism serving as a precursor of various steroid hormones, e.g., sex hormones and adrenal corticoids. Cholesterol is also used for absorption of vitamin D, bile production, and cellular membrane structure and is also used as a part of the covering (the myelin sheath) on some of the nerves in the body. The liver creates all the cholesterol that we require to function normally. This is called **endogenous cholesterol** because it is manufactured within the body. Exogenous cholesterol comes from our diets. Because the basic nature of cholesterol fat, it is insoluble in water. Hence it attaches to proteins to move within the body through the blood. These carriers are called **lipoproteins.** These lipoproteins again are of two types—high density and low density, and thus the names high density lipoprotein (HDL) and low density lipoprotein (LDL). When they are attached to cholesterol, they give rise to two different kinds of cholesterols:

- High density lipoprotein cholesterol (HDL-cholesterol)
- Low density lipoprotein cholesterol (LDL-cholesterol)

Clinical significance

We have heard the mantra "watch your cholesterol" for a healthy body. If you have heart disease you are repeatedly warned against fat and cholesterol. Of the two types of cholesterols (HDL and LDL), the type that puts your heart at risk is LDL, also called "bad cholesterol". Higher levels of LDL in blood put you on a greater risk for a heart attack than from a sudden formation of a blood clot (blockage).

In any case, elevated levels of cholesterol (total) undoubtedly increase the risk of coronary heart disease (CHD). The cholesterol collects in the walls of our blood vessels, where it can cause blockages. Cholesterol is measured to help assess the patient's risk status and to follow the progress of patient's treatment to lower serum cholesterol concentration. Desirable cholesterol levels are considered to be those of 200 mg/dL in adults and below 170 mg/dL in children.

Serum cholesterol (total) is found in two forms—**esterified cholesterol** and **non-esterified** (free) **cholesterol.** As esterification of cholesterol is closely related to **liver function,** a fall of esterified cholesterol reflects liver disorder. The level of cholesterol is affected by stress, age, sex, hormonal balance and pregnancy.

Normal range

Normal values vary with age, diet, sex, and geographic or cultural region.

Test	Category	Adults (fasting)	Children and Adolescent (12–18 years)
Total cholesterol (mg/dL)	Desirable level	140–199	<170
	Borderline high	200–239	170–199
	High	>240	>200
HDL (mg/dL)*	Men	35–65	
	Women	35–80	
LDL (mg/dL)	Desirable level	<130	<110
	Borderling high	140–159	110–129
	High	>160	>130

Note HDL (good cholesterol) above 75 mg/dL is most desirable. The risk of coronary heart disease increases as the level drops below 45 mg/dL.

Conversion factor to International Unit: Cholesterol concentration (mg/dL) = 38.7 × (mmol/L)

Principle

Total cholesterol is measured in serum or plasma in a series of coupled reactions that hydrolyse cholesterol esters and oxidize the 3-OH group of cholesterol. One of the reaction by-products, H_2O_2 is measured quantitatively in a peroxidase catalyzed reaction that produces a colour. Absorbance is measured at 500 nm. The colour intensity is proportional to cholesterol concentration. The reaction sequence is as follows:

The first step is an enzymatic hydrolysis of cholesterol ester by cholesterol esterase (CE):

$$\text{Cholesterol ester} + H_2O \xrightarrow{CE} \text{Cholesterol (free)} + \text{Fatty acid}$$

The non-esterified free cholesterol is then oxidized by cholesterol oxidase (CO) to a ketone, with the production of hydrogen peroxide (H_2O_2).

$$\text{Cholesterol (Free)} + O_2 \xrightarrow{CO} \text{Cholester-4-en-3-one (a ketone)} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{P} \text{Coloured compound} + 4\ H_2O$$

Hydrogen peroxide can be measured in a peroxidase-catalyzed reaction that oxidizes a reduced dye (4-aminophenazone) with phenol to a coloured product that absorbs at 500 nm. The commercial reagent kits generally combine all enzyme and other required components, and the formulations vary. The method has been optimized in various automated systems.

Laboratory assay

The enzymatic methods have largely replaced the older methods and the laboratories are able to get the commercial kits from local vendors. Considering the difficulties of peripheral laboratories in remote places, an easier colorimetric method is suggested here.

Reagents

All reagents are supplied with the kit. The method described here is taken from Roche/Boehringer-Manhein Diagnostics kits. Similar kits are also available with local vendors. A visible range colorimeter is needed to perform the test. Normal laboratory supplies and distilled water will be required.

Procedure

1. Assemble necessary equipment, and verify that all reagents. Do not use reagents beyond their expiry date.
2. Allow all reagents to come to room temperature before use.
3. Prior to patient testing, quality control run must be completed and recorded. Follow instructions from the manufacturer of the kit.
4. Carefully compare the identification of the specimen and laboratory request.
5. Carry on all steps of analytical procedures in separate test tubes labelled as reagent blank (B), standard (S) and tests (T_1, $T_{2,}$ etc.) for the specimens. Serum without other reagents can be used as reagent blank. This compensates for any colour or turbidity of the serum due to high lipid content. Standard solution, supplied by the manufacturer, will be used as reference.
6. Mix the contents to the test tubes and proceed to the measurement of the intensity of the colour developed.
7. Transfer the contents of the test tubes in two matched cuvettes marked as ‘T’ and ‘S’.
8. Read the absorbance of ’T’ and ‘S’ solutions at 500 nm using water to set the zero.
9. Calculate and report the result in patient’s chart and also keep and record in the laboratory register with identification of the specimen.

Triglycerides

Fats, or lipids, are the third main class of the **macronutrients needed in human nutrition;** the other two are carbohydrates and proteins. Oils are also fats but liquid at room temperature. Most of the fat in our bodies is made up of triglycerides which are simple fat. When hydrolyzed by lipase enzyme (LPS), triglycerides yield glycerol and fatty acids. This forms the basis of triglyceride assay.

Clinical significance

Patients with high carbohydrate diet have elevated triglyceride level. When a person takes in more calories than are needed for energy, the excess calories are converted into triglycerides and stored as fats. Elevated level of triglycerides in serum is a risk factor related to **atherosclerotic disease** that causes thickening of the walls of larger arteries. This may lead to a heart attack. Some of the clinical conditions that cause hyperlipaemia are glycogen storage disease, nephrotic syndrome, diabetes mellitus, chronic hepatitis and alcoholism. Excessive elevation of triglycerides can cause acute pancreatitis by causing activation of pancreatic amylase in side pancreatic cells hence causing cell death and inflammation.

Normal range

10–190 mg/dL serum (0.11–2.15 mmol/L)

Specimen

Fasting (12–14 h) specimens are recommended for triglyceride analysis. Although serum is preferred, plasma can also be used for determining triglycerides. Anticoagulants such as EDTA should be used in case of the enzymatic method. For the colorimetric method, fluoride-oxalate can be used. Analyse the specimen on the same day. If this is not possible, the specimens should be stored in the refrigerator for not > 2–3 days. Specimens should be brought to room temperature prior to use.

Interfering substances: Haemoglobin and bilirubin in high concentrations may interfere with the triglyceride analysis. Hence avoid a haemolyzed serum specimen.

Laboratory assay

As with cholesterol testing, the advent of enzymatic method has proved to be a relief. Reagents are now available that contain all enzymes, buffers, and cofactors needed for the assay.

Principle

Here triglycerides are measured enzymatically in serum or plasma using a series of coupled reactions in which triglycerides are hydrolyzed to produce glycerol, using lipase (LPS) enzyme.

$$\text{1 Triglyceride} + 3H_2O \xrightarrow{\text{LPS}} \text{1 Glycerol} + \text{3 Fatty acids}$$

In the following steps, glycerol is taken through a series of chemical reactions with the mediation of three enzymes:

- glycerol kinase (GK)
- glycerol phosphate oxidase (GPO)
- horseradish peroxidase (P)

In this method, the peroxidase frees oxygen radical that **oxidizes a reduced dye to a coloured end product.** This can be read in the visible wavelength of light. The three enzymatic reactions, after the hydrolysis of triglycerides, are shown here.

$$\text{Glycerol} + \text{ATP} \overset{\text{GK}}{\rightleftarrows} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \overset{\text{GPO}}{\rightleftarrows} \text{Dihydroxy acetone phosphate (DHAP)} + H_2O_2$$

$$H_2O_2 + \text{Reduced dye} \overset{\text{P}}{\rightleftarrows} \text{Oxidized dye*} + \text{HCl}$$

*Absorbance is measured at visible wavelength (500 nm) of light.

An **alternative method** is to use glycerol phosphate dehydrogenase (GPD) in place of glycerol phosphate oxidase. In this method NAD is used which is reduced to NADH, allowing the measurement of absorbance at 340 nm. Various automated systems adapt the same procedure for triglyceride assay. The major problem with this type of assay is the ability of the spectrophotometers to distinguish absorbance differences at that wavelength. Sample blanks are necessary to reduce interferences from other UV-absorbing pigments in the sample.

Reagent, equipment and supplies

- Use commercial kit which supplies all the required reagents
- Spectrophotometer
- Laboratory supplies for chemical analysis

Procedure

Follow manufacturer's direction in performing the test.
Conversion to International Unit: mg/dL × 0.0113 = mmol/L

Note Conversion factor is based on triolein (molecular weight 885).

Subgroups of Cholesterol

Subgroups of cholesterol (HDL and LDL) have been discussed earlier. In this section we will deal with them more closely. Of the two, **HDL** (High Density Lipoprotein)-cholesterol has a protective effect and higher than 40 mg/dL is desirable. Very low density lipoprotein **(VLDL)** cholesterol and **LDL** cholesterol affects coronary risk adversely. HDL and LDL are now analyzed by enzymatic methods. And kits are available through local vendors. We have used Roche/Boehringer-Mannheim Diagnostic kit here.

High density lipoprotein (HDL)

If the concentration of HDL-cholesterol (good cholesterol) in serum drops below 40–30 mg/dL, the risk of coronary heart attack (CHD) increases markedly. A low HDL-cholesterol concentration is considered to be a value below 35 mg/dL and high HDL ≥60 mg/dL.

Laboratory assay: Direct HDL method

Principle

HDL is measured directly in serum. The basic principle of the method is described here. The apoB containing lipoprotein in the specimen is reacted with a blocking reagent that renders them non-reactive with the enzymatic cholesterol reagent under conditions of the assay. The apoB containing lipoproteins are thus effectively excluded from the assay and only HDL-cholesterol is detected under the assay conditions.

Roche/Boehringer-Mannheim Diagnostics market the kit with all necessary reagents. The method uses sulphated alpha-cyclodextrin in the presence of Mg^{+2} which forms complexes with apoB containing lipoproteins, and polyethylene glycol-coupled cholesteryl esterase and cholesterol oxidase for the HDL-cholesterol measurement. The reactions are as follows:

1. ApoB containing lipoproteins + α-cyclodextrin + Mg^{+2} + dextran $SO_4 \rightarrow$ Soluble non-reactive complexes with apoB-containing lipoprotein
2. HDL-cholesteryl esters $\xrightarrow{\text{Cholesteryl esterase (CE)}}$ HDL-unesterified cholesterol + Fatty acid
3. Unesterified cholesterol + O_2 PEG $\xrightarrow{\text{Cholesteryl oxidase}}$ Cholestenone + H_2O_2
4. H_2O_2 + 5 aminophenazone + N-ethyl-N-(3-methylphenyl)-N succinyl ethylene diamine + H_2O + H^+ peroxidase → **quononeimine dye** + H_2O

Absorbance is measured at 600 nm.

Low density lipoprotein (LDL)

LDL cholesterol in serum is desirable. Value between 100 and 129 mg/dL (2.6 and 3.3 mmol/L) is close to ideal, while between 130 and 159 mg/dL (3.4 and 4.1 mmol/L) is borderline **elevated.** From physician's viewpoint, **LDL levels** are the very best gauge of a risk of cardiac arrest.

Most of the circulating cholesterol is found in **three major lipoprotein fractions:** very low density lipoproteins (VLDL), LDL and HDL.

Total cholesterol = VLDL-cholesterol + LDL-cholesterol + HDL-cholesterol

LDL-cholesterol is calculated from measured values of total cholesterol, triglycerides and HDL-cholesterol according to the relationship:

LDL-cholesterol = Total cholesterol – HDL cholesterol – Triglyceride/5 (TG/5)

Where, TG/5 is an estimate of VLDL-cholesterol and all values are expressed in mg/dL.

THYROID FUNCTION TESTS

Thyroid is a large **ductless gland** in the neck that secretes hormones regulating growth, development, differentiation, cellular metabolism, and general hormonal balance of the body. By regulating the metabolic activity it affects the developments of the skeletal and organ systems of the body. It is the major component of the **endocrine system.**

Free Triiodothyronine (fT3)

Thyroid gland produces two major **thyroid hormones:** T3 (triiodothyronine) and T4 (thyroxine). Of these two, T4 is the principle thyroid hormone which will be discussed later. Most of the T3 that circulates in the blood is bound to carrier proteins such as TBG, pre-albumin and albumin. The **free fraction of T3** (fT3), which represents only 0.25% of the total amount, is considered to be the physiological active fraction.

Clinical significance

Laboratory assay of free T3 (or total T3 – bound plus unbound) is requested in order to assess **thyroid function.** A low value is indicative of hypothyroidism (poor function) and high value may indicate hyperthyroidism (over reactive). T3 tests are requested only in outpatient settings. Thyroid stimulating hormone or TSH stimulates the production and release of T4 (primarily) and T3. T4 is converted into T3 by liver and other tissues. The free or total T3 test is usually ordered following an abnormal value of TSH. Physicians often consider **free T3 value more reliable than total T3.** Hence measurement of free T3 is generally recommended for patients with symptoms of **hyperthyroidism** and **hypothyroidism.**

Normal range

As for all assays each laboratory should collect data and establish their own range of expected normal values. Total T3 (bound and free): 75–200 ng/dL
Adults: 2.3–4.2 pg/mL (mean = 3.2 pg/mL).
SI units: 3.5–6.5 pmol/L

Specimen

Collect 4–5 mL of blood into an appropriately labelled tube and allow it to clot. Centrifuge and remove the serum layer. Store at 4°C for up to 24 h, or at –10°C or lower if the analyses are to be done at a later date.

Caution Consider all human specimens as possible biohazardous materials and take appropriate precautions when handling.

Laboratory assay

Principle

Most kits in the market provide materials based on Enzyme-Linked-Immunoassay (ELI) which is typical of **competitive binding.** Here the unlabelled antigen (the analyte T3) present in the specimen and an enzyme-labelled antigen (conjugate) compete for a limited number of antibody binding sites on the solid surface. The washing and decanting procedures remove unbound materials. After the washing step, the enzyme substrate is added. The enzymatic reaction is terminated by addition of the stopping solution. The absorbance is measured on a micro-well plate reader. The intensity of the colour formed is **inversely proportional** to the concentration of free T3 in the sample. A set of standards is used to plot a standard curve from which the amount of free T3 in patient samples and controls can be directly read.

Use of reliable kit is recommended.

Procedural caution

1. Follow the instructions of manufacturer given in the insert.
2. Control materials should be included in every run. It may be pooled sera whose results are known or "control" supplied by the manufacturer.
3. If micro-wells are not provided by the manufacturer, make minor changes to suit to your own laboratory.
4. The substrate solution (TMB) is sensitive to light and should remain colourless if properly stored. Instability or contamination may be indicated by the development of a blue colour, in which case it should not be used.
5. Avoid contamination of reagents, sample, standard, and control.
6. Do not accept grossly haemolyzed, grossly lipaemic, icteric or improperly stored sera.

Safety precautions

Consider the specimens as potential biohazardous and all chemicals as poisonous. Use gloves as recommended. Avoid contact with reagents containing TMB, hydrogen peroxide and sulphuric acid. TMB is a suspected carcinogen.

Reagents and their storage (typically provided in kit)

1. Antibody coated tubes or wells (microplate): Keep in a refrigerator when not in use. Stability is indicated on label (usually 12 months).
2. fT3-Horseradish Peroxidase (HRP) Conjugate Concentrate: To be reconstituted.
3. fT3-HRP conjugate in a protein-based buffer with preservative. Follow manufacturer's instructions for preparation.
4. Calibrators (Free T3 or fT3) and controls.
5. Wash buffer concentrate: Requires preparation.

6. Assay Buffer: Ready to use.
7. Stopping Solution: 1M sulphuric acid.

Reagents and equipment needed but not provided in the kit

- Precision pipettes
- Disposable pipette tips
- Distilled or deionized water

Procedure

1. All reagents must reach room temperature before use.
2. Calibrators, controls, and specimen samples should be assayed in duplicate.
3. Once the procedure has been started, all steps should be completed without interruption.
4. Follow manufacturer's instructions given in the kit.

Calculations

- Calculate the mean optical density of each calibrator duplicate.
- Draw a calibrator curve on semi-log paper with the mean optical densities on the Y-axis and the calibrator concentrations on the X-axis (Figure 33.7). Calculate the mean optical density of each unknown duplicate.
- Read the values of the unknowns directly off the calibrator curve.

Typical tabulated data

Calibrator	Mean OD	Value (pg/L)
A	2.764	0
B	2.250	2
C	1.668	4
D	0.979	8
E	0.444	16
F	0.242	40
Unknown	1.450	4.8

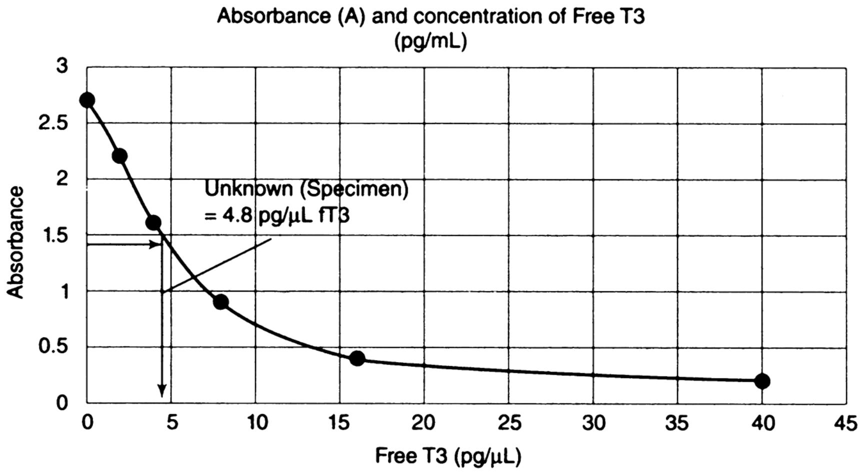


FIGURE 33.7 *Calibrator curve*

Total Triiodothyronine (T3)

The thyroid hormones are a major component of the endocrine system. They circulate in blood in the forms of T3 (triiodothyronine) and T4 (thyroxine). They are ordered to diagnose thyroid disorder. These hormones are mostly bound to the plasma protein, thyroxine binding globulin (TBG) but a small portion is also free or not bound. Blood tests can measure total T4 (unbound plus bound), free T4, total T3 (bound plus unbound), or free T3. Most professional guidelines, however, recommend total and free T3 along with T4 for evaluating thyroid function.

Clinical significance

A free or total T3 is used to assess thyroid function. If it is ordered in conjunction with T4 it becomes especially valuable to distinguish euthyroid and hyperthyroid subjects.

Normal range

Total Triiodothyronine (T3): 80–220 ng/dL
Free Triiodothyronine (fT3): 260–480 pg/d

Specimen

Collect blood by routine laboratory procedure. Avoid grossly haemolytic, lipaemic, or turbid samples. Plasma samples are collected in tubes containing anticoagulant (follow instructions of the kit manufacturer). Specimens are stored up to 48 h at 2–8°C prior to assaying. Specimens held for a longer time (up to 6 months) should be frozen only once at –20°C prior to assay. Thawed samples should be inverted several times prior to testing.

Laboratory assay

Principle

Most of the kits are based on the principle of ELA (Enzyme-linked immunoassay) for the determination of T3. The manufacturer supplies anti-body (IgG) coated on the solid surface (in micro-wells) which is used to capture the antigen (T3) present in the specimen along with its competitor T3-antigen which is conjugated with horseradish peroxides (tagged). The antibody sites compete with these antigens—tagged and untagged. Finally, when colour reaction is set-up to interact with the tagged antibody the amount of analyte will be known from its inverse relationship. For example, if T3 is high tagged then it will bind less. At the end, after repeated washing, the colour producing tagged antibody (with peroxidase) gives a colour reaction with tetramethylbenzidine (TMB).

Reagents provided in the kit (contents may vary with kits)

1. Antibody-coated solid phase
2. T3-conjugate with peroxidase
3. Reference standard solution
4. Tetramethylbenzidine (TMB)
5. Stop solution (IN HCl)

Materials required but not provided in the kit

1. Distilled or deionized water
2. Precision micropipettes
3. Disposable pipette tips
4. Spectrophotometer to read absorbance at 450 nm
5. Quality control material

Warning and precautions

1. Treat all chemicals as poison and all specimens as potential biohazard.
2. Do not use kits after their expiration date.
3. Replace caps on reagents immediately. Do not switch caps.
4. Do not pipette reagents by mouth.

Storage conditions

- Store kits at 2–8°C upon receipt. Refer to the package label for the expiration date.
- Keep microtitre plate in a sealed bag with desiccant to minimize exposure to damp air.

Reagent preparation

1. All reagents should be reconstituted according to manufacturer's instructions.
2. Store in refrigerator when not in use and allow it to reach room temperature (18–25°C) before use.
3. Prepare the working T3-HRPO conjugate reagent before use. The amount of conjugated diluted depends on the assay size. The working conjugate reagent is stable in the refrigerator (4°C) for at least 24 h.

Instrumentation

For reading the colour absorbance (A) or optical density, the microscope must have a proper reader that can read 450 nm with a bandwidth of 10 nm or less.

Procedure

Follow the procedure carefully as given in the insert.

1. Pipette standards and specimens (antigens). These will react with the antibody on the surface.
2. Dispense T3 antibody reagent. Mix thoroughly. These will react with the antigens held on the surface.
3. Add working conjugate reagent into each well. Mix thoroughly.
4. Incubate at room temperature (18–25°C) for 60 min.
5. Remove the incubation mixture by flicking plate contents into a waste container.
6. Rinse and flick the wells several times.
7. Strike the wells sharply onto absorbent paper or paper towels to remove all residual water droplets.
8. Dispense TMB reagent into each well. Gently mix for few seconds.
9. Incubate at room temperature in dark for a defined period.
10. Stop the reaction by adding stop solution (HCl) to each well.
11. Gently mix for 30 s. Ensure that the entire blue colour changes completely to yellow.
12. Read absorbance at 450 nm with a microtitre plate reader within 15 min.

Calculation

- Calculate the mean absorbance value (A_{450} or OD_{450}) from the duplicate set of reference standards, controls and samples.
- Construct a standard curve by plotting the mean absorbance obtained for each reference standard against its concentration in ng/mL on linear graph paper, with absorbance on the vertical (Y) axis and concentration on the horizontal (X) axis.
- Using the mean absorbance value for each sample, determine the corresponding concentration of T3 in ng/mL from the standard curve. Depending on experience and/or the availability of computer capability, other methods of data reduction may be employed.
- Any diluted samples must be further corrected by the appropriate dilution factor.

Example of a standard curve

Results of a typical standard run with optical density readings at 450 nm shown on the Y-axis against total T3 concentrations (ng/mL) shown on the X-axis, are presented below (Figure 33.8). ***Note*** The standard curve is for illustration only and should not be used to calculate unknowns. Each laboratory must provide its own data and standard curve for each assay run. Additionally, the absorbance (450 nm) values can be varied due to incubation at different room temperature in different laboratories.

Total T3 (ng/mL)	Absorbance (450 nm)
0.0	2.685
0.75	2.381
1.5	2.028
3.0	1.502
6.0	0.992
10.0	0.518

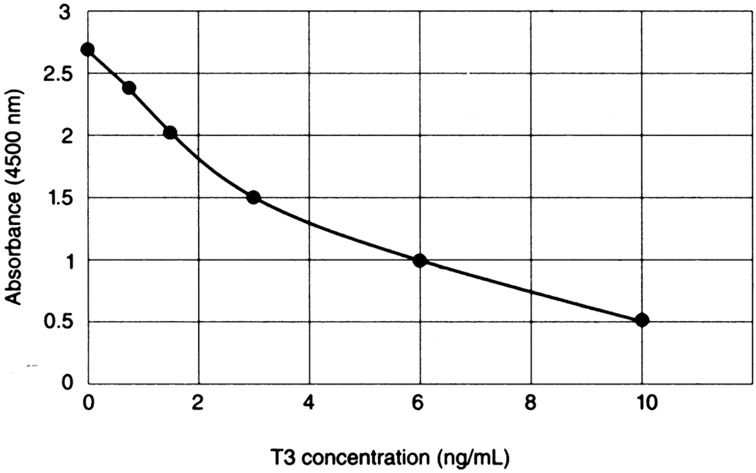


Figure 33.8 *A typical standard run*

Free T4 (Thyroxine)

Thyroxine (T4) and **triiodothyronine (T3)** are produced by the thyroid gland. They are important components of the endocrine system. T4 is found both in free and bound forms.

Clinical significance

Free thyroxine (free T4) tests are used to help evaluate thyroid function and diagnose thyroid diseases. They are requested along with the thyroid stimulating hormone or TSH.

Laboratory assay

Free T4 (Thyroxine) ELISA Assay kits are available in the market. The following account is from one such kit.

Principle

The solid surface holds the monoclonal anti-thyroxin antibodies. When sample is laid on it along with the conjugated-antigen, they compete for the binding site. As a result, if T4 is less, more conjugated-T4 will bind on the site. Following washing, and colour reaction (peroxidase), one can determine the amount of T4—more is the colour, less is the amount of T4 in the sample. This is the "one step immunoassay".

In the **first incubation,** a **competition between horseradish peroxidase-Iabelled thyroxin (conjugated) and free thyroxin of serum sample is set up.** Here both compete for the sites of immobilized antibody on the solid surface.

In the **second incubation** (after wash), a substrate is added to react with horseradish peroxidase and converts the **colourless substrate solution of 3,3′,5,5′-tetramethylbenzidine (TMB) added into a blue product.**

Finally, the enzyme reaction is stopped by dispensing an acidic solution (HCl). Care is taken to fully complete the colour reaction—turning the solution from blue to yellow. The optical density (OD) or absorbance of the solution at 450 nm is indirectly proportional to the amount of thyroxin. The standard curve is established by plotting the concentrations of the standards (X-axis) and their corresponding OD values (Y-axis) measured. The concentration of the specimen is directly read off the standard curve.

Specimen

- Blood is collected by routine procedure of venepuncture. After clotting, the serum is separated by centrifugation. **Do not use plasma, haemolyzed or lipaemic sera.**
- Fresh specimen is recommended. Specimen can be stored in the refrigerator for 2 days; for extended storage, put in the freezer at –20°C. Repeated freezing and thawing should be avoided.
- Prior to assay, allow the samples to reach room temperature. Take care to agitate serum samples gently in order to ensure homogeneity.
- If the specimen has particles, centrifuge and use the supernatant.

Materials provided with the kit

- Anti-T4 coated solid phase
- T4 reference standards: 2–25 µg/dL, ready to use.
- Enzyme conjugate
- TMB reagent
- Stop solution (1N HCl)

Precautions

- All reagents should be allowed to reach room temperature (18–25°C) before use.
- Prepare only the amount of conjugate that is required each time.
- Working conjugate reagent should be used within 24 h.
- Discard the excess after use.

Materials required but not provided with the kit

- Precision pipettes: 25 µL, 100 µL and 1.0 mL.
- Deionized distilled water.
- Spectrophotometer for 450 nm and 620 nm or 690 nm.

Procedure

1. Bring all reconstituted reagents to room temperature (20–25°C).
2. Follow instructions of the manufacturer.

Data Processing: Prepare a standard curve with reagents supplied by the manufacturer.

Typical Example

A typical standard curve is presented here (Figure 33.9).

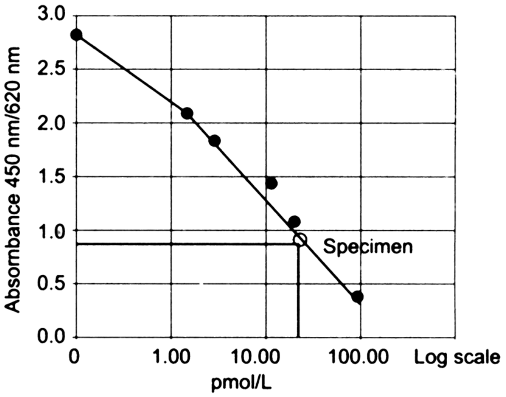


Figure 33.9 *Standard curve*

Sample	Mean OD (450 nm)	Thyroxine pmol/L
Calibrator 0	2.7	0.1
Calibrator 1	2.27	3.4
Calibrator 2	1.9	6.3
Calibrator 3	1.5	12.5
Calibrator 4	1.1	26
Calibrator 5	0.4	100
Specimen	0.9	29.7

Note Concentrations of calibrators 0–5 may vary from lot-to-lot.

Standard curve: Any extrapolation of the standard curve to free T4 concentration above the nominal value of calibrator 5 is not permitted.

Reference Values

The laboratory may collect reference values from healthy subjects. This will minimize errors.

Conversion factors

pmol/L × 0.78 = pg/mL
pg/mL × 1.28 = pmol/L

Safety Precautions

- Read the instructions from manufacturer very carefully.
- Do not use expired kits.
- Do not use or mix reagents from different kits or lots.
- Avoid time shift during pipetting of reagents.
- All reagents should be stored according to manufacturer's specification.
- All reagents must be considered as poisonous and the specimen biohazardous.
- Do not contaminate the reagent.

Total T4 (Thyroxine)

L-Thyroxine (T4) is the main hormone of the endocrine system that is **synthesized and stored in the thyroid gland.** It provides energy needed for growth and development. Most of the T4 (99%) is reversibly bound to plasma proteins in blood. Approximately 0.03% of T4 is in the free, unbound state in blood at any one time.

Clinical significance

Determination of total T4 is the most reliable and convenient screening test available to determine the presence of thyroid disorders in patients. Increased levels of T4 have been found in hyperthyroidism and in acute thyroiditis. Low levels of T4 have been associated with congenital hypothyroidism.

Normal value

A typical normal range is 4.5–11.2 mcg/dL.

Specimen and its preparation

Collect blood specimens by routine venepuncture and separate the serum immediately. Specimens may be stored refrigerated at (2–8°C) for 5 days. If storage time exceeds 5 days, store frozen at (–20°C) for up to one month.

Laboratory assay

Use of ELISA kit in determining total thyroxine T4 is most widely accepted laboratory procedure. Call reputable local vendors of laboratory assays.

Principle: It is same as described in T3 and T4 assays.

Storage: Store the kits according to manufacturer's specification.

Reagents (provided in kit)

- Sheep anti-T4 coated microtitre wells, 96 wells
- T4 reference standards
- Enzyme conjugate concentrate
- Enzyme conjugate diluent
- TMB reagent (one-step)
- Stop solution (1N HCl)

Materials required but not provided in the kits

- Precision pipettes
- Common laboratory supplies
- Disposable pipette tips
- Spectrophotometer

Procedure

Note

- Before proceeding with the assay, bring all reagents to room temperature (18–25°C).
- Follow instructions from the manufacturer.
- Do not start the assay without first comprehending the complete procedure. Secure the desired number of coated wells in the holder.
- Calculate the average absorbance values (A450) for each set of reference standards, control, and samples.
- Construct a standard curve by plotting the mean absorbance obtained for each reference standard against its concentration in µg/dL on linear graph paper, with absorbance on the vertical (Y) axis and concentration on the horizontal (X) axis.
- Using the mean absorbance value for each sample, determine the corresponding concentration of T4 in µg/dL from the standard curve.
- Each laboratory must provide its own data and standard curve in each experiment (Figure 33.10).

T4 (µg/dL)	Absorbance (450 nm)
0	2.667
2	1.786
5	1.060
10	0.778
15	0.591
25	0.384

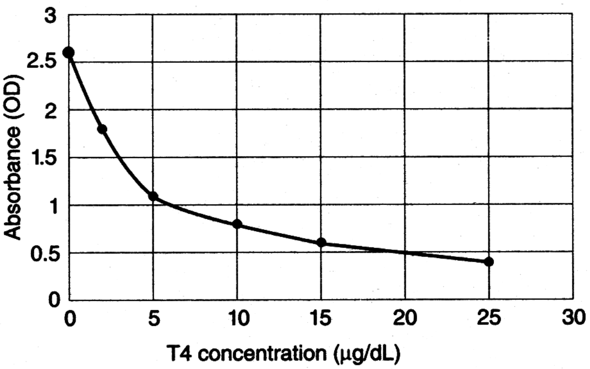


Figure 33.10 *Standard curve*

Results of a typical standard run with optical density readings at 450 nm shown in the Y axis against T4 concentrations shown in the X axis (see the attached curve).

Note This standard curve is for the purpose of illustration only, and should not be used to calculate unknowns. Each laboratory must provide its own data and standard curve in each experiment.

Thyroid Stimulating Hormone (TSH)

Thyroid Stimulating Hormone (TSH) is a glycoprotein hormone secreted by the pituitary gland and regulates the synthesis/release of T3 and T4 by thyroid gland.

Clinical significance

TSH measurement helps in diagnosing thyroid disorder. Its increase or decrease reflects hyperthyroidism and hypothyroidism respectively.

Normal range

Each laboratory should establish its own normal values from pooled specimens.
Adults: 0.4–4.2 µIU/mL
Newborn (1–4 days): 1.0–39 µIU/mL
Six months – 20 years: 0.7–6.4 µIU/mL

Specimen and its preparation

1. Collect blood specimens and separate the serum immediately.
2. Specimens may be stored in a refrigerator for 5 days or in a freezer (–20°C) for one month.
3. Prior to assay, frozen sera should be completely thawed and mixed well.
4. Do not use grossly lipaemic specimens.

Laboratory assay

ELISA is the standard assay procedure for TSH. The method is simple with good reproducibility of results.

Reagents and things provided in the kit

- TSH-coated solid phase
- Conjugate reagent
- Substrate
- Stop solution
- Wash concentrate

Reagents and Equipment Required but Not Provided with the kit

1. Distilled or deionized water
2. Precision pipettes
3. ELISA reader capable of reading absorbance at 450 nm.

Caution: All reagents should be considered as poison and the specimens as potential biohazard.

Storage: Store the kit in a refrigerator. Follow manufacturer's instructions.

Special Notes

- The components in the kit are intended for use as an integral unit. The components of different lots should not be mixed.
- It is recommended that standards, control and serum samples be run in duplicate.
- Prior to assay, allow reagents to stand at room temperature (18–26°C). Gently mix all reagents before use.

Principle

TSH is measured by the **solid phase sandwich ELISA method.** TSH in the sample forms a sandwich between two specific antibodies to TSH. Other materials are washed off. Upon the addition of the substrate, the intensity of colour is proportional to the concentration of TSH in the samples. A standard curve is prepared relating colour intensity to the concentration of the TSH.

Procedure

Follow manufacturer's instructions given inside the kit.

1. Pipette 50 μL of TSH standards, control, and specimens into designated wells.
2. Read absorbance at 450 nm within specified time.

Calculations

The standard curve is constructed as follows (Figure 33.11):

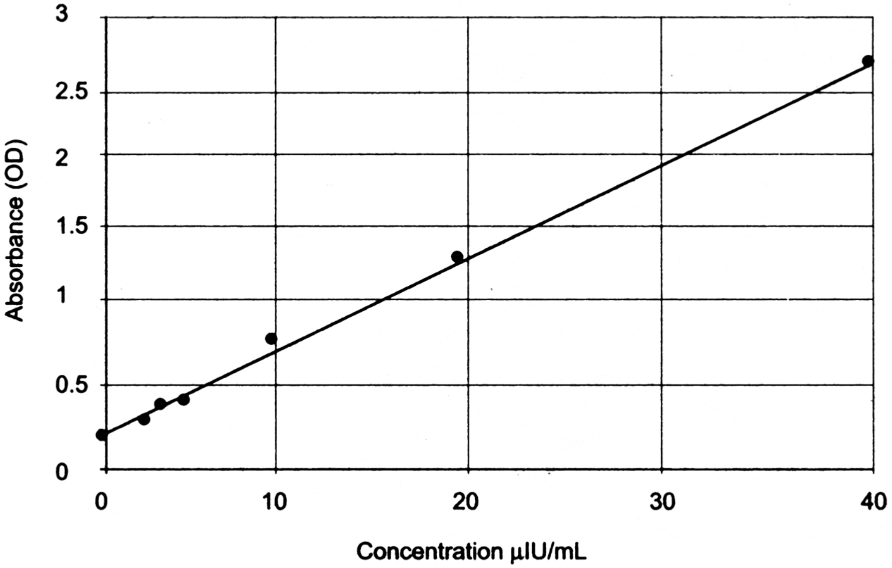


FIGURE 33.11 *Standard Curve*

	OD (450 nm)	Concentration (µIU/mL)
Std 1	0.033	0
Std 2	0.062	0.5
Std 3	0.21	2.5
Std 4	0.41	5
Std 5	0.75	10
Std 6	1.37	20
Std 7	2.61	40

ELECTROLYTES

Routine blood screening often includes an order for electrolytes. **Electrolytesare substances that ionize in solution** and bear a positive or negative charge and conduct electricity. Acids, bases and salts are electrolytes. Electrolyte imbalance in the body or disturbance in homeostasis can bring about serious consequences. Electrolytes enter the body through diet and leave the body primarily through kidney, although other routes are also important such as sweat and the gastrointestinal tract. **Hormones, secreted by the endocrine glands, control the absorption of electrolytes** by the renal tubules and thus play a vital role in maintaining homeostasis. Some of the electrolytes also form important constituents of the body structure (calcium and phosphorus) while others act as catalysts or form a part of some enzymes (magnesium, zinc and copper). Electrolytes include several minerals which are essential for normal body function. Most commonly requested tests for electrolytes include **sodium, potassium, chloride, phosphorus and bicarbonate HCO_3^-**. Additional tests may include magnesium and calcium under special conditions.

Sodium and Potassium

Sodium is the major **extracellularcation** (Na^+) of the body. Sodium salts are necessary to preserve a balance between calcium and potassium to maintain normal heart action and equilibrium of the body. Sodium salts regulate the osmotic pressure in the cells and fluids and guard against an excessive loss of water from the tissues. Almost all the blood sodium is found in the plasma; there is very little in the red cells. Conditions in which extracellular fluid is lost (e.g., vomiting, diarrhoea), lead to a salt deficiency and hence to low plasma sodium values which may be exaggerated if water losses are replaced without adequate salt therapy. A nonspecific fall in plasma sodium values also occurs in many chronic diseases.

Potassium, unlike sodium, is the major **intracellular cation** of the body. It is high inside red blood cells and hence, haemolyzed blood is inappropriate for potassium analysis. In addition, the serum should be promptly separated, or it may yield a false, elevated value. Potassium in conjunction with sodium and chloride, aids in the regulation of osmotic pressure and acid–base balance. A proper balance of potassium, calcium and magnesium is accessory for the normal function of heart and muscle tissues. Potassium is also known to play a significant role in the conduction of nerve impulses.

Clinical significance

Low values are found in excessive use of diuretics and profuse loss in diarrhoea. Insulin causes inward shift of glucose and potassium and used in very high level of potassium in conjunction with intravenous glucose water. Reduced plasma potassium concentration often causes electrolyte shifts with persistent alkalosis which is restored to normal by potassium replacement.

Electrolyte assays may provide information to the physician about the **acid-base balance of the body,** causes for oedema (oedema), or additional information about the status of kidney dysfunction. Electrolyte levels may also be altered by certain drugs, dehydration or over hydration. The clinical condition that may very likely bring about electrolyte imbalance in the body is fluid loss through diarrhoea, vomiting and sweating. **Disturbance in endocrine function is one of the most important causes of electrolyte imbalance.** This is particularly true for sodium and potassium. Analysis of electrolytes is a stat (emergency) procedure. Severe drift from the homeostatic level of electrolytes may result in disordered cardiac rhythm and cardiac arrest or other serious complications.

Normal range

The reference ranges for normal individual for any analyte may vary according to the method of testing. Hence each laboratory must establish its own range with normal specimen.

Sodium: 135–145 mmol/L
Potassium: 3.5–5.0 mmol/L
Chloride: 98–105 mmol/L
Bicarbonate: 22–30 mmol/L

Specimen

Specimen collection for electrolyte testing does not require any specific preparation, but it is important to collect the specimen using appropriate phlebotomy technique to avoid erroneous results. Hospital and reference laboratories call for **plasma or serum specimens.** In case of plasma, **lithium heparin is used as an anticoagulant** (not sodium heparin). In case of serum specimen, the clot must be separated within one hour of collection. Electrolyte results can easily be affected by poor pre-analytical procedures. Mild haemolysis does not affect the results of sodium but excessive haemolysis will yield false low value of sodium and chloride (dilution error). Red cells are high in potassium. Hence haemolysis will result in false high value of potassium. Specimen for potassium determination should be kept at room temperature and assayed promptly. Contamination of the specimen with IV fluids (which patient was receiving at the time of specimen collection) will also alter the plasma electrolyte results. Because of the emergency situation and stability in findings, peripheral laboratories are often not involved in the determinations of sodium and potassium.

Laboratory assay

Ion selective electrode

Use of **ion selective electrode** (ISE) is the most common way to determine sodium and potassium. In recent years, stat electrolyte analyzers have considerably helped the physicians to make the diagnosis on the spot using venepuncture. In the following pages we will describe the electrolyte testing using Abbott i-STAT chemistry analyzer.

Procedure

1. Wash hands and apply gloves.
2. Verify the test ordered and examine the specimen.
3. Assemble all necessary equipment and verify all the reagents are within their expiration dates. For an electrolyte panel to be performed, it may be necessary to use a cartridge that tests for other analytes in addition to those in the panel requested. This is due to cartridge packaging.
4. Turn on the analyzer. If necessary, use the electronic simulator cartridge to verify that the instrument is operating correctly.
5. Prior to performance of test, verify whether a quality control (QC) specimen needs to be tested, and if so, complete that QC test before patient's test is performed. It is recommended that two levels of QC be tested every time QC is run.

6. Remove the cartridge from the foil pouch and place it flat on a clean surface until use. Always handle the cartridge by the edge.
7. If the technician has to collect the specimen, perform capillary puncture, using appropriate technique. Plain capillary may be used if the test is done immediately.
8. Wipe away the first drop of blood which may be contaminated with interstitial fluids, resulting in an erroneous result.
9. Hold the end of the filled capillary tube to the sample well. The well will fill with blood through capillary action. Fill the well to the indicated fill mark. (The specimen may also be taken from a lithium heparin blood tube or sterile syringe without additives).
10. The analyzer will request an operator ID and the specimen ID number. Enter these according to laboratory policy.
11. After the analysis has completed, the AutoAnalyzer will display the results on the LED screen for 45 s.
12. Take a note of it in case the results are not automatically recorded. Directly record on patient's log sheet and transferred to patient's request form when ready.
13. Remove the used cartridge from the instrument and discard it as biohazardous trash."
14. Turn off the instrument or prepare to run the next specimen.
15. Dispose of the capillary puncture device or used venepuncture supplies in a sharp container immediately after use. Any other equipment or supply that may be contaminated with blood must be disposed of as a biohazard.
16. Disinfect the work area.
17. Remove gloves and sanitize hands.

Calcium

Calcium and inorganic phosphate are the major constituents of bone and their relationship, however, is reciprocal. Calcium is also the primary constituent of teeth and plays many other roles like helps nerves to work, muscles to squeeze together, blood clot, and pumping of the heart.

The level of calcium in blood is delicately controlled. In case of hypocalcemia when calcium level of body falls, the bones release calcium to bring it back to a good blood level. When the calcium level is high (hypercA1cemia), the extra calcium is stored in the bone or passed out of the body through urine or stool. Calcium of the body is largely maintained by vitamin D and specific hormones. The blood level of phosphate is closely linked to calcium level of the blood and they work in opposite directions. If the blood calcium increases, the phosphate levels get low and vice versa.

Calcium level of the body is tightly controlled (8.4–11.4 mg/dL) by various hormones and vitamin D. Causes of low calcium can be related to chronic kidney failure, vitamin D deficiency and low blood magnesium which can occur in severe alcoholism.

Clinical significance

The calcium blood test measures the level of calcium in the blood that is free, unattached to protein. The value of calcium level in blood can diagnose parathyroid disease and helps in its treatment. The measurement is also used in a variety of bone diseases, chronic renal failure and tetany. Increased serum calcium levels are associated with primary hyperparathyroidism, multiple myeloma, metastatic bone lesions and hypervitaminosis D. Hypocalcaemia is associated with hypoparathyroidism, nephrotic syndrome, rickets and renal failure.

Normal range

8.5–10.4 mg/dL or 2.2–2.7 mmol Ca/L

Specimen

Serum is obtained by routine venepuncture. Let the blood clot at room temperature for 10 min, spin and separate the serum. This is preferred over plasma. Avoid haemolyzed and lipaemic serum. If unavoidable, use a serum blank. Samples can be assayed without any prior treatment. Calcium in serum is stable for 12 h at room temperature, one week in refrigerator and for a longer period frozen (–20°C) up to 3 months.

Laboratory assay

Calcium can be determined by different methods—titrimetric, colorimetric, fluorometric or by atomic absorption. The method best suitable for the laboratories of developing countries is **colorimetric method,** which is described here. Various chromogenic complexes are used by the manufacturer. Stick carefully to the instruction provided in the kit. Most kits use *o*-cresolphthalein whose absorbance is read at 575 nm. The cresolphthalein complex one method is highly sensitive which sometimes makes the method in adaptable to the conditions existing in developing countries. The use of calcium-free glassware and water are important. We have a commercial kit to describe the procedure. It may vary with different manufacturer. Always follow the instructions laid by the manufacturer and inserted in the kit.

Principle

The metal-complexing dye ***o*-cresolphthalein complexone** (CPC) forms a red complex with calcium in alkaline solution:

$$Ca^{++} + o\text{-CPC} \rightarrow Ca^{++} - o\text{-CPC (red)}$$

In some procedures, HCl is included which helps to release calcium bound to proteins. In subsequent steps, **8-hydroxy-quinoline** eliminates the interference by magnesium. 2-amino-2-methyl-l-propanol (AMP) provides the proper alkaline medium for the colour reaction. The intensity of the colour is measured at 540 nm/yellow green filter.

Method using micro-well plate

The manufacturer may provide micro-well plate otherwise make necessary arrangement to perform the test. It, however, must yield a reliable standard curve. Microplates have the advantage of using small amount of reagents and incubation is easy, but at the same time, the laboratory must have the optical reader and other arrangements that go with it.

Reagents

All necessary reagents are provided in the kit.

- Calcium assay buffer
- Chromogenic reagent
- Calcium standard

Things required but not supplied in the kit

- Spectrophotometer as plate reader (540 nm/yellow green filter)
- Common laboratory supplies
- Timer

Reagent Storage Conditions

- Store the kit in the refrigerator when not in use.
- Reconstitute as per manufacturer's direction.

- Warm to room temperature before use.
- Protect from light.

Procedure

Standard curve preparations: Follow instructions from the manufacturer or modify to suit your condition.

Here is an example:

10 µL (500 mM) + 990 µL = 1000 µL
Dilution = 100×

Concentration of diluted standard = 5 mM (20 mg/dL)

Amount of Standard (µL)	0	2	4	6	8	10
Amount of Calcium (µg Ca)	0	0.4	0.8	1.2	1.6	2.0
Amount of Water (µL)	50	48	46	44	42	40
Concentration of Standard (mg/dL)	0	4	8	12	16	20

1. Prepare a series of dilutions of standard.
2. Perform the test in accordance with manufacturer's direction.
3. Incubate as per instructions.
4. Protect from light.
5. Measure the absorbance at 575 nm. The chromophore is unstable and will fade slightly over time, so read the standard and samples within 30 min.

Calculations

- Correct background by subtracting the value derived from the 0 Calcium control from all sample and standard readings (***Note*** The background reading may be significant and must be subtracted from sample readings).
- Plot standard curve µg/well vs absorbance 575 nm readings.
- Then apply the sample readings to the standard curve to get calcium concentration. Calcium concentration in your sample can be expressed as mg/mL, mg/dL or mM (mmol/liter). 1 mg/mL = 100 mg/dL; 1 mM = 4 mg/dL.

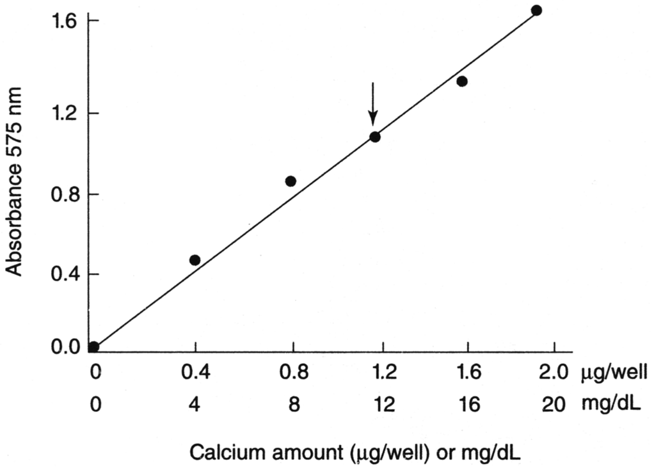


FIGURE 33.12 *A typical standard curve*

Alternatively, apply the formula:

$$C_T\,(\text{mg/dL}) = A_T/A_S \times C_S$$

Where,

C_T = Concentration of calcium in specimen (test) in mg/dL
A_T = Absorbance reading of specimen (test)
A_S = Absorbance reading of standard
C_S = Concentration of standard

It is recommended to compare absorbance of specimen against the absorbance of standard at 12 mg/dL which is close to normal range. Then the above formula reads as follows:

$$C_T\,(\text{mg/dL}) = A_T/A_S \times 12$$

Test tube method

Classical test tubes are still in use. These, however, need more reagents and specimen. It is described here for the laboratories where microplate or microplate readers are not available. In such case the authors recommend that the reagent is made inside the laboratory in order to make the procedure cost effective.

Reagents

All chemicals must be pure grade.

- *Concentrated hydrochloric acid (HCl)* Concentrated hydrochloric acid is usually 12 N. See the bottle for actual strength. Dilute with distilled water to make 6 N strength.
- *AMP Buffer pH 10.7:* Follow instructions from the manufacturer. Store the buffer solution in refrigerator in a brown coloured glass bottle. Label with date. This is stable for 3 weeks.
- *o-Cresolphthalein complexone solution:* Follow manufacturer's instructions in preparing the colour reagent.
- *Stock Calcium standard 50 mg/dL:* Provided by the manufacturer. Dilute when needed.
- *Working standards:* Follow instructions from manufacturer. An example has been presented.

Note Use 10 mg/dL as single working standard.

Equipment and supplies

- Spectrophotometer or filter photometer (540 nm/yellow green filter)
- Common laboratory supplies of volumetric glassware. All glassware used in this method must be scrupulously cleaned, soaked overnight in 3% (V/V) HCl to remove traces of calcium, thoroughly rinsed with distilled water and dried before use.
- Test tubes (18 × 150 mm) and test tube racks
- Timer

Procedure

1. Mark three test tubes as 'B', 'S' and 'T', representing blank, standard and test respectively.
2. Follow instructions from the manufacturer for calcium assay.
3. Set the spectrophotometer/filter photometer to zero using blank at 540 nm/yellow green filter.
4. Measure the absorbance of standards (A_S) and test (A_T).

Calculation

The following calculation is valid only when the standard curve is linear. Check the linearity once a month. If a new reagent is made, recheck the standard curve. If the linearity is good, one can apply the formula:

$$\text{Serum calcium concentration (mg/dL)} = A_T/A_S \times 10\ (\text{mg/dL})$$

Quality control serum with its corresponding calcium concentration is commercially available.

Chloride

Chloride is the **major extracellular anion of the body.** It is needed for the proper functioning of many critical aspects of metabolism, including aiding in maintenance of the body's **acid—base balance.** In Cystic Fibrosis, mutations to the CF transmembrane conductance regulator (CFTCR) gene result in altered sodium and chloride ion transport channels. Blood serum contains 99–108 mEq/dL or 351–383 mg/dL, principally as sodium chloride. Its primary role in the body is to maintain proper water distribution, osmotic pressure, and normal anion–cation balance in the external fluid (plasma). A decrease in plasma chloride occurs in conditions where there is low plasma sodium or when the bicarbonate is raised as in metabolic alkalosis. Chloride concentration of red cells is lower than that of whole blood. The chloride ion is ingested through the food and filtered or re-absorbed by the kidney according to the body's need. Chloride, to some extent, may be lost through sweating. Prolonged vomiting, from any cause, may result in a significant loss of chloride and ultimately a decrease in serum and body chloride.

Clinical significance

Loss of serum chloride is associated with nephritis, diabetic acidosis or excessive fluid loss. Increased chloride concentration of serum is seen in case of congestive heart failure and decreased renal blood flow. Estimation of chloride are of value as a check on the accuracy of the other plasma electrolytes, in case of electrolyte imbalance (disturbance in homeostasis), since the sum of sodium and potassium (in mEq/L) is usually 20 more than the sum of chloride and bicarbonate.

Normal range

Serum: 98–106 mmole/L (or mEq/L)
Sweat: 5–35 mmole/L (or mEq/L)
Urine: 110–250 mmole/day (varies with chloride intake)

Note These values should only be taken as guidelines. It is recommended that each laboratory should establish its own range of expected values by pooling normal sera. This eliminates differences between instruments, laboratories and local population.

Specimen

Serum is separated from the blood clot soon after drawing. **Grossly haemolyzed serum should not be used** as it may create false decreased values. Avoid contamination of blood with tissue fluid. Store the serum in tightly capped tubes. Chloride is stable in serum for one day at room temperature, up to one week in refrigerator and for three months if frozen. The method described here does not require protein free filtrate to determine serum chloride concentration.

Laboratory assay

Chloride assay by **colorimetric method is described here** although there are several other methodologies available for measuring chloride including **ion selective electrode** (ISE) which is most commonly used by reference laboratories. Colorimetric assay of chloride is most suitable to peripheral laboratories of developing countries. The kits are now available and it is easy to follow.

Principle

The chloride assay kit provides a simple and direct procedure for measuring chloride in a variety of samples, including blood and urine. Chloride ions form a soluble non-ionized compound with mercuric ions and displace thiocyanate ions from non-ionized mercuric thiocyanate. The released thiocyanate ions react with ferric ions to form a colour complex. This colour complex absorbs optimal light at 480 nm. Thus, intensity of colour is directly proportional to the chloride concentration in the medium.

$$Hg(SCN)_2 + 2Cl^- \rightarrow Hg^{2+}Cl_2 + 2,4,6\text{-TPTZ} + Fe^{2+} \rightarrow \mathbf{4\ FeTPTZ\ (red\ complex)}$$

Micro-well procedure

This is now more popular than test tube procedure. Disposable nature of the test tube and use of small quantities of reagents and specimen make it easily adoptable. Many manufacturers supply the flat bottom multi-well plates otherwise laboratory has to procure them. We have picked here a typical kit for describing the procedure but the technician must follow the instruction provided by the manufacturer with the kit.

Reagents

Chloride Reagent	15 mL
Chloride Standard	1 vial

Reagents and Equipment Required but Not Provided with the Kit

- 96-well flat-bottom plate. It is recommended to use clear plates for colorimetric assays.
- Spectrophotometric multi-well plate reader set at 620 nm
- Automatic pipettor
- Standard laboratory glassware

Precautions

This kit contains small amounts of mercury. Waste generated from using this kit should be disposed of properly.

Preparation Instructions

- Briefly centrifuge vials before opening. Use ultrapure (deionized-distilled) water for the preparation of reagents.
- Chloride reagent
- Chloride standard: Reconstitute according to manufacturer's direction (10 mM standard). Mix well by pipetting, then aliquot and store, protected from light, at room temperature.

Storage/Stability: The kit is shipped and storage at room temperature, protected from light, is recommended.

Specimen Preparation

Dilute the specimens—urine or serum, in accordance with manufacturer's direction.

Procedure for preparing standard curve

1. Prepare a series of chloride standards for colorimetric assay.
2. Add 0,2,4, 6, 8, and 10 uL of the 10 mM standard solution in different test tubes. These correspond to following standards—0 (blank), 20, 40, 60, 80, and 100 nmole/tube.
3. Add water to each tube to bring the volume to 50 µL (as indicated in the table).

µL of standard solution (10 mM) added	0	2	4	6	8	10
Water added to make a total volume of 50 µL	50	48	46	44	42	0
Final amount of standard in the well (nmole/well)	0	20	40	60	80	100

Procedure

1. Add 150 uL of the chloride reagent to each tube. Mix well using a horizontal shaker or by capillary.
2. Incubate the reaction for 15 min at room temperature. Cover the tube and protect from light during the incubation.
3. Measure absorbance at 620 nm (A_{620}) or 480 nm in accordance with the direction of the kit.

Calculations

1. The background for assay is the value obtained for the 0 (blank) chloride standard. Correct for the background by subtracting the blank value from all readings.
2. Use the values obtained from the appropriate chloride standards to plot a standard curve. ***Note*** A new standard curve must be set up each time the assay is run.
3. Using the corrected measurement, the amount of chloride present in the samples may be determined from the standard curve. ***Note*** There is slight nonlinearity below 20 nmoles of chloride. Samples falling below 20 nmoles should be repeated with 3 to 5-fold higher sample.

Determination of concentration of chloride from standard curve

$$C\ (\text{nmole}/\mu\text{L}, \mu\text{mole/mL, or mM}) = S_a/S_v \times D$$

Where,

C = Concentration of chloride in sample
S_a = Amount of chloride in unknown sample (nmole) from standard curve
S_v = Sample volume (µL) added to reaction well
D = Dilution of original sample

Amount of chloride in specimen sample (S_a) = 45.8 nmole (from standard curve)
Sample volume (S_v) = 50 µL
Concentration of chloride in sample 45.8 nmole/50 µL = 0.916 nmole/µL
C = Concentration of chloride in sample expressed in different units
0.916 nmole/µL x 35.5 ng/nmole = 32.52 ng/µL

Determination of concentration of chloride by formula method

As the standard curve is basically linear, formula method for the determination of chloride concentration can make the job easy. All absorbance readings, however, should be against blank. The instrument can be zeroed with the blank. Alternatively, the absorbance value of the blank should be subtracted from the absorbance readings of test and standard.

Absorbance of the standard, which is close to the normal range, i.e., 350 mg/dL (100 mEq/dL) is chosen here. Absorbance of the test specimen (T) varies proportionately. This is reflected in the following formula:

$$C_T\ (\text{mEq/dL}) = A_T/A_S \times 100$$

Where,

C_T (mEq/dL) = Concentration of chloride in test specimen
A_T = Absorbance of test specimen
A_S = Absorbance of chosen standard (100 mEq/dL)
100 = Concentration of standard (C_S)

Additional Note

- If the chloride concentration of the specimen is higher than 140 mEq/L, dilute the sample with distilled water, rerun the assay and multiply with the dilution factor.
- Control sera of known concentrations of chloride should be analyzed routinely with each group of unknown samples.
- Hydrochloric acid fumes and fumes from chloride bleach may cause high results.
- Bromide and fluoride can cause falsely elevated chloride values.
- Lipaemic and/or icteric sera do not interfere in the reaction.

ACID–BASE BALANCE AND BLOOD GASES

Acid–base and electrolyte balance govern the **homeostatic** mechanism of the body. Two organs are closely associated with this balance—lungs and kidney. They communicate through the mediation of hormones. The acid–base balance is finally reflected by the pH, which is delicately maintained at pH 7.4 in blood. These biochemical information, obtained through blood analysis, are used to assess patients in life-threatening situations (Table 33.3). Although the technicians working in peripheral laboratories are rarely involved with the analyses of blood gases and the parameters of acid–base balance, but a physiological knowledge of body's complicated balancing act help them to understand the crucial role played by the laboratories. This section is devoted towards the understanding of acid–base physiology and the way the laboratories interact with the attending physicians. Before we pitch into this complicated discussion, we need to require a review of several basic concepts: acid–base, buffer, pH, pK, the principles of equilibrium and the law of mass action.

TABLE 33.3 *Laboratory findings in acidosis and alkalosis*

Clinical Condition	Bicarbonate	PCO_2	pH	PO_2	Cause
Metabolic acidosis	Fall	Fall*	Fall	Normal	Excessive discharge of urinary bicarb. High anion gap.
Metabolic alkalosis	Rise	Rise	Rise	Rise	Poor discharge of urinary bicarb.
Respiratory acidosis	Rise* Or normal	Rise	Fall	Fall	Hypoventilation and CO accumulation
Respiratory alkalosis	Fall*	Fall	Rise	Normal	Hyperventilation and fall of CO_2 level

*May be late in compensation.

Definitions

Electrolytes, such as acids and bases, when in solution, are ionized. The ions carry an electrical charge, + or – and migrate towards the opposite poles when held in an electrical field. For example, the electrolyte sodium chloride (NaCl) ionizes into Na^+ and Cl^-, the hydrochloric acid (HCl) ionizes into H^+ and Cl^- and the alkali sodium hydroxide (NaOH) or a base, ionizes into Na^+ and OH^-. **An acid is a substance that can yield a hydrogen ion (H^+)** or hydronium ion when dissolved in water. Hydrochloric acid H^+Cl^- is a familiar example. **A base is a substance that can yield hydroxyl ions (OH^-)**. Sodium hydroxide (Na^+OH^-) is a base. The base with OH^- ion is capable of accepting the H^+ ion of the acid. The union of these two ions—H and OH—produces water, with no charged ions.

$$\begin{array}{lllllllll} HCl & + & NaOH & \rightarrow & NaCl & + & H(OH) \\ \text{Acid} & + & \text{Base} & \rightarrow & \text{Salt} & + & \text{Water } (H_2O) \end{array}$$

Total strength of an acid or base is determined by its **normality** (N). Thus equal volumes of hydrochloric acid and sodium hydroxide, both of 1 N strength, will neutralize each other, yielding water and sodium chloride. They have lost their powers as acid or base. However, the total strength (normality) does not truly express their reactivity. To give an example, if you put your finger into a 6 N hydrochloric acid it will burn your finger but if you do the same with same strength (6 N) boric acid, you will not get hurt.

To make things clear, let us take an example from our daily life. Imagine two friends and both own $100. One of them (A) has $98 in the wallet and $2 in bank while the other (B) has $6 in the wallet and $94 in the bank. Now they arrive in a shop, which only accepts cash for any purchase. Who is richer in this situation? Obviously, it is the friend A. The same situation is with hydrochloric acid and boric acid. 98% of H^+ ions of hydrochloric acid are in ionized state while with boric acid it is only 6% although both of them may be of same strength of normality ($100). Therefore, boric acid is **weak acid** and hydrochloric acid is a **strong acid.** In other words, the concentration or activity of hydrogen ions in a solution determines the acidity or reactivity of the acid. The same holds good for alkali. Sodium hydroxide is a strong alkali whereas bicarbonate is a weak alkali. The degree of ionization is defined by a constant, **K,** which characterizes the strength of the acid. The larger the value of K, the stronger the acid and the greater is the tendency of it to dissociate.

Hydrogen ion activity of a solution also determines the pH of the solution. In other words, pH is a symbol relating to the hydrogen ion (H^+) concentration. Numerically the **pH** is approximately equal to the **negative logarithm of the hydrogen ion (H^+) activity** in aqueous solution.

$$\text{pH} = \log_{10}\frac{1}{H^+}$$

By virtue of its logarithmic nature, **pH is a dimensionless quantity.** Thus, pH decreases as $[H^+]$ increases and, conversely, pH increases as $[H^+]$ decreases. The pH ranges from 1 to 14, where 1 is the strong acid and 14 is the strong base. The water (neutral) has a pH of 7.0 where H and OH ions are in equal proportion. As the pH goes above 7.0, it gets increasingly alkaline which can be scaled as decreasing H^+ ion (increasing OH). On the other hand, when the pH goes below 7.0, there is increasing amount of H^+ ion (decreasing OH). Since this relationship is also logarithmic in nature, the correlation between pH an $[H^+]$ is not linear. As illustration of this point, a decrease of 1 pH unit represents a 10-fold increase in the hydrogen ion activity.

A **buffer,** composed of a weak acid or weak base and its corresponding salt, is a system that resists change in pH. The **law of mass action** applies to the dissociation reaction equilibrium is established between the weak acid and its dissociated ions. Like pH, pK is defined as the negative log of the **ionization constant** (K). Strong acids have pK values less than 3.0, whereas strong bases have pK values greater than 9.0. For acids, raising the pH above the pK will cause the acid to dissociate and yield a $[H^+]$. For bases, lowering the pH below the pK will cause the base to release $[OH^-]$. The lower the value of pK, the stronger is the acid; likewise, the higher the pK, the weaker is the acid.

Bicarbonate is not usually tested by itself. It may be done on a blood specimen taken from a vein as part of a panel of tests that looks at other electrolytes, such as sodium, potassium, and chloride. It can also be done as part of an arterial blood gases test. For the arterial blood gas study, the blood sample is taken from an artery.

Handerson-HasselbA1ch equation explains how the buffer system works to stabilize the pH of the body fluids in circulation:

$$\text{pH} = \text{pK} + \log\left(\frac{\text{Bicarbonate}}{\text{Carbonic acid}}\right)$$

Carbonic acid (H_2CO_3) is a weak acid with bicarbonate (HCO_3^-) serving as its conjugate base. When acid is added to this system, the hydrogen ions react with bicarbonate to form more carbonic acid and thus minimize changes in pH.

Note Both bicarbonate and carbonic acid can be expressed in mEq/L. Carbonic acid, however, can also be expressed as PCO_2. The mathematical relationship is given in this equation:

$$H_2CO_3 = 0.03 \times PCO_2 \text{ mm Hg.}$$

At body temperature, the pK of the buffer pair bicarbonate–carbonic acid is 6.1 and the ratio of bicarbonate (HCO_3^-) and carbonic acid (H_2CO_3) is maintained by the body precisely at 20 : 1. Thus, under normal conditions, the above equation can be written as:

$$\text{pH} = 6.1 + \log 20 \text{ Or, pH} = 6.1 + 1.3^* = 7.4$$

$$^*\text{The value of } \log 20 = 1.3$$

The variation of this ratio is the cause of pH imbalance. The bicarbonate level of the blood is maintained by the kidney (metabolic), whereas the carbonic acid, or PCO_2 level is regulated by the lungs (respiratory).

There are four **clinically important buffers systems** that maintain the normal pH balance in the body between the narrow range of 7.35 and 7.45. These buffers, in order of decreasing physiologic importance, are the **bicarbonate–carbonic acid buffer system, haemoglobin, plasma proteins, and phosphates.** Although not considered to be the most important physiologically, haemoglobin accounts for the majority (80%) of the chemical buffering capacity of the blood. Plasma proteins are the next most abundant buffer, representing approximately 14% of the chemical buffering capacity of the blood. The remainder of the buffering capacity is represented by the bicarbonate–carbonic acid system (~ 5%) and inorganic phosphates (~1%). In the body, most buffering takes place inside the cell, where all of the four major buffer systems are found. Outside of the cell, plasma buffer include bicarbonate, proteins and inorganic phosphates.

Lungs participate in the intake of oxygen and removal of carbon dioxide, the product of oxidative respiration. **Oxygen** is transported primarily through the **haemoglobin of the red cells** and it also releases the carbon dioxide. Both these gases are bound to haemoglobin to meet the demand of numerous cells of the body. Partial pressure of the oxygen in blood determines to a great extend how much oxygen the haemoglobin will carry. The other two factors are the normal effective haemoglobin and affinity of the available haemoglobin. The affinity of haemoglobin for oxygen shifts, increased–decreased, with change in pH, PCO_2, 2,3-DPG (2,3-diphosphoglycerate) and temperature. The analysis of PCO_2 helps to understand the H_2CO_3 status which is controlled by lungs. **Blood gas analyzers** determine PCO_2. The value of **pH gives the end result of the acid–base imbalance** and is determined by **potentiometry.** Bicarbonate level (HCO_3^-) is regulated by the kidney. Thus ventilation by lungs and discharge of bicarbonate by kidney regulate the bicarbonate–carbonic acid **ratio of the body's buffer system.** For the normal function of the body, this ratio must stay close to 20 : 1.

The **acid–base (pH) imbalance** in blood leads to four major clinical conditions—metabolic acidosis, metabolic alkalosis, respiratory acidosis and respiratory **alkalosis**. The trend of laboratory reports and the causes of these pathologic conditions are shown in Table 33.3. The fall of plasma bicarbonate is often associated with metabolic acidosis. **Metabolic acidosis** can also be caused under untreated diabetic conditions due to building up of **ketone bodies** (Chapter 29 in this Volume). This leads to increased anion gap.

Anion gap is a concept used to estimate electrolyte (anion and cation) levels in the serum and conditions that influence them. The anion gap ranges from 8 to 15 mEq/L in normal patients. This is estimated by subtracting the sum of the anions chloride and bicarbonate ($Cl^- + HCO_3^-$) which is (103 + 27) mEq/L from the sum of the cations sodium and potassium ($Na^+ + K^+$) which is (140 + 4) mEq/L. Because the potassium cation is low in serum (4.5 Eq/L), the anion gap is calculated by the formula $Na^+ – (Cl^- + HCO_3^-)$ or 140 –(103 + 27) = 10 mEq/L of anion gap. With the building up of ketone bodies (which contains organic acids), the acid level of blood increases (pH falls below 7.4) and the anion gap increases. This is called diabetic acidosis.

The laboratory determinations of pH, PCO_2 (partial pressure of carbon dioxide in mmHg), PO_2 (partial pressure or tension of oxygen in mmHg), oxygen saturation of haemoglobin (%) and bicarbonate concentration (mEq/L) help in the understanding of the acid–base imbalance. The concentration of 2,3-DPG (diphosphoglycerate), an intermediate metabolite in red cells, is not determined because of technical difficulties. With the easy availability of blood gas analyzers, bicarbonate determination by titrimetry has practically become obsolete. Because of cost involvement, most peripheral laboratories in developing countries cannot afford blood gas analyzers. Hence, titrimetric assay of bicarbonate is described here.

Determination of Blood Gases

Clinical significance of PO_2 determination is to assess the oxygen carrying capacity of haemoglobin. An elevated PO_2 may be related to decreased oxygen affinity of the haemoglobin, low plasma pH, and high PCO_2. Because of the non-availability of the blood gas analyzer in routine laboratories of developing countries, we will confine our further discussion to the determination of pH and bicarbonate. Blood gases and pH are determined electrochemically. Determinations of pH and PCO_2 are done by the potentiometric method. Carbon dioxide (CO_2) is a gaseous waste product from metabolism. The blood carries carbon dioxide to your lungs, where it is exhaled. More than 90% of carbon dioxide in your blood exists in the form of bicarbonate (HCO_3). The rest of the carbon dioxide is either dissolved carbon dioxide gas (CO_2) or carbonic acid (H_2CO_3). Your kidneys and lungs balance the levels of carbon dioxide, bicarbonate, and carbonic acid in the blood.

Only the blood gas analyzers can determine the partial pressure of oxygen (PO_2). If the blood analyzers are not available, the laboratory can adopt the alternate procedure by determining pH and bicarbonate and consult the nomogram (Figure 33.13) to find out the PCO_2. We will discuss here the colorimetric procedure to determine bicarbonate.

Determination of Bicarbonate

In the body, most of the CO_2 is in the form of a substance called bicarbonate (HCO_3^-). Therefore, the CO_2 blood test is really a measure of your blood bicarbonate level. Bicarbonate is the second largest fraction of the anions in plasma. Included in this fraction are the bicarbonate (HCO_3^-) and carbonate (CO_3^{-2}) ions. Carbon dioxide is in physical solution, as well as carbamino compounds. At the physiological pH of blood, the concentration of carbonate is 1/1000 that bicarbonate.

Clinical significance

The **bicarbonate measurement** of serum or plasma is used in the diagnosis and treatment of numerous potentially serious disorders associated with acid–base imbalance in the respiratory and metabolic systems. An elevation of the bicarbonate level may be observed in compensated respiratory acidosis and metabolic alkalosis. Low bicarbonate levels may be observed in compensated respiratory alkalosis and metabolic acidosis. Additional laboratory determinations will permit differentiation between metabolic and respiratory conditions. Other clinical conditions include diarrhoea, renal tubular acidosis, carbonic anhydrase inhibitors, hyperkalemic acidosis, renal failure, and ketoacidosis.

Normal range

Adult: 21–31 mEq/L or mmole/L

Expected values may vary with age, sex, diet and geographical location. Each laboratory should determine its own expected values as dictated by good laboratory practice.

Laboratory assay

The determination of bicarbonate (HCO_3^-) is used in conjunction with other clinical and laboratory information for the evaluation of acid–base status. Additional laboratory determinations will permit differentiation between metabolic and respiratory conditions.

Principle

The bicarbonate reagent utilizes the enzymatic method to determine the bicarbonate level of the serum. In this procedure, in the first step, bicarbonate (HCO_3^-) and phosphoenolpyruvate (PEP) are converted to oxaloacetate and phosphate in the reaction catalyzed by

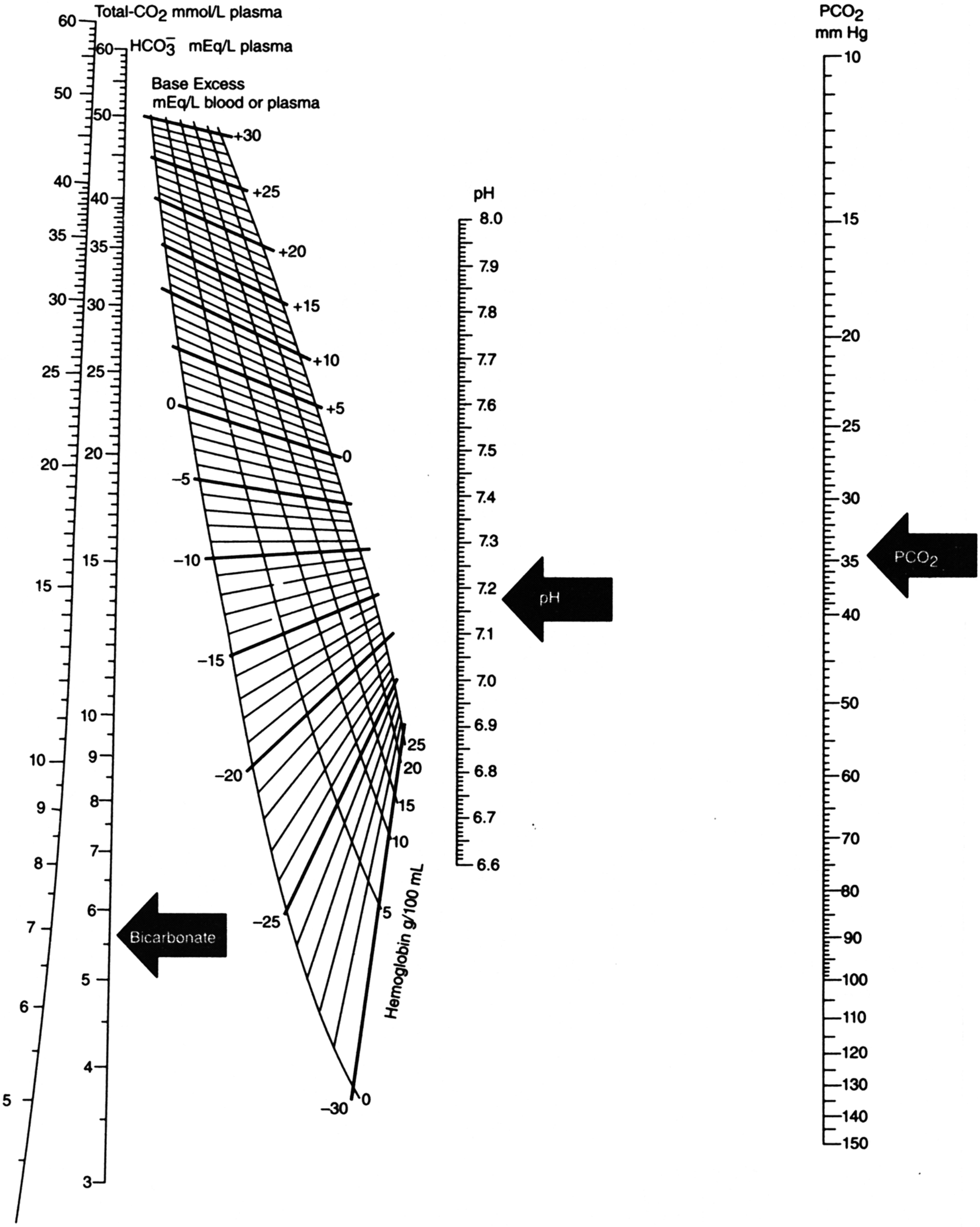


FIGURE 33.13 *Siggaard Andersen alignment nomogram for the determination of acid–base values. Determine the bicarbonate and pH. Locate the values on the left and middle scales, join the points with a ruler and extend the line to the PCO_2 scale to determine the corresponding value.*

phosphoenolpyruvate carboxylase (PEPC). In the second step, malate dehydrogenase (MD) catalyses the reduction of oxaloacetate to malate with the concomitant oxidation of reduced nicotinamide adenine dinucleotide (NADH). This oxidation of NADH results in a decrease in absorbance of the reaction mixture at 340 nm which is proportional to the bicarbonate content of the sample. The chemical reaction is overviewed in the following equations.

$$\text{PEP} + HCO_3^- \xrightarrow[\text{Magnesium}]{\text{PEPC}} \text{Oxaloacetate} + H_2PO_4$$

$$\text{Oxaloacetate} + \text{NADH} + H^+ \xrightarrow{\text{MD}} \text{Malate} + \text{NAD+}$$

Specimen

Heparinized venous blood is commonly submitted to the laboratory on ice for the analysis of blood gases. Serum and plasma samples, free from haemolysis, are the recommended specimens. Arterial blood is recommended but is difficult to obtain.

Obtain blood specimen aseptically by venepuncture or skin puncture. Separate serum or plasma from cells promptly to minimize haemolysis. Oxalate, citrate and EDTA have been reported to interfere with assay results. If plasma is used, the recommended anticoagulant is heparin. Prepare sample in the following way:

- After collecting the specimen, avoid contamination with CO_2. Do not blow into pipette. Do not open tubes unnecessarily. Keep container tightly stoppered.
- Dilute serum samples, if necessary, with CO_2-free water.

Sample Storage and Stability: Once separated from cells, bicarbonate in serum is stable for several hours when stored at 2–8°C and protected from exposure to air.

Special Instruction: Carefully read the user's guide or protocol published by the manufacturer. It is important that the technician must fully comprehend the procedure before starting the assay.

Carbon Dioxide (Bicarbonate) Enzymatic Assay: Carbon dioxide enzymatic assay kit is now available. Closely follow manufacturer's instruction. It is based on the principle of colorimetric enzymatic assay. Serum is the recommended specimen.

Principle

The coupled enzyme assay of the kit detects CO_2 (as HCO_3^-) by the following equation.

$$\text{Phosphoenolpyruvate} + HCO_3^- \xrightarrow{\text{PEPC}} \text{Oxalate} + H_2PO_4$$

$$\text{Oxalate} + \text{NADH} \xrightarrow{\text{MDH}} \text{Malate} + \text{NAD+}$$

In the first step, the bicarbonate condenses with phosphoenolpyruvate to form oxalate (and phosphoric acid); this reaction is catalyzed by the enzyme Phosphoenolpyruvate Decarboxylase, PEPC. In the second step, the oxalate is reduced by the enzyme Malate Dehydrogenase (using an NADH cofactor) to form malate and NAD+. Since the NADH molecule absorbs light at 340 nm but the NAD^+ does not, the decrease in absorbance is dependent on the presence of CO_2 in the reaction. Therefore, the CO_2 analyte causes a decrease in absorbance at 340 nm that is directly proportional to the CO_2 concentration in the samples. The kit comes with a CO_2 standard that contains 30 mmol/L of sodium bicarbonate in an aqueous solution.

Kit Contents, Storage and Shelf Life

Store the kit in refrigerator (4°C). Reconstitute the reagents as instructed by the manufacturer. All reconstituted reagents must be stored in refrigerator (4°C) unless advised by the manufacturer. The usual shelf life of reconstituted reagents is 6 months but some can be frozen and stored in the freezer. CO_2 free water is provided in the kit for making dilutions.

Procedure

Follow instructions of the manufacturer.

Calculation

A typical standard curve is presented here with the diluted standards of CO_2.

Tube#	Volume Carbon Dioxide Standard (30 mmol/L)	Volume CO_2-free water	CO_2 Concentration
1 (blank)	0 µL	60 µL	0 mmol/L
2	20 µL	40 µL	10 mmol/L
3	30 µL	30 µL	15 mmol/L
4	40 µL	20 µL	20 mmol/L
5	50 µL	10 µL	25 mmol/L
6	60 µL	0 µL	30 mmol/L

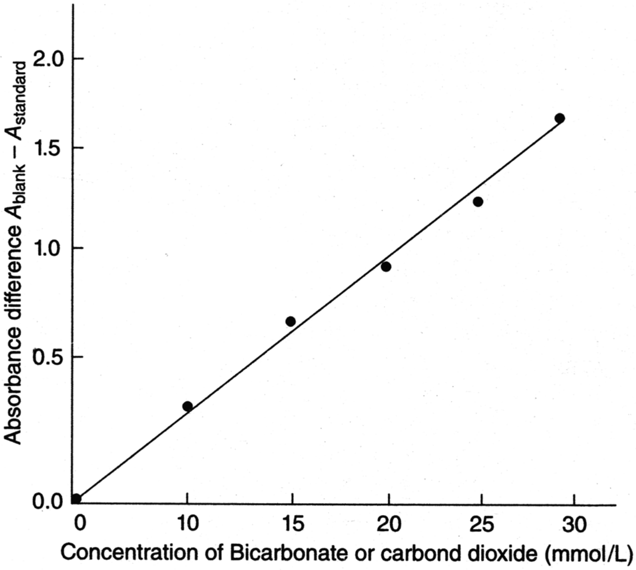


FIGURE 33.14

The formula method will be as follows:

$$C \text{ (mmole/L)} = A_{dt}/A_{ds} \times 30$$

Where,

C = Concentration of CO_2 or bicarbonate in test specimen (mmole/L)
A_{dt} = Absorbance difference for test or specimen (340 nm)
A_{ds} = Absorbance difference for standard (340 nm)
30 = Concentration of standard (mmole/L)

REVIEW QUESTIONS

1. List the routine biochemical analyses of serum and give the principle on which the analyses are based.
2. What is the clinical significance of each of the following biochemical analysis of serum? Glucose, Protein, Bilirubin, Phosphorous and Cholesterol

3. What is the clinical significance of determining the transaminase level of serum? How are the results correlated with other biochemical findings?
4. How would you determine true glucose? What is the clinical significance of true glucose determination?
5. Under which clinical condition would you expect a rise of serum protein level?
6. Why do you need fluoride as the anticoagulant for the determination of plasma glucose and BUN?
7. What are the laboratory findings in case of obstructive jaundice?
8. How are diabetes mellitus, anion gap and metabolic acidosis related?
9. What is the significance of serum bicarbonate determination?
10. A haemolyzed sample will not cause falsely increased levels of which of the following? Potassium, Sodium, Phosphate and Magnesium
11. What is the significance of Henderson-Hasselbalch equation?
12. What is the role of lungs in maintaining pH balance in blood?
13. Name some of the important buffer system of the body.
14. How is SO_2 determined? What is its significance?
15. Name the clinical conditions that arise from acid–base imbalance.
16. *Case study*: A 15-year-old girl was brought to emergency in coma. She was diabetic and insulin dependent. The parents stated that she had several episodes of hypoglycaemia and ketoacidosis in the past and often skipped insulin. Her blood and urine studies revealed the following abnormalities:

Venous blood:	Potassium ↑ Chloride ↓ Bicarbonate ↓ Glucose ↑ Urea ↑ Lactate ↑
Arterial blood:	pH ↓ PCO_2 ↓
Urine:	Glucose ↑ Ketone ↑

What is the reason for the fall of chloride and bicarbonate? What is the significance of elevated potassium? What is the diagnosis?

Chapter

34

Biochemical Test Profiles

Ashoke Khanwalkar

Chapter Outline

- Diagnostic Clinical Chemistry
- Analytes Commonly Tested in Chemistry Profiles
    - Protein
    - Electrolytes
    - Mineral Metabolism
- Kidney (Renal) Function Tests
    - Creatinine
    - Blood Urea Nitrogen (BUN)
    - Uric Acid
- Liver Function Tests
    - Bilirubin
    - Liver Enzymes
- Cardiac Function Tests
- Lipid Metabolism
    - Cholesterol
    - Triglycerides
- Carbohydrate Metabolism
- Thyroid Function Tests
- Other Tests of Organ Functions
    - Creatinine Clearance for Kidney Function
- Gastric Function Tests
    - Gastric Fluid Laboratory Investigation of Gastric Fluid
- Pancreatic Function Tests
    - Blood Test
    - Faecal Test
    - Laboratory Investigation of Pancreatic Juice

- Test for Malabsorption
    - D-xylose absorption test
- Review Questions

DIAGNOSTIC CLINICAL CHEMISTRY

A physician may order biochemical laboratory tests either as individual tests or as a **chemistry profile.** A chemistry profile is a group of tests performed simultaneously on a patient specimen to provide an assessment of the patient's general condition. The physician can use the results of the chemistry profile, in conjunction with the physical examination, to assess the overall health of the patient.

Tests included in a **routine chemistry profile** reflect the state of carbohydrate and lipid metabolism, as well as kidney, thyroid, liver, and cardiac function. Profiles or panels that assess one particular biological system, such as renal or liver function, are also performed. Examples of chemistry panels are shown in Table 34.1. These tests are requested when a particular diagnosis is suspected or treatment must be monitored. Many automated instruments used in advanced laboratories are designed to provide **'panel test'** results. In developing countries, however, individual tests are handled independently.

TABLE 34.1 *Biochemical test profiles*

Panels	Tests
General (1) (Overall check up)	Glucose Total protein BUN Bilirubin (Total) Albumin Calcium Phosphorus Uric acid Alkaline phosphatase SGOT LDH Cholesterol A/G ratio Globulin
Thyroid (2) (Thyroid function)	T3, T4, T7
Renal (3) (Kidney function)	Creatinine BUN Uric acid Sodium Potassium CO_2 Chloride Glucose

(Contd.)

Hepatic (4) (Liver function)	Albumin Total bilirubin Direct bilirubin Alkaline phosphatase Total protein ALT/SOFT AST/SGOT LDH Globulin
Lipid (5)	Cholesterol Triglyceride HDL LDL
Cardiac (6) (Heart function)	SGOT LDH CK (Isoenzymes done if CK is elevated)
Iron (7)	Total iron Total iron binding capacity Unsaturated iron binding capacity
Electrolytes (8)	Sodium Potassium Chloride Carbon dioxide

ANALYTES COMMONLY TESTED IN CHEMISTRY PROFILES

Nearly 15 biochemical tests are routinely done in basic laboratories of developing countries. Others must be sent to reference laboratories. Some tests are part of more than one panel. Before we discuss the **'organ function tests',** an overview of the basic tests may be helpful. ***(Note*** For various tests, reference normal healthy value ranges will be provided in parentheses.)

Protein

Two major groups of serum proteins are the **albumins** and the **globulins.** Albumins comprise approximately 60% of the total serum protein and globulin about 40%. The albumins are produced in the liver, serve as transport proteins, and help maintain the fluid balance in the body. Antibodies, blood coagulation proteins, enzymes, and proteins that transport iron (transferrin) are all examples of serum globulins.

Total serum protein

Total serum protein (6.0–8.0 g/dL) represents the sum of many different proteins. Total protein levels are elevated in patients with dehydration, chronic inflammatory conditions, or certain blood cancers. A decrease of serum protein can be related to malnutrition, loss through urine (proteinuria caused by a kidney disorder), or lack of synthesis due to liver disorder. Most serum proteins are made in the **liver.**

Albumin

Albumin is produced by the **liver** and is the main protein in the blood. It gives the blood its **oncotic pressure** or colloid osmotic pressure, which prevents leakage of plasma fluid from the small blood vessels or capillaries into the tissues, thereby causing edema. Albumin also functions to bind drugs to **transport** them via the blood to body tissues. Decreased albumin levels may be due to its reduced synthesis in liver disease, or from loss through the gastrointestinal tract or urine in diseases of the bowel or kidneys respectively. High levels can occur with dehydration.

A/G ratio

The **albumin/globulin ratio** is often computed from the values of total protein and albumin. Globulin is the difference between total protein and albumin (Total protein – albumin = globulin). A fall of A/G ratio may be due to an increase in globulin concentration in the serum (viral infection) or a decrease in albumin concentration due to loss (proteinuria) or poor synthesis (liver damage). Thus, a change in the A/G ratio indicates the presence of many possible disorders whose identification requires further investigation.

Electrolytes

In clinical chemistry, the term electrolytes refers to the **major cations** in the blood, sodium (Na^+) and potassium (K^+), and the **major anions,** chloride (Cl^-) and bicarbonate (HCO_3^-). Electrolytes have a significant effect on acid–base (pH) balance, as well as on heart, brain, and muscle function. Fluid volume status in the body can affect electrolyte levels. Measurement of electrolytes is included in most routine chemistry and renal profiles.

Mineral Metabolism

Minerals are necessary for good health. Calcium, phosphorus (phosphate), and iron are examples of minerals often measured in chemistry profiles. **Calcium and phosphorus** are necessary for proper bone and tooth formation. Calcium is also required for blood coagulation. **Iron** is essential for haemoglobin production and is an integral component of some enzymes.

Calcium

Of all the minerals found in the body, **calcium** is present in the highest concentration. Approximately 99% of the body's calcium is bound in calcium complexes in the skeleton and is not metabolically active. Only unbound calcium ions are metabolically active and are measured in the calcium assay. Calcium is required for proper blood coagulation and for normal neuromuscular excitability. Vitamin D, parathyroid hormone, oestrogens, and calcitonin influence the calcium balance. These control dietary absorption of calcium, calcium excretion by the kidneys, and calcium movement in and out of bone.

Hypercalcemia (increased level of blood calcium) occurs in hyperparathyroidism, malignancies, certain hormonal disorders, excessive calcium and vitamin D intake, sarcoidosis, renal failure, and immobilization. It can cause calcium to be deposited in soft tissues, vascular walls, heart valves, and other organs, leading to complications such as calcification and **kidney stones.** It may also cause musculoskeletal pain, gastrointestinal upset and psychiatric manifestations. **Hypocalcemia** (decreased level of blood calcium) can be **life threatening** due to cardiac complications and should be reported to the physician immediately. This may be caused by hypoparathyroidism, hypothyroidism, vitamin D deficiency, poor calcium absorption due to intestinal disease, and kidney disease.

Phosphorus

Most phosphorus in the body is in the form of inorganic phosphate. Approximately 80% is in the bone and the rest is mostly in **high-energy compounds** such as adenosine triphosphate (ATP). Calcium and certain hormones influence phosphorus levels. Elevated blood phosphorus levels may be seen as an artefact of sample haemolysis but may also signify kidney diseases, hypoparathyroidism, severe muscle injury, or certain conditions affecting the bones.

Iron

Iron is essential for **haemoglobin synthesis.** Iron is absorbed from dietary sources and is highly conserved by the body. Iron levels differ with age, gender, and time of day, being higher in the morning than in evening or night.

Iron deficiency can lead to **anaemia.** The deficiency can be due to insufficient iron in the diet, poor iron absorption, impaired release of stored iron, or increased iron loss due to bleeding. Serum iron levels can be elevated with haemolytic anaemias, increased iron intake, or blocked synthesis of iron-containing compounds, such as in lead poisoning.

KIDNEY (RENAL) FUNCTION TESTS

The primary functions of kidneys are to remove the waste materials of metabolism and to preserve an optimum internal environment for cells (pH, water balance, electrolyte balance, and fluid osmotic pressure). Thus, substances are excreted into and reabsorbed from the urine to help maintain **homeostasis.** These physiological processes are largely controlled by hormones. Apart from this, kidneys are also the production sites for certain essential hormones of the body. The serum and plasma concentration of certain substances such as creatinine, BUN, sodium, potassium, bicarbonate, phosphorus, and uric acid are altered in certain kidney diseases.

Currently used **kidney function tests** include:

- **Serum creatinine:** It is a waste product formed in muscles. It has a relatively constant excretion rate and its concentration in serum is a fairly good measure of glomerular filtration rate (GFR), which characterizes kidney function. Creatinine is not reabsorbed by the renal tubule; therefore, any condition which reduces glomerular filtration rate will result in reduced creatinine excretion in urine with a consequent increase in plasma concentration.
- **Blood urea nitrogen (BUN):** Urea is a waste product of protein breakdown which appears in the glomerular filtrate but approximately 40% is reabsorbed in the tubule. In addition, its plasma concentration is strongly influenced by diet and other physiological conditions not connected with renal function. Hence, serum creatinine is considered a better indicator of renal function. However, the serum concentration of urea (or BUN) rises in impaired renal function just as creatinine does. Moreover, its ratio with creatinine (BUN : Creatinine ratio) can help characterize the type of renal pathology.
- **Uric acid:** Formed from the **breakdown of nucleic acids** and excreted by the kidneys, often used to diagnose **gout.**
- **Glomerular filtration rate (GFR):** This is also known as a clearance test. It determines the filtration capacity of the nephrons in the kidney. Marked decrease of GFR indicates a kidney disorder. This will be discussed separately with 'Other Functional Tests'.
- **Urine analysis or urinalysis (physical and microscopic):** This provides a general picture of kidney function and health and is excellent for screening purposes. Presence

of casts (on microscopic examination), proteinuria, decreased volume, and increased specific gravity indicate kidney failure. This test is also performed to look for bacteria and white blood cells that may indicate an infection. This is further discussed under Urinalysis (Chapter 25 of Vol. II).

- **Blood electrolytes:** Blood electrolytes (especially chloride) tend to accumulate if they are unable to exit the circulation through the kidneys. Thus an increased concentration of blood electrolytes may indicate renal disorder. This will be discussed in the chemistry section.

Here we will focus on three commonly analysed serum components that directly indicate kidney disease—creatinine, BUN, and uric acid. We will discuss the methodology for detennining GFR separately.

Creatinine

Creatinine is a waste product of **creatine phosphate,** a substance stored in muscle and used for energy. Creatinine is excreted by the kidneys. When renal function is impaired, blood creatinine levels rise. The creatinine level is not affected by diet or hormone levels. Hence, a rise in blood creatinine indicates the impairment of urine formation or excretion, which occurs in renal disease, shock, water imbalance, and/or urinary blockage.

Blood Urea Nitrogen (BUN)

In the human body, the breakdown products of **amino acids** (the building blocks of proteins) are converted to **urea,** which is excreted by the kidneys. Urea levels are measured by the **BUN.**

Diet, hormones, and kidney function influence the BUN level. Therefore, BUN is not as good an indicator of kidney disease as is the **creatinine level.** The BUN level may be low during starvation, pregnancy, and a low-protein diet. Increased BUN concentration may occur during a high-protein diet, after administration of steroids, and in kidney disease.

Uric Acid

Uric acid is formed from the **breakdown of nucleic acids** and is excreted by the kidneys. It has low solubility and may precipitate as uric acid crystals, or urates. Uric acid measurement is principally used to diagnose and treat **gout,** a disease in which uric acid precipitates in tissues and joints, causing pain. Uric acid levels can also increase after massive radiation or chemotherapy because of increased cell destruction.

LIVER FUNCTION TESTS

The liver is both a **secretory** and **excretory** organ of the body and has numerous metabolic functions. The liver is closely associated with **carbohydrate metabolism** (synthesis of glycogen from glucose as well as breakdown of glycogen into glucose), **protein metabolism** (most plasma proteins are made in liver), and **lipid metabolism** (source of cholesterol). The liver stores a number of essential substances and makes them available when needed—glycogen, vitamins, iron, and others. It also destroys old cells by phagocytosis and detoxifies many substances.

Significant liver function must be lost or impaired before some laboratory tests show abnormality. Numerous tests are used to evaluate liver function. Most are not specific for a particular disease but only reflect liver tissue damage or liver dysfunction. The tests which are routinely followed for assessing liver function are as follows:

- **Serum bilirubin (total and direct):** Diagnoses conjugation disorders and bile duct obstruction.

- **Serum enzymes:** Transaminases or amino transferases (SGOT or AST, SGPT or ALT) and ALP (alkaline phosphatase); confirms damage in the liver that causes the release of liver enzymes.
- **Serum total protein and albumin:** Examines the liver's synthetic function in producing proteins.
- **Urine bilirubin:** Confirms bile duct obstruction, which results in the rise of conjugated bilirubin in serum and its excretion through the kidney.
- **Urine urobilinogen:** Suggests increased haemolysis, bilirubin production, and discharge of urobilinogen through the kidney.
- **Serum cholesterol (total):** Assesses the liver's function in lipid metabolism.
- **Prothrombin time:** Assesses the liver's synthetic function in producing coagulation factors.

In the following sections, we will focus on **bilirubin** and **liver enzymes,** which are routinely used to assess liver function.

Bilirubin

Bilirubin is a waste product from **breakdown of haemoglobin.** It is formed in the tissues, then transported to the liver and excreted in the biliary system. In the liver, most bilirubin becomes bound to glucuronide and is then excreted into the bile—this is called **conjugated** or **direct bilirubin.** Bilirubin that is not conjugated is called **indirect bilirubin.** Total serum bilirubin equals direct bilirubin plus indirect bilirubin. Bilirubin assays usually measure both total and direct bilirubin. Indirect bilirubin is then calculated from those two numbers.

Bilirubin is measured to screen for or to monitor liver or gallbladder and **biliary tract** dysfunction. Since bilirubin levels are normally low, only increases in serum bilirubin are significant. Bilirubin can be increased when there is excessive destruction of haemoglobin such as in the haemolytic anaemias, impaired excretion by the liver such as in biliary obstructions including gallstones, or impaired bilirubin processing as in hepatitis.

LIVER ENZYMES

Arise in liver enzymes generally reflects injury to tissue, since most enzymes are intracellular. Some enzymes are widely distributed in many body tissues, whereas others are found in only a few tissues. The measurement of enzyme levels is not always specific for damage to a particular organ but is most helpful when used with other tests, clinical symptoms, and patient history.

Enzymes used to assess **liver function** include the following:

- Alkaline phosphatase (ALP)
- Alanine aminotransferase (ALT): Formerly called serum glutamic pyruvic transaminase (GPT or SGPT).
- Aspartate aminotransferase (AST): Formerly called serum glutamic oxaloacetic transferase (GOT or SGOT).
- Gamma glutamyl transferase (GGT)
- Lactate dehydrogenase (LD or LDH)

Alkaline phosphatase (ALP or AP) is widely distributed in the body, especially in bone and the liver ducts. Elevated serum is often noted with obstruction or damage to the biliary system. ALP level can also greatly increase with liver tumours and lesions and can show a moderate increase with diseases such as hepatitis.

Aminotransferases (ALT and AST) are found in abundance in liver tissues. When liver tissues are injured, these enzymes are released into the circulation. Serum concentrations of these enzymes change with time; rises during acute liver disease and falls as recovery occurs. Generally, only one enzyme needs to be measured—ALT or AST. **ALT** levels are low in cardiac tissue and high in liver tissue. This enzyme usually rises higher than AST in liver disease, with moderate increases (up to 10 times normal) in cirrhosis, infections, or tumours, and increases up to 100 times normal in viral or toxic hepatitis. **AST** is present in many tissues, particularly cardiac, muscle and liver. It is elevated after myocardial infarction, as well as in liver disease. Elevated AST and ALT with an AST : ALT ratio of greater than 2 : 1 may be associated with alcoholic liver disease.

Gamma glutamyl transferase (GGT) is found in kidneys, pancreas, liver, and prostrate tissue. GGT can be more helpful than ALP in determining liver damage because GGT remains normal in bone disease. It is more useful than AST because it remains normal in muscle disorders. GGT measurement is often used to monitor recovery from hepatitis.

Lactic dehydrogenase (LD or LDH) is widely distributed in body tissue. The serum LDH level increases during liver disease and following myocardial infarction. Haemolysis of a blood sample will cause increased LDH levels in the serum because of LDH release from red blood cells.

A summary of the biochemical findings and their possible diagnosis for liver disorders is presented in Table 34.2.

TABLE 34.2 *Findings of biochemical profile in the diagnosis of liver disorders*

Test	Acute hepatitis	Chronic hepatitis	Obstructive jaundic
Alkaline phos. (ALP)	1+	1+	3+
AST (SCOT)	3+	1+	1+
ALT (SGPT)	3+	1+	1+
Serum bilirubin			
Conjugated	2+	V	2+
Unconjugated	2+	V	2+
Urine bilirubin	2+	2+	2+
Cholesterol	0	0/1–	2+
Icterus index	2+	V	2+
Protein			
Total	0	0/2–	0
Albumin	2–	2–	0
Prothrombin time	2+	2+	2+
Urobilinogen			
in urine	2+*	2–	2–

Note 1+, slight increase; 2+, increase; 3+, marked increase; 1–, slight decrease; 2–, decrease; 3–, marked decrease and 0, no change

V, variable

*In early stage

Special Note

Some of the tests are now considered to be obsolete and these include the thymol-turbidity test and BSP (bromsulphthalein test). **Thymol turbidity** and other flocculation tests reflect on the albumin-globulin ratio of serum which is maintained by the liver. The **BSP test,** on the other hand, assesses the excretory function of the liver. As BSP occasionally results in allergic and anaphylactic shock problems, Cardiac green is used in place of BSP.

CARDIAC FUNCTION TESTS

The most commonly used enzyme assay for the diagnosis of myocardial infarction is **creatine kinase** (CK), also called **creatinine phosphokinase** (CPK). It is present in large amounts in muscle and the brain, but in small amounts in organs such as the liver and kidneys. Following a myocardial infarction, or **heart attack,** CK is released from the damaged heart muscle. The serum CK level peaks in about 24 h, reaching 5–10 times the upper limit of normal (30–170 U/L). It falls rapidly back to normal levels within 3–4 days. Serum CK levels also increase following skeletal muscle damage and brain injury. Significant elevation in CK is seen following rhabdomyolysis, which is associated with significant skeletal damage, and can lead to acute renal failure. Determination of isoenzymes helps to determine the source of the injury, which may be requested if the CK is elevated. CK-MB is a more specific biomarker for cardiac damage and its proportion of overall CK may indicate cardiac pathology. CK is routinely measured in emergency room patients with chest pain and when acute renal failure is suspected. For the differential diagnosis of heart and liver pathology, CK is requested along with SGOT and LDH. Low CK can be an indication of alcoholic liver disease and rheumatoid arthritis.

Troponin levels are another test that may be ordered. This biomarker tends to persist much longer than CK following myocardial infarction and can be used to determine whether one occurred several days earlier. TnI and TnT are subtypes used to evaluate for cardiac pathology.

LIPID METABOLISM

Lipids are synthesized in the body from dietary fats. The commonly measured lipids are **cholesterol and triglycerides.** These are of interest primarily because of their association with cardiovascular disease (CVD).

Cholesterol

Cholesterol is present in all tissues, and the serum concentrations tend to increase with age. Elevated cholesterol levels can increase the risk of coronary, cerebral, and peripheral artery disease. It is recommended that total serum cholesterol levels be maintained below 200 mg/dL. Cholesterol fractions such as low-density lipoprotein **(LDL)** cholesterol, high-density lipoprotein **(HDL)** cholesterol, and very low density lipoprotein **(VLDL)** cholesterol are also measured.

Triglycerides

Hyperlipidemia is an excess of fatty substances called **lipids,** largely cholesterol and triglycerides, in the blood. Triglycerides are the main form of lipid storage in humans, comprising approximately 95% of fat (adipose) tissue. Triglycerides are **transported** in the plasma bound to **lipoproteins,** molecules composed of lipid and protein. This is the only way that these fatty substances can remain dissolved while in circulation.

The best-known lipoproteins are **LDL** and **HDL.** Excess LDL cholesterol contributes to the blockage of arteries, which eventually leads to cardiovascular events including heart attack and stroke. Population studies have clearly shown that the higher the level of LDL cholesterol, the greater the risk of heart disease. This is true in men and women, in different racial and ethnic groups, and in all adult age groups. Hence, LDL cholesterol has been labelled as **'bad' cholesterol.** HDL cholesterol, on the other hand, is associated with improved cardiovascular health. It has the ability to retrieve lipid depositions in the arteries and return them to the liver. Hence, it has been labelled as **'good' cholesterol.**

Increased blood levels of triglycerides cause the plasma to have a milky appearance. Blood to be tested for triglycerides should be collected when the patient has been fasting for 12–14 h, such as in the morning before breakfast.

CARBOHYDRATE METABOLISM

Carbohydrate or glucose metabolism is largely regulated by **insulin** which is a hormone produced by the pancreas. Other hormones such as growth hormone, glucagons, and cortisol also influence glucose metabolism. Glucose is a commonly tested blood constituent. **Diabetic patients** have high glucose levels in blood (hyperglycaemia) which needs to be regulated in order to avoid complications.

THYROID FUNCTION TESTS

Endocrine glands are ductless and produce and secrete hormones into the bloodstream that regulate many physiological processes of the body. The thyroid gland is one of those sites. It produces thyroid hormones that stimulate metabolism by increasing protein synthesis and oxygen consumption by the tissues. Thyroid hormones are synthesized from **iodide** and the amino acid **tyrosine.** In the blood, more than 99% of thyroid hormones are bound to serum proteins and are metabolically inactive.

The two major thyroid hormones are **thyroxine,** also called T_4, and **triiodothyronine,** also called T_3. Measurement of thyroid hormones is a special test done through specialized laboratories, but is mentioned here because of its importance in clinical biochemistry. Thyroid profiles or endocrine panels will include measurement of free or total T_4, free or total T_3 and thyroid stimulating hormone (TSH) levels. TSH is an anterior pituitary hormone that regulates thyroid gland activity. Measurement of the minute quantities of these hormones is usually done through immunological techniques.

Grave's disease is an example of a disease caused by **hyperthyroidism,** or excessive secretion of thyroid hormone. On the other hand, decrease of thyroid function **(hypothyroidism)** causes a condition called myxedema, as well as several other clinical manifestations.

OTHER TESTS OF ORGAN FUNCTIONS

Creatinine Clearance for Kidney Function

The best currently available method for measuring kidney function is the **creatinine clearance test.** It is also known as **'Measurement of glomerular filtration rate (GFR) for creatinine'.** It estimates the amount of blood which passes through the glomerulus in 1 min with complete removal of creatinine. That amount of creatinine is then measured in a timed collection of urine. The method is sensitive and can be easily followed in any laboratory with facilities to measure creatinine. Decreased GFR is associated with worsening renal failure.

The expression of GFR in **'millilitres per minute'** indicates the amount of substance cleared by the kidney during a period of 1 min. Ideally the GFR is determined by measuring the clearance of a completely filterable substance that is neither absorbed nor excreted by the renal tubules, such as inulin. Creatinine is the best clinically useful substance. Urea clearance is of no value as a measure of GFR, because it is influenced by too many variables.

Calculation of clearance rate

Clearance calculated without considering the body weight is **uncorrected.** Except for children, the correction is not ordinarily done.

Uncorrected

Clearance (uncorrected) can be mathematically expressed by the following formula:

$$\text{Uncorrected clearance (mL/min)} = \frac{U_{\text{cr}} \times V}{P_{\text{cr}}}$$

where, U_{cr} is concentration of creatinine in urine, P_{cr} is concentration of creatinine in plasma (or serum), and V is the volume of urine passed per minute, expressed in mL.

Corrected

The **GFR** is roughly proportional to the size of the kidney and the **body surface area** of the individual. Therefore, the calculation for the clearance of any given substance should provide for correction for deviations from the average adult body surface. This is done by multiplying the clearance by the factor 1.73/A, where 1.73 is the average body surface in square metres and ***A*** is the body surface of the patient under investigation. The formula for calculating the **renal clearance,** therefore, expands as follows:

$$\text{Corrected clearance rate (mL/min)} = \frac{U_{\text{cr}} \times V}{P^{*}_{\text{cr}}} \times \frac{1.73}{A}$$

The body surface area (A) may be determined conveniently from the nomogram (Figure 34.1).

Example

A patient discharged 228 mL of urine in a 5 h period. The plasma concentration of creatinine was found to be 2.1 mg/dL and that of urine was 110 mg/dL. If the weight and height of the patient are 70 kg and 150 cm ($A = 1.65$) respectively, calculate the creatinine clearance rate.

Calculation

$$V = \frac{228}{5} \times 60 = 0.76 \text{ mL/min};\ U_{\text{cr}} = 110;\ P_{cr} = 2.1$$

$$\text{Uncorrected GFR}_{\text{cr}} = \frac{110 \times 0.76}{2.1} = 39.8 \text{ mL/min}$$

$$\text{Corrected GFR}_{\text{cr}} = \frac{39.8 \times 1.73}{1.65} = 41.7 \text{ mL/min}$$

Thus, with average height and weight, the uncorrected value is not too different from the corrected value. Hence, it is not necessary. However, the same data for a child will be much higher, as shown in the following example.

Example

If the body weight of an infant is 4 kg and height is 37 cm, the body surface (A) will be 0.17.

$$\text{Corrected clearance rate (mL/min)} = \frac{39.8 \times 1.73}{0.17} = 405$$

This shows that correction for body surface is absolutely mandatory if the body surface of the patient differs greatly from that of the average adult person. The error otherwise introduced is substantial in case of infants.

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*The uncorrected value of creatinine clearance.

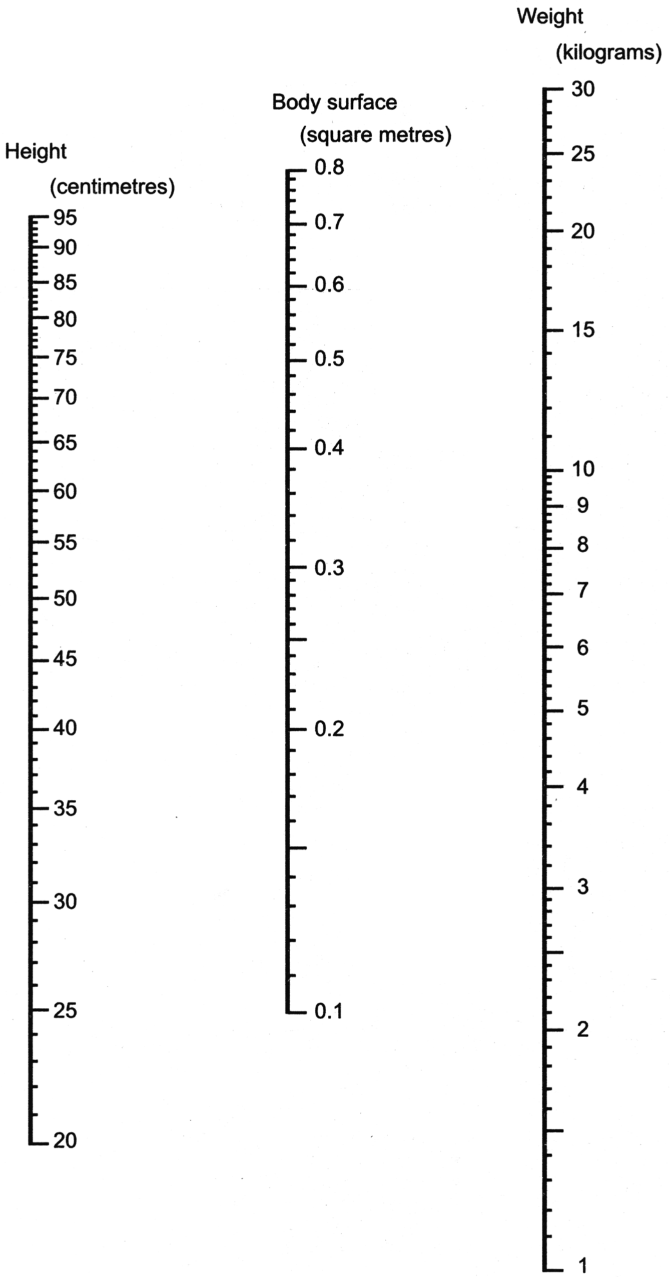

Figure 34.1 *Nomogram to determine the body surface (A) of children: Take a scale and place it across the values of height (cm) and weight (kg). The point where the middle scale (body surface) intercepts is the body surface in square metres*

Normal GFR value

Men: 140 mL/min (± 27)
Women: 112 mL/min (±20)

Procedure details

Perform the test in the morning. Give the patient three glasses of water to drink to guarantee adequate urine flow during the test period. Ask the patient to empty the bladder completely at the beginning, discarding this urine. Note the exact time (including minutes). Thereafter, all urine should be collected for the next 5 h. Immediately afterwards, obtain a blood sample for the creatinine determination. Send specimens, urine, and blood, to the laboratory, where the urine volume is accurately measured and the creatinine levels of serum and urine are determined. Calculate the clearance rate by the above formula.

Sources of error

- Faulty timing or improper collection of the urine specimen (most common).
- Improper hydration of the patient.
- Exercise during the test.

GASTRIC FUNCTION TESTS

The stomach is a reservoir that holds food, begins digestion, and produced a pasty **chyme** that is squeezed into the intestine. The use of **gastric analysis** as a diagnostic method has fallen out of favour, and while descriptions of new modifications continue to appear, they receive at best a half-hearted reception. The reason is relatively clear—variations in gastric secretion are so wide in healthy people that very little specific diagnostic information can be elicited by measurement in disease. Recent studies, however, indicate that basal acidity in patients with duodenal ulcer often exceeds the highest control values; hence, this measurement has diagnostic value. Measurement of **basal secretion** is suggested as the simplest and most useful procedure in the study of clinical gastric physiology. If basal elevated acidity exists, the test should be supplemented by a histamine stimulation test.

Although many textbooks have eliminated this material because of its questionable utility, we have chosen to describe the test here because of renewed interest. One should keep in mind that the physician or an experienced nurse collects the specimen. The laboratory technician is only involved in the visual physical examination, microscopic examination, and chemical examination, which include screening tests and titration for acidity. Microscopic examination of gastric juice is presented in Chapter 26 of Vol. 2.

Clinical significance

Gastric secretion contains hydrochloric acid, intrinsic factor, digestive enzymes (pepsin and lipase), and electrolytes. The secretion is under hormonal control. Analysis of gastric juice is important in the diagnosis of gastric ulcers, carcinoma of the stomach, pernicious anaemia, **Zollinger-Ellison syndrome,** and other disorders.

Specimen evaluation

Patient preparation: The patient should fast (no food or liquid for 12 h), is not permitted to smoke in the morning of or during the test, and should avoid any form of exercise.

Collection of specimen: A gastric juice specimen is obtained by inserting a lubricated gastric Levin tube, orally or through the nose. The attending physician or an experienced nurse collects the specimen. Two types of specimens arrive in the laboratory—gastric juice without stimulation (basal secretion), and following the subcutaneous administration of histamine dihydrochloride (maximal secretion).

Normal value

Gastric acidity:

Basal acidity (H^+): <2 mEq/L
Maximal acidity: 1–20 mEq/L

Gastric Fluid Laboratory Investigation of Gastric Fluid

Physical examination

Physical examination of the gastric juice is done without filtration. Report the physical conditions in the following format:

Amount

Measure the volume in a graduated cylinder. The normal volume is 50–80 mL. Over 100 mL volume is considered pathologic and could indicate a condition such as gastroparesis. Decreased volume is also considered to be abnormal.

Colour

Normal gastric juice is colourless. **Bile** will stain it **yellow-green** and **blood** will produce a **red-brown colour.** Blood may also appear black if digested, leading to a **"coffee-ground"** appearance. Presence of blood suggests a gastric ulcer or some other potential source of bleeding, e.g., trauma. If the presence of blood is suspected, perform the occult blood test (see chemical screening). Microscopic examination of gastric juice may also reveal the presence of red blood cells, which suggests bleeding. Usually the red cells are haemolysed.

Odour

Normal gastric juice has a sour odour. A faecal or rancid odour should be reported.

Fluid character

Standing gastric juice separates into three layers—mucus (top), opalescent fluid (centre), and sediment (bottom). The amount of mucus increases in gastric carcinoma, in gastritis, and pyloric obstruction. Sediment represents undigested food.

Chemical screening

Test for starch

Supplies

- Pasteur pipette
- Watch glass

Reagent

Potassium iodide solution (1% aqueous)

- You can dilute the iodine solution, sold as 'antiseptic agent, 2%' (see the label), two-folds with water in order to make it 1% strength. This is good for the spot test. This, however, contains some amount of **alcohol.**
- If you want to make it yourself, take a few potassium iodide crystals in a beaker with 100 mL water. Add to this 1 g of iodine. ***Caution*** Iodine is highly volatile.

Procedure

1. Place two drops of gastric juice in a watch glass.
2. Add iodine reagent on gastric juice held in the watch glass.
3. Observe for an instantly discernable blue colour that indicates presence of starch.

Test for blood

Reagent

***o-tolidine reagent* (4% aqueous)**

Dissolve 4 g *o*-tolidine in 95% ethyl alcohol (just sufficient to dissolve) in a 100-mL volumetric flask; add water to reach total solution of 100 mL.

Procedure

1. Add 4 drops of *o*-tolidine reagent in the gastric juice held in a watch glass.
2. Add 4 drops of glacial acetic acid and 4 drops of water. If colour develops, it is due to contamination.
3. Add 1–2 drops of hydrogen peroxide. A green or blue colouration suggests the presence of blood. Presence of blood can be due to trauma or gastric ulcer.

Determination of gastric acidity

The physician occasionally requests quantitative determination of gastric acidity by **titration.** Many laboratories prefer a centrifuged specimen, although others feel that this procedure removes some of the hydrogen ions through the sediment. In order to remove the gross food particles, filter the specimen through two layers of gauze. Use the filtrate for the determination of free acid by titration.

Principle

A known amount of gastric residue is titrated with 0.1 N NaOH to a pH of 3.5. If a **pH meter** is not available, add two drops of **Topfer's reagent,** which changes to a salmon colour when all the free hydrochloric acid is neutralized. The total acidity, however, is determined by titration using phenolphthalein as the indicator.

Reagents

- ***Sodium hydroxide solution (0.1 N):*** Stock sodium hydroxide (1.0 N) is diluted 10-fold. Alternatively, dissolve 4 g of NaOH in fresh distilled water to make 1000 mL of solution.
- ***Phenolphthalein solution (1%, alcoholic):*** Dissolve 1 g of phenolphthalein in 100 mL of 95% alcohol.
- ***Topfer's reagent (dimethylaminoazobenzene)-0.5% alcoholic solution:*** Dissolve 0.5 g of Topfer's reagent in 100 mL of 95% alcohol.

Procedure

1. Measure 10 mL of gastric juice specimen using a graduated cylinder and transfer to a porcelain-evaporating dish. In case of high acidity, mix 1 mL of gastric juice with 5 mL of distilled water.
2. Add 1–2 drops of Topfer's reagent.
3. Observe for colour change; a bright red colour will appear if free hydrochloric acid is present.
4. Add 1–2 drops of phenolphthalein to the gastric juice with Topfer's reagent.
5. Titrate with 0.1 N NaOH from a burette, mixing after each addition until the last trace of red colour disappears and is replaced by a canary yellow colour.
6. Read from the burette the number of millilitres of NaOH used. This represents the amount of free hydrochloric acid.
7. Continue the titration until the red/pink colour of phenolphthalein appears, titrating to the point at which further addition of alkali does not deepen the colour.
8. Take the burette reading (mL NAOH) for the total acidity, counting from the original reading.

Calculation

(a) Free hydrochloric acid (mEq/L)

$$X \times 10 \text{ (acid)} = 100 \times \text{mL alkali } (0.1 \text{ N NaOH} - 100 \text{ mEq/L})$$

or, X = mL 0.1 N NaOH × 10

Where, X = strength of free hydrochloric acid, 10 is volume of acid (gastric juice) taken for titration and 100 is conversion of 0.1 N NaOH to mEq/L.

(b) Total acidity (mEq/L)

$$Y = \text{mL of } 0.1 \text{ N NaOH} \times 10$$

Note If the volume of gastric juice (V) taken for analysis is different from 10, or the normality of NaOH is not exactly 0.1 N, apply the following formula for calculating free acidity and total acidity:

$$X \text{ (or } Y) \times V = N \times 100$$
$$X \text{ (mEq/L)} = (N \times 100)/V$$

Where, X is free hydrochloric acid (mEq/L), Y is total acidity (mEq/L) and V is the volume of gastric juice (mL).

(c) Combined acidity (mEq/L)

Total acidity (Y) – Free hydrochloric acid (X) = Combined acidity

Combined acidity is due to HCl and/or organic acids (e.g., lactic acid, amino acids, and acid salts).

Note Free and total acidity are determined for both basal secretions as well as for maximal secretion (stimulated).

Interpretation

Increased values of **basal acidity** (free and total) suggest the presence of a **gastric ulcer** (2–5 mEq/L), duodenal ulcer (>5 mEq/L), or **Zollinger-Ellison syndrome** (>20 mEq/L). Chronic gastritis may be associated with an increase in free HCl while achlorhydria is diagnosed by the lack of maximal total acidity (0 mEq/L). Higher values of **maximal total acidity** may be related to duodenal ulcer (20–60 mEq/L) and Zollinger-Ellison syndrome (>60 mEq/L). If there is no free HCl after administering maximal histamine, pernicious anaemia or cancer of the stomach may be considered. Presence of blood is a significant sign of ulcer.

PANCREATIC FUNCTION TESTS

The pancreas has both endocrine functions (enzyme secretion into the bloodstream, as in the case of insulin for carbohydrate metabolism) and exocrine function (enzyme secretion into the GI tract, as in the case of digestive enzymes). Pancreatic function tests may be performed to determine pancreatic function in people with diseases that affect the pancreas (e.g., pancreatitis, cystic fibrosis, or pancreatic cancer). In developed countries, use of computed tomography (CT) scans, abdominal ultrasound, endoscopy, and magnetic resonance imaging (MRI) have replaced much of the old-fashioned laboratory tests. They are less invasive, more comfortable for patients, and provide better information for treatment. However, these are expensive procedures and hard to adapt to the existing conditions of underdeveloped countries. The following are some of the laboratory tests currently used and many of them are effective for preliminary diagnosis.

Blood Test

Blood tests can evaluate the function of the gallbladder, liver, and pancreas. Levels of the pancreatic enzymes **amylase** and **lipase** can be measured. Elevated lipase is more specific for damage to the pancreas, as in the case of acute pancreatitis. Blood tests can also check for signs of related conditions including infection, anaemia (low blood count), and dehydration. The physician makes special requests for the above enzymes to the biochemistry laboratory.

Faecal Test

Faecal trypsin

Trypsin is a digestive enzyme secreted by pancreas. It is present in the stool of a normal person. Trypsin has the ability to dissolve **gelatine** (a protein), which is used on X-ray films. Small strips of exposed X-ray films are dipped into a test tube holding patient's stool suspension and left overnight. If the stool clears off the film after overnight exposure, the patient is considered normal. Lack of clearance of the film is considered as abnormal and is related to the deficiency of trypsin or dysfunction of pancreas.

Faecal elastase test

The **faecal elastase test** is another test of pancreatic function. The test measures the levels of elastase, an enzyme found in fluids secreted by the pancreas. Elastase digests (breaks down) proteins. In this test, a patient's stool sample is analysed for the presence of elastase. Commercial kits are used which are based on EIA procedure.

Laboratory Investigation of Pancreatic Juice

Pancreatic juice (duodenal content) analysis can assess the **exocrine function** of the pancreas. The sample is obtained from a fasting patient in a similar way as described under the gastric function test after inserting the stomach tube further down into the duodenal region. An experienced nurse, under the supervision of a physician, may perform this procedure.

The basic method of pancreatic juice analysis has been further improved by the **secretin stimulation test.** The secretin stimulation test measures the ability of the pancreas to respond to secretin. Secretin is a hormone made by the small intestine. Secretin stimulates the pancreas to release a fluid that neutralizes stomach acid and aids in digestion. Secretin is administered after the initial pancreatic juice sampling. The contents of the duodenal secretions are aspirated (removed with suction) and analysed over a period of about 2 h. The laboratory examines and analyses the duodenal secretions and report the results to the physician for diagnosis. People with diseases involving the pancreas (e.g., pancreatitis, cystic fibrosis, or pancreatic cancer) might have abnormal pancreatic function.

Laboratory investigation involves physical examination of the specimen, measurement of specific gravity, microscopic examination, and chemical screening. Normal pancreatic juice is colourless or yellow with a specific gravity of 1.007–1.042 and pH of 8.0–8.3. Presence of **trypsin** and **amylase** is expected.

Physical examination

The report should include colour, specific gravity (use hand refractometer), and pH as done for other body fluids (Chapter 26 of Vol. 2).

Chemical screening for pancreatic enzymes

Deficiency of pancreatic digestive enzymes (trypsin, amylase, and elastase) indicates a pathologic condition of the pancreas.

Trypsin Make the duodenal content slightly alkaline with 2% (aqueous) sodium carbonate. Evaluate with **phenolphthalein,** which gives a faint pink colour. Place small amounts of the duodenal fluid in three test tubes labelled 'N' (negative control), 'P' (positive control), and 'T' (test). Boil the first tube ('N') and add trypsin to the second ('P'). Place small pieces of gelatine square in each. Incubate overnight at room temperature or in an incubator (35°C). Gelatine will dissolve in the tubes marked 'P' and 'T' (if normal). Alternatively, cut-strips of exposed X-ray films can be used in place of gelatine strips. Clearing of the film indicates normal trypsin activity.

Amylase Dilute the duodenal content 10-fold with saline. Mix equal portions (2 mL each) of the diluted duodenal content and 1% starch solution in a test tube. Incubate for 30 min at 37°C. Use 1 drop of **iodine solution** (1 g iodine and 2 g potassium iodide dissolved diluted to produce 100 mL of solution). A blue colour indicates amylase deficiency.

Elastase Several reports have indicated that faecal elastase determination is a new, sensitive, and specific non-invasive pancreatic function test. Commercially available kits are now available which are based on immunologic principles (ELISA, Roche Diagnostics).

TEST FOR MALABSORPTION

Malabsorption tests are performed to determine if a patient has dietary malabsorption or maldigestion and to help differentiate between these two conditions. Malabsorption occurs when the gastrointestinal (GI) tract cannot absorb up a dietary compound. This may be caused by malfunction of the organ or lack of important digestive enzymes. Genetic disorders could lead to this situation or may be caused by injury to the tissue that provides the enzyme (i.e., the **pancreas** in cystic fibrosis), alterations in pH that make the enzymes inactive, or due to surgery. In general, clinicians speak of both disorders as malabsorption disorders since they both result in a lack of absorption of nutrients.

D-xylose Absorption Test

The D-xylose absorption test measures the level of D-xylose, a type of sugar, in a blood or urine sample. This test is done to help diagnose problems that prevent the small intestine from absorbing nutrients in food. The intestines normally absorb the **pentose** sugar D-xylose easily. When problems arise with absorption, the intestines do not absorb D-xylose. Hence, its level in blood and urine remains low due to the abnormality. The purpose of the test is to:

- Check to see if malabsorption syndrome is causing symptoms, such as chronic diarrhoea, weight loss, and weakness. A person with malabsorption syndrome is unable to absorb nutrients, vitamins, and minerals from the intestinal tract into the bloodstream.
- Find the cause of a child's failure to gain weight, especially when the child seems to be eating enough food.

The D-xylose absorption test is useful in the differential diagnosis between malabsorption that is intestinal versus pancreatic in origin. In malabsorption due to pancreatic insufficiency, absorption of the D-xylose will be essentially normal. In intestinal dysfunction, D-xylose absorption will be substantially decreased. The test results are abnormal in 80% of patients with malabsorption syndrome. The test may be invalidated by renal retention, myxedema, or incomplete urine collection.

Principle

The D-xylose absorption test is a convenient method of **diagnosing malabsorption.** The amount of **D-xylose** in urine samples is measured before and after the patient drinks 25 g of a D-xylose solution, and the amount excreted in the urine over the following 5-h period is determined for comparison. Chemical determination depends on dehydration of the **pentose D-xylose** to **furfural** in the presence of acid and heat followed by condensation of furfural with **p-bromoaniline** to form a pink coloured compound. At 70°C, very little furfural is formed from precursors other than the available pentose. Thiourea acts as an antioxidant, which helps prevent the formation of interfering coloured compounds.

Equipment and supplies

- Spectrophotometer
- Water bath (70°C)

Reagents

- ***p-Bromoaniline colour reagent:*** Saturate glacial acetic acid with thiourea by adding approximately 4 g of thiourea to 100 mL of glacial acetic acid, then decant. The clear supernatant is stable and may be prepared in advance. The colour reagent is prepared fresh before use by adding 2 g of p-bromoaniline to 100 mL of glacial acetic acid saturated thiourea.
- ***Saturated benzole acid solution:*** Dissolve 2.5 g benzole acid (CP) in 1000 mL distilled water by boiling in a 1500 mL beaker; cool and make up loss by evaporation.
- ***D-xylose stock standard (2 mg/mL):*** Place 500 mg of D-xylose into a 250 mL volumetric flask. Add sufficient benzole acid solution to dissolve the D-xylose and dilute to the mark with saturated benzole acid. Keep in a refrigerator.
- ***D-xylose working standards:*** Dilute stock D-xylose standard with saturated benzole acid as given below. Keep the solutions refrigerated.
- 5 mL of stock standard diluted to 100 mL. This is equivalent to 0.1 mg/mL.
- 10 mL of stock standard diluted to 100 mL. This is equivalent to 0.2 mg/mL.

Standardization procedure: With each run, two standard control levels are analysed in the same manner as are the test unknowns. The standard used in the calculations should be that one whose net absorbance is closest to the net absorbance of the unknown test. To check the linearity and chemistry of the procedure, treat the remaining standard as an unknown and calculate its concentration to see how well it checks with its known concentration.

Patient preparation

The health professional must note the **routine medications** taken by the patient. Some of them may **interfere** with the test. Instruct the patient to refrain from eating or drink anything except water for 8–12 h before the test. Children younger than 9 years old should not eat or drink anything except water for 4 h before the test. Asking patients to collect urine or faeces over a long time period may decrease adherence. It is important to make sure that the patient understands the test and the necessity of his/her adherence to the instructions. Clinical laboratories will reject any samples that appear to have been collected or stored incorrectly.

Aftercare

Some amount of **aftercare** is needed when the test is done. Some patients may feel sick after the procedures since they are being exposed to compounds that they may have trouble absorbing. Nurses should be careful to discuss any side effects with the patient beforehand, and the patient should be given the smallest amount of substance possible to avoid problems. In addition, patients may be malnourished and need something to eat and drink once the procedure is over.

Specimen collection

1. In the morning, the patient should void to empty the bladder after having fasted, as described earlier. This urine is kept as a sample for the pre D-xylose.
2. The patient is then given 25 g of D-xylose dissolved in 250 mL of tap water. Immediately the patient is given another 250 mL of water. Note the time.

   Note For children weighing less than 25 kg, give 0.5 g D-xylose per 225 g body weight.
3. Collect and pool all urine over the next 5 h (some labs collect over 24 h). Keep the urine on ice or refrigerated prior to sending to the laboratory.
4. Many laboratories conduct blood analysis simultaneously with the urine analysis. Blood is collected by venepuncture. For adults, a blood sample is usually taken 2 h after drinking the solution. For children, a blood sample may be taken 1 h after drinking the solution. Another blood sample may be drawn 5 h after drinking the solution.

Specimen Processing

1. Measure the volume of urine collected during the 5 h period.
2. Prepare two dilutions of urine specimen:
   - 1 : 50 = Mix 0.1 mL of urine with 4.9 mL of water.
   - 1 : 200 = Mix 0.1 mL of urine with 19.9 mL of water.
3. Pipette the following into four tubes labelled as 'SB', 'ST', 'UB', 'UT' that correspond to standard blank, standard test, unknown blank, and unknown test, respectively.

Reagent	Standard		Unknown	
	Blank (SB)	Test (ST)	Blank (UB)	Test (UT)
Working Standard	0.5	0.5	–	–
Dilute urine	–	–	0.5	0.5
p-Bromoaniline	2.5	2.5	2.5	2.5

4. Mix and place all of the test reaction tubes in a 70°C water bath for 10 min and all blank reaction tubes in dark at room temperature.
5. Following incubation, cool the test reaction tubes in running water until they reach room temperature.
6. Place all tubes (tests and blanks) in dark for 70 min.
7. Read the absorbance at 520 nm of all the tubes (standard blank and test plus unknown blank and test) against the water blank.

Calculation and results

$$\text{Excretion of D-xylose (g)} = \frac{A_t}{A_s} \times C_s \times F \times \frac{V}{1000}$$

where, A_t is the net absorbance of the test solution; A_s is the net absorbance of the standard; C_s is the concentration of the standard; F is the dilution factor (50 or 200), V is volume of urine during 5-h period, 1000 is used to convert milligrams to grams in the calculation.

Note

- Consider only that standard (0.1 or 0.2 mg) whose net absorbance reading is closest to the net absorbance reading of the unknown or test solution.
- Urine with net absorbance greater than ~ 0.9 should be repeated on a greater dilution (more than 200), as the colorimetric chemical reaction is not linear at higher concentrations.

Interpretation

Normal values

Adult

Dosage: 25 g
Discharge:
(a) Under 65 years: 4 g/5 h urine
(b) Above 65 years: 3.5 g/5 h urine

Child

Dosage: 0.5 g/225 g body weight
Discharge: 16–33% of ingested D-xylose

D-xylose absorption should be greater than 4.0 g/5 h in an adult given a 25 g dose of D-xylose (or 1.2 g/5 h with a 5 g dose). The D-xylose test will be normal if the patient has normal absorptive capacity in the intestine, or if the patient has malabsorption that is caused by a pancreatic problem. It will be low if the patient has celiac disease, tropical sprue, Crohn's disease, advanced ATDs, or pellagra **(niacin** deficiency). Falsely low results with the D-xylose test will be seen if the patient has been vomiting, has gastric stasis, fluid build up (ascites), fluid retention (edema), or bacterial overgrowth. There is a decrease in urinary excretion of D-xylose with aspirin, colchicines, digitalis, MAO inhibitors, food consumption, neomycin, and opiates. In addition, excretion is lower in those with impaired renal function and in elderly patients.

REVIEW QUESTIONS

1. What are the functions of the following organs? Liver, Kidney, Pancreas, and Stomach
2. At clinic, a patient had total protein and albumin assays performed on his blood. When the physician was given the test results, total protein 6.5 g/dL and albumin 3.0 g/dL, she asked the technician to calculate the A/G ratio. What is the ratio?
3. Dr. Tiwari ordered the following chemistry tests for a patient: creatine kinase, AST, and cholesterol fractions. Which of the following do you think Dr. Tiwari suspects – heart disease, renal disease, liver disease, or thyroid disease?
4. Name three enzymes that are useful in diagnosing liver disease.
5. How would you assess the malfunction of the liver, pancreas, kidney, heart, and stomach in a clinical biochemistry laboratory?
6. What are the two major types of serum proteins and what are their functions?
7. What is glomerular filtration rate? What information would you require to calculate creatinine clearance rate?
8. Which clinical conditions require gastric function tests? How would you report gastric acidity?
9. What are the enzymes secreted by pancreas? Why is it necessary to make the duodenal content slightly alkaline before testing for trypsin?
10. Which enzymes are most useful in diagnosing myocardial infarction?
11. What is the significance of D-xylose test? How is it done?
12. Why are two standards selected for testing the D-xylose excretion?
13. Define the following regarding the gastric function test: basal acidity, maximal acidity, free acidity (HCl), total acidity, combined acidity.

Chapter

35

Therapeutic Drug Monitoring and Clinical Toxicology

Kanai L Mukherjee and Piyali Basu

Chapter Outline

DRUG AND DRUG ADDICTION

Drug is a chemical substance used to treat a patient or prevent diseases. Many of the drugs are also used for well-being of a patient or a normal person. The chemical substance may be synthesized or extracted from plant. Some of the drugs may be abused that leads to addiction. Drug addiction is a chronic and relapsing brain disease due to which the patient undergoes compulsive drug seeking. Use of these drugs is illegal. But some of the legal habit-forming substances include cigarette smoking and drinking. The obvious sign of drug addiction is the strong craving, even knowing its harmful effects which make it difficult to stop. Drug addiction often leads to a drastic change in a person's thinking and judgement. Hence a person under drug influence should not drive or operate machines that require right judgement. Drug addiction can cause serious, **long-term consequences;** this includes family relation, employment, law or mental status.

Drug abuse during pregnancy can affect the unborn baby and may even lead to neonatal abstinence syndrome where the baby can suffer from dependence and withdrawal symptoms after birth. Drug addiction is a social nuisance. The drug addict may be referred to a doctor, counsellor, or support group to overcome drug addiction and stay drug-free.

Gross Drug Abuse Symptoms

- Behavioural changes
- Cravings
- Spending money to buy drugs beyond your means
- Withdrawal symptoms when you attempt to stop taking the drug
- Risk everything to get the drug

Specimen in Drug Testing

The most common specimens for drug testing (as applied to employees) are:

- **Urine:** The urine composition reflects drug abuse over last 2 or 3 days. This includes amphetamines, cocaine, and opiates. Other drugs like marijuana and cannabinoids will be detectable for several weeks.
- **Saliva:** It is another favorable specimen which is easy to get. It detects the drugs used in the previous 24 h.
- **Hair:** Hair samples are taken for detective investigation like on dead personnel. It is good for 2–3 months. Many employers use hair sample in place of urine in order to meet their mandatory drug testing. Urine needs to be attended promptly as it decays with time, while hair does not.

Hair samples may be used as an alternative to urine testing for employment or accident drug testing. Sweat testing may be used as a court-ordered monitoring tool in those who have been convicted of drug use, while saliva is often used by the insurance industry to test insurance applicants for drug use. Blood is most frequently used for alcohol testing.

Sports Testing

Use of drugs for performance enhancement, often referred as "Dope", has become an important topic in virtually every sport. Doping is an effort to gain a competitive advantage. It helps to cope stress, injury and physical pain. But in the long run it causes mental illness. Importantly, these performance enhancing drugs are no longer restricted to prescription medications (legal or illegal), such as anabolic steroids, but is extended to "dietary supplement" available in health food stores and online.

DIAGNOSTIC SCREENING IN EMERGENCY

Many employers want their work place to be "drug free". Hence a pre-employment drug testing has become a routine. The usual practice is that the employer hires the person conditionally as long as his/her drug testing comes out negative. This policy, however, is not always implemented by independent employers but is mandated by many government agencies. Yet jobs like taxi driving, airplane pilot, truck driver, etc., must be strictly screened for safety reasons before their license is issued.

"Urine testing" is the most common procedure for drug screening. The procedure is easy to follow and is cost effective. In addition, it is typically able to screen a wide variety of illicit or prescribed drugs.

The alternate procedure is the oral fluid (saliva) testing. This is becoming more popular as the specimen can be collected on site thereby reducing cost and time associated with other collections.

Hair testing is a good choice for the employer with minimal facilities. Specimen collected can be subjected to laboratory assay later and does not deteriorate during storage. In many cases, more than one specimen is taken for confirmatory report.

Drug testing enables an employer to hire a risk-free dependable candidate. In addition, the procedure improves the morale and productivity of the employee, and decreases the accidents, theft, absenteeism, and down time. If testing reveals no drugs, or drugs in amounts below the established cutoffs, then the results are usually reported as **"none detected."** A negative result does not necessarily mean that the person did not take a drug at some point. The drug may be present but below the established cutoff, **the drug may have been already metabolized and eliminated** from the body, or the test method does not detect the particular drug present in the sample.

Emergency Drug Screening

Drug overdose is an emergency. The person may be found unconscious, or in a life-threatening state, or a state of bizarre behaviour. Until the cause is established, toxicological tests are recommended. These tests confirms overdose due to drug or toxic substances which covers broader area of substances beyond just self-administered drugs. If the person is dead, the cause of death needs to be established and hence these toxicological tests can be done post-mortem during an autopsy. In addition, the clinical laboratory may have to handle cases of forensic investigation and accordingly will collect and handle samples to meet their needs of testing and reporting. To establish the cause of death, the tests are extended up to 4 days (96 h). In such a case urine and blood samples may also be tested.

A group of emergency **toxicology testing** is first activated whose report is available within **one hour.** This includes the assay of acetaminophen, salicylates, and ethyl alcohol which may or may not have been prescribed. Many of the findings may be nonintentional overdose of a prescription drug. This includes iron toxicity in children, carbamazepine (prescribed for seize and nerve pain), digoxin (heart medication), lithium (psychiatric drug), phenobarbital (prevent or treat seizure), theophylline (shortness of breath), valproic acid (bipolar disorder and migraine headaches). So, the test result must be tallied with history of medication.

After the initial emergency testing, a second round of tests will include other substances of drug abuse. These include cocaine, opiates, barbiturates, antidepressants and amphetamines. Before rushing into conclusion, the healthcare personnel may also test for glucose in order to determine whether the person is hypoglycaemic or hyperglycaemic. Additional tests will include kidney function tests, liver function tests and electrolytes. A composite image can only determine the cause of coma of a patient.

COMMENTS ON COMMONLY USED DRUGS

Nicotine is a well-accepted **mild drug,** found in **tobacco** that has sedative and vasoconstrictive properties. Similarly, **caffeine,** found in coffee, tea, chocolate, and cola, is a commonly used "stimulants". In the same spectrum, **alcohol** is a sedative that comes from the natural fermentation process of various grains such as rye, barley, corn, and potatoes, as well as grapes. Yet they are not considered "dangerous" if used within limits. This is especially true for alcohol.

Many of the **mind-altering drugs** have medicinal values. But they are often misused by public and hence declared **"illegal"** unless prescribed by a doctor. Some of these drugs are plant-based while others are synthesized. For example, heroin, a sedative-hypnotic, comes from the opium poppy; cocaine, a stimulant, from coca leaf (a shrub called *Erythroxylon coca)* and marijuana, a sedative, from hemp plant *(Cannabis sativa).* These plant compounds have some medicinal properties. When refined, heroine becomes morphine, which is used to relieve severe pain. Cocaine was used medically in the 20th century as a local anaesthetic. Argument continues today over the medicinal uses of marijuana, which has been shown in some experiments to reduce the intraocular pressure of glaucoma, as well as the nausea caused by some cancer treatments. In recent years, more dangerous synthetic drugs have merged into the market of illegal drug trade and the manufacturers are constantly trying to alter the composition of the drug in order to avoid prosecution and outreach the illegal consumers.

Illegal drug trade is a global problem. Drug trafficking has increased crimes and various illegal activities. This black market economy has disturbed tranquillity in all phases of social living, and hence an individual family feels insecure. In short drug-related activities have placed additional demands on the healthcare system. Consequently, clinical toxicology laboratories have moved beyond their traditional activities and now are routinely performing urine testing of drug abuse for other clinical services such as obstetrics for pregnant drug abusers, paediatrics for their newborns and drug-dependency treatment programmes. Such testing usually focuses on a small panel of the most frequently abused drugs such as cocaine, cannabinoids and the benzodiazepines, rather than the broad-spectrum screening performed in emergency toxicology. In the following sections, we chose to discuss only the most important ones. Facilities for the analysis of drugs may not be available in peripheral laboratories but the technicians may be involved in specimen collection and the account presented here attempts to make them conscious of their crucial role. Further discussion about laboratory analysis of illegal drugs will be presented later in this chapter.

CLASSIFICATION OF ILLEGAL DRUGS AND THEIR USES

Following is the classified listing of illegal drugs.

Hallucinogens

- Marijuana/Cannabis
- Ecstasy/MDMA
- K2/Spice
- Ketamine
- LSD
- Peyote and Mescaline
- Psilocybin
- Steroids
- inhalants

Depressants

- Barbiturates
- Benzodiazepines
- GHB
- Rohypnol

Stimulants

- Amphetamines
- Cocaine
- Khat
- Methamphetamine

Narcotics

- Fentanyl
- Heroin
- Hydromorphone
- Methadone
- Morphine
- Opium
- Oxycodone

Plant products

Marijuana, hashish and other cannabis-containing substances are obtained from plants. They are consumed in more than one way: raw paste in drinks, smoking, eating, or inhaling the vaporized form of the drug. These may be used alone or along with alcohol or other illegal drugs. In drug testing they are often the first drug tried.

Synthetic drugs

This class of drugs includes imitation (or synthetic) **cannabinoids** and **substituted cathinones.** These two illegal synthetic drugs are common in drug addicts. These are taken orally or through injection. The effects of these drugs can be dangerous and unpredictable, as there is no quality control and some ingredients may not be known. Synthetic cannabinoids (also called "K2" or "Spice") are also smoked after sprayed on dried herbs or can be prepared as a herbal tea. Despite manufacturer claims, these are chemical compounds rather than "natural" or harmless products. Irrespective of the source, plant or synthetic, all harmful habit-forming drugs are capable of producing the "high" state and can become life threatening. Some of them are sold in the market with a false pretext like "bath salts" which can be psychoactive similar to amphetamines or cocaine. Substituted cathinones can be taken orally, inhaled or injected. They can be addictive and can bring severe intoxication and may even bring death through overdose.

Inhalants

Some commonly inhaled substances include glue, paint thinners, correction fluid, felt tip marker fluid, gasoline, cleaning fluids and household aerosol products. One may find kick by inhaling these substances but they are harmful for the body and can be addictive. Due to the toxic nature of these substances, users may develop brain damage. Signs and symptoms of inhalant use vary, depending on the substance.

Hallucinogens

These are the drugs, like LSD, that causes **hallucination.** Use of hallucinogens can produce different signs and symptoms, depending on the drug. The most common hallucinogens are lysergic acid diethylamide (LSD) and phencyclidine (PCP). Hallucinogenic drugs create most of the same physical effects as stimulant drugs. They increase heart rate, dilate the pupils and increase the blood pressure leading to sleeplessness.

Barbiturates and benzodiazepines

Barbiturates and benzodiazepines are prescription drugs used for the treatment of depression. But they are also abused in search for a sense of relaxation and combat stress. The examples of barbiturates are phenobarbital, amobarbital (Amytal) and secobarbital (Seconal Sodium). Various sedatives are examples of benzodiazepines. This includes substances such as diazepam (Valium), alprazolam (Xanax, Niravam), lorazepam (Ativan), clonazepam (Klonopin) and chlordiazepoxide (Librium).

Meth, cocaine and other stimulants

Stimulants are the drugs that **"stimulate"** the central nervous system of the body. Stimulant is used in treating many clinical conditions like depression, asthma, obesity and narcolepsy. Though each stimulant has its unique effect but all have one common feature as to increase the heart rate, blood pressure and body temperature. It also increases alertness, decreases fatigue and prolong physical activity. This is the reason they are commonly referred as "uppers". Common stimulants include amphetamines, meth (methamphetamine), cocaine, and methylphenidate (Ritalin). They are often used and abused in search of a "high", or to boost energy, to improve performance at work or school, or to lose weight or control appetite.

Club drugs

These drugs are commonly used in clubs and parties but it is the generic term for mind-bogging effect. Most of these drugs are illegal. The most commonly used club drugs are MDMA (ecstasy), LSD, gamma-hydroxybutyric acid (GHB), flunitrazepam (Rohypnol, or roofie) and amphetamine. These drugs are mostly illegal and they have different effects and possible complications. Many of these drugs are mixed with other intoxicants, making them more dangerous.

Narcotic painkillers

There are a variety of strong medicines, including narcotic drugs, which can help relieve pain. Often these pain relievers are combined with acetaminophen (Tylenol). Always keep in mind that **Tylenol does not mix with alcohol.** It causes liver damage. Many of the pain-killing drugs are produced from opium or are made synthetically. The use of narcotics for minimizing pain has become a common practice in hospital situation. This includes heroin, morphine, codeine, methadone and oxycodone. Some people who've been using opioids over a long period of time may need physician-prescribed temporary or long-term drug substitution during treatment.

TOXICOLOGY LABORATORY AND FORENSIC MEDICINE

Clinical toxicology in laboratory medicine is defined as the **analysis of poisonous substances** in human biological fluids for the purpose of patient care. The poisonous substances are drugs that were taken beyond therapeutic limits, have been ingested accidentally or may be related to criminal or suicidal intent. Hence the technician must bear in mind that the investigation may have legal implications.

Poisoned patients account for approximately 10% of all acute admissions to medical wards in hospitals. These include self-poisoning attempts, accidental poisoning or due to administration of illicit drugs. Many victims succumb to the poison before even reaching the hospital. Pregnant patients with a chemical dependency particularly that of cocaine, are at high risk for obstetric complications. **Urine testing for drugs** of abuse provides evidence for the diagnosis of drug dependency and subsequent monitoring of drug use during pregnancy. The majority of common drug interactions are known, and data should be available through hospital records.

In case of **therapeutic drug monitoring,** the biochemistry laboratory carefully tracks the level of the drug in order to avoid overdose. The latter may lead to various side effects or may even threaten the life of the patient.

Toxicological investigations are not routinely performed in the biochemistry laboratory. In this chapter only a few common procedures will be discussed. Within the next few years, as the **'potently dangerous drugs'** are increasingly used for heart patients, patients under chemotherapy and for difficult-to-cure diseases, many of these procedures may become routine in the laboratory.

Forensic medicine, however, goes into greater detail in order to identify the wrongdoer, while the goal of the biochemistry laboratory is to establish the cause in order to assist the clinician to resolve a diagnostic dilemma and indicate the prognosis of the poisoned patient. It is a common incidence to find a patient in the emergency room in coma and the laboratory has to provide some kind of clue towards the possible identification of the toxicant so that appropriate measures can be taken. Forensic drug chemistry is simply chemistry as it is applied to the identification of illegal substances, explosives and poisonous substances.

This practice uses a variety of chemical analysis methods to conduct both presumptive and confirmatory tests on seized material suspected to be or contain illegal substances. Results from this analysis often serve as the basis for criminal proceedings and help to determine sentencing for convicted offenders. This practice uses a variety of chemical analysis. Results from this analysis often serve as the basis for criminal proceedings and help to determine sentencing of the convicted offenders.

DRUG SCREENING IN CLINICAL CHEMISTRY LABORATORY

A **drug screen** is a compromise among rapid turnaround time, analytical specificity and sensitivity. It may be a combination of qualitative and quantitative analyses. Following five methods for initial screening suit to the existing conditions of peripheral laboratories with limited facilities:

- Spot test with chromogenic reaction that can be done on paper or tile.
- Microdiffusion tests for volatile compounds.

- Quick chemical test in test tubes with visual observation.
- Colorimetric assay for chemical reactions that yields colour for quantitative assay.
- Immunoassays with agglutination or colour reaction as a result of immunologic reaction.

Reference laboratories have better capabilities to detect smaller quantities of the analytes as well as to their better identification for specific treatment. These analyses require sophisticated instruments such as Thin Layer Chromatography (TLC), Gas Chromatography (GC) and High Pressure Liquid Chromatography (HPLC). These techniques have been described in the Chemistry section of this book.

Specimens

The specimens most frequently submitted for analysis are urine, whole blood/plasma, clotted blood/serum and gastric juice. Drug screen in most clinical laboratories are performed on urine. The advantage of urine is that a large volume can be obtained, allowing analysis of drugs in low concentration. However, urine contains metabolites which may complicate identification. For those drugs that are extensively metabolized, the parent drug may not be present. In addition, urine tests are qualitative, as drug concentrations in urine correlate poorly with clinical effects. Blood (serum and plasma) although limited by sample volume, is the specimen of choice for quantitative analyses of those drugs for which there is a correlation between drug level and toxicity. However, quantitative tests are usually more time consuming.

Blood is obtained by venepuncture and urine specimen is collected by ‘random clean catch’. **Gastric aspirate** is obtained through **gastric lavage.** Gastric aspirate has advantages in that the unchanged drug or poison may be found in the aspirate, often in much higher concentrations than the blood or urine. The process of cleaning out the contents of the stomach by gastric lavage has been used earlier which successfully eliminates poisons from the stomach. Such devices are normally used on a person who has ingested a poison or overdosed on a drug. Gastric aspirate specimen should be obtained before, not after washout. Gastric lavage is performed only by experienced nurse or by the physician.

Presumptive and Confirmatory Tests in Drug Investigation

There are two kinds of tests: **presumptive** and **confirmatory.** Presumptive tests are less precise and used in the initial screening. Use of handheld simple colorimeter in the field is recommended. A simple colour reaction may lay the guideline for further laboratory investigation. Once the presumptive test reveals that a suspected substance is present by colour reaction, the technician finds a clue for further investigation with a definitive approach. Presumptive testing by law enforcement is typically followed up with laboratory tests that confirm with certainty the presence of the suspected substance.

The presumptive test is followed by **confirmatory testing.** This further confirms the involvement of drug. The test is repeated in a well-equipped laboratory, using more precise instruments like gas chromatograph, mass spectrophotometer, and infrared spectroscope capable of identifying newer substances. During confirmatory testing the technician may require a multistep process to separate the individual compounds, determine the chemical characteristics of the compounds, and perhaps make a positive identification.

LABORATORY ASSAY OF DRUGS AND POISONING

Microdiffusion Analysis

For the routine laboratory, the **microdiffusion technique** is most helpful in the screening of **volatile compounds.** When this is supplemented with colorimetry, a wide range of drug-related investigations can be covered.

Microdiffusion analysis is a simple procedure in which a volatile poison is absorbed on a solvent. The solvent with the poison is then subjected to laboratory analysis in order to identify and quantitate the volatile poison. A simple Conway microdifrusion dish (Figure 35.1) is used which consists of two round, concentric chambers moulded into a porcelain or glass dish that can be sealed by a glass plate. The inner well has a lower wall than the outer rim of the dish so that the plate can be properly sealed. The outer well holds the sample (1–5 mL, of blood or urine), and the inner well holds the 'absorbent' which traps the volatile substance. The absorbent is a solvent capable of capturing volatile substances. The specimen and the trapping solution are in contact with the same atmosphere when the chamber is sealed. This allows the microdiffusion to occur. After the sample and absorbent are added to the proper well, the dish is sealed with a viscous sealant (stopcock grease or petrolatum jelly) material and a ground-glass cover plate. The substance to be separated, because of its vapour pressure, leaves the specimen and enters the atmosphere, from which it is absorbed by the trapping solution.

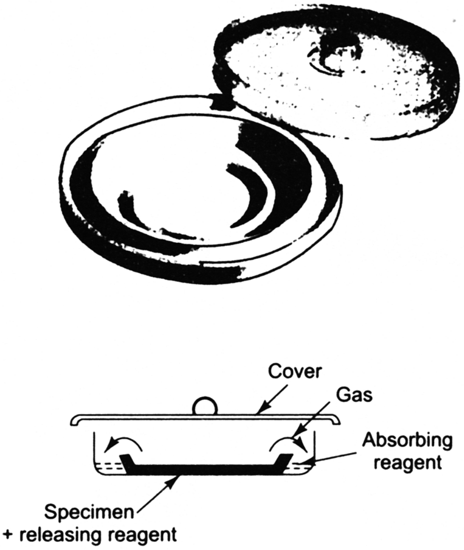

Figure 35.1 *Conway microdiffusion dish*

The microdiffusion process is usually carried out at room temperature for the desirable length of time as recommended in the procedure. The process of diffusion can be expedited by applying gentle heat. The process of gaseous diffusion continues until equilibrium is reached. The absorbent may show a colour change which is noted and this may help in the identification of the volatile substance under study. The microdiffusion procedure is applied in the analysis of carbon monoxide, cyanide and ethanol (Table 35.1).

Table 35.1 *Summary for analysis of volatile substances by microdiffusion method*

Toxic Substance	Absorbing Solution	Test (Colour Change)
Volatile alcohols and acetone	Potassium dichromate	Orange colour to green orange
Carbon monoxide	Palladium chloride	Black film of palladium
Cyanide	Sodium hydroxide	Pyridine-barbituric acid: red

Screening for Ethanol

Alcohol (ethanol, C_2H_5OH) is among the most widely consumed drinks. It is also volatile. Low doses of alcohol may be stimulating but heavy alcohol consumption leads to various forms of diseases. At the same time alcohol is undoubtedly the most commonly abused drug. During the past decade the consumption of alcohol in India has increased many-fold. This also includes 'country-made' alcohols. If the alcohol is contaminated with more harmful alcohols (e.g., methyl alcohol), which are unfit for consumption, it may lead to dangerous consequences on a mass scale.

Alcohol has a short half-life, thus most patients will show a fall in plasma concentrations between 2 and 5 mmol/L/h unless there is impairment of hepatic blood flow.

Specimen

The commonly used specimens are blood and urine.

Chemical oxidation of ethanol (and acetone) with dichromate

This method can be adopted easily by any laboratory.

Principle

Alcohols, aldehydes and other volatile substances are released from the sample and absorbed in acid dichromate solution placed in the central well of a microdiffusion dish. The dichromate is reduced producing a colour change from orange to green-coloured chromic ion (for alcohol) or blue (for acetone). This colour change constitutes a rough qualitative indication of the presence of alcohol or acetone in the specimen (blood or urine). ***Note*** The method is non-specific. The reaction occurs with other alcohols and paraldehyde. More specific **enzymatic method** for the analysis of alcohol is presented later in this chapter.

Reagents

- ***Potassium dichromate reagent:*** Dissolve 1 g of potassium dichromate in 500 mL of water in a 2-L beaker with graduations. Dissolve by stirring with a glass rod. Add 0.1 g of silver nitrate to the solution. Then carefully and slowly add 500 mL of concentrated sulphuric acid. Add a little at a time and mix before adding more. Store the solution in a tightly capped dark bottle. This solution is stable for 1 year.
- Saturated potassium carbonate solution in water.
- Control solution

Procedure

1. Take two microdiffusion dishes and label them as ‘T’ (test) and ‘C (control).
2. Add 0.5 mL of potassium dichromate reagent to the centre well of each microdiffusion dish.
3. Place 1–2 mL of the specimen in the outer well of the plate marked ‘T’ and 1–2 mL of the control solution in the outer well of the plate marked ‘C.
4. Add 1 mL of saturated aqueous potassium carbonate solution in the wells containing the sample and control.
5. Place the covers on the dishes and heat at 50°C in an oven or on a hot plate (not more than 70°C).
6. Observe the colour change in the potassium dichromate solution.
7. A green colour developing in 30 min indicates the presence of alcohols (methanol, isopropanol and aldehydes) or other reducing substances. The control should show a positive reaction.
8. A positive reaction may be due to methanol, isopropanol, aldehyde or acetone. Rule out methanol by its specific test described later in this chapter. Perform the acetone spot test with the urine specimen, as described here. If positive, presence of isopropanol may be suspected. ***Note*** **Acetone** is a metabolite of **isopropanol** and its presence in urine suggests the ingestion of isopropanol (an alcohol).

Analysis of alcohol (ethanol) by enzymatic method

Commercially available **ethanol** (alcohol) **assay kit,** that uses enzymatic method for the assay, provides a simple, rapid, and sensitive method for accurate quantification of ethanol concentration in a variety of biological samples like serum, plasma, and other body fluids. The method is highly specific to ethanol.

Principle

Buffered enzyme mixture contains the enzyme alcohol dehydrogenase (ADH) and NAD. ADH catalyses the oxidation of ethyl alcohol to acetaldehyde. During this reaction, NAD is reduced to NADH. The increase in absorbance at 340 nm (UV) is proportional to the concentration of alcohol in the specimen. It is measured with a UV spectrophotometer.

$$\underset{\text{Ethanol}}{C_2H_5OH} + NAD^+ \xrightarrow{ADH} \underset{\text{Acetaldehyde}}{CH_3CHO} + NADH$$

The amount of NADH produced can also be measured by its fluorescence at 535 nm. The method detects 0.1–10 ppm alcohol.

Follow instructions provided by the manufacturer. It consists of four major steps as given below:

- Sample preparation
- Making of standard curve
- Preparation of reaction mixture
- Measurement of optical density or fluorescence

Reagents

The kits provide all the reagents. Follow manufacturer's advice in storing. The kit is usually stored in a refrigerator. Always keep the enzyme in a refrigerator or in freezer as advised by the manufacturer.

Special precaution: Make sure that no alcohol vapour (ethanol, methanol, or propanol) is present in the laboratory air. If the laboratory benches are cleaned by alcohol, it is inappropriate to work in that area.

Procedure

1. Prepare the specimen promptly for the assay.
2. Prepare a standard curve using the standard provided in the kit. Strictly follow manufacturer's directions. Usually the ethanol standard is 0.1 mM (1 mole = 46.07 g/mol). This is diluted to obtain the curve.
3. Prepare a reaction mixture that contains the following:
   - Requisite amount of reaction mixture
   - Ethanol assay buffer
   - Ethanol enzyme mixture (AO, ADH)
   - Measured amount of specimen
4. Take the optical reading after specified time suggested by the manufacturer.

Calculation

Background correction: Either take away the reading of the blank (background) from the reading of the standard/specimen or set the blank at zero in the instrument.

$$\text{Concentration of alcohol (nmol/}\mu\text{L or mM)} = \frac{S_a\text{(nmol)}}{S_v\ (\mu\text{L})}$$

S_a = Sample amount (nmol) determined from the standard curve
S_v = Sample volume added into the sample well (µL)

Carbon Monoxide Poisoning

Carbon monoxide is a poisonous odourless and colourless gas. Poisoning is caused by the inhalation of exhausts from automobiles and other vehicles using petrol. Because there is no governmental control maintaining the emission standard of automobiles driven in most developing countries, it is difficult to assess the damage done to the public and street dwellers. In industrial countries, it is still the leading cause of death and may be responsible for more than half of all fatal poisonings worldwide. Unfortunately, for most patients poisoned by CO, poisoning is not immediate and obvious. Variable symptoms, a wide range of patient sensitivity and unsophisticated detection systems often result in misdiagnosis and treatment delays.

The common symptoms of CO poisoning are headache, dizziness, weakness, upset stomach, vomiting, chest pain, and confusion. It is often described as "flu-like." Inhaling excessive amount of CO might kill a person. People who are sleeping or drunk can die from CO poisoning before they have symptoms. The affinity of carbon monoxide to haemoglobin is lot more than oxygen (210 times). Carboxyhaemoglobin is the haemoglobin-carbon monoxide complex. This markedly reduces the oxygen-carrying capacity of blood. The same situation arises in cigarette smoking.

By measuring the carboxyhaemoglobin (COHb) levels in a person's blood, one can assess the damage done by the exposure to CO. Thus, assessment of a patient's COHb level first provides an accurate diagnosis of CO poisoning and then guides treatment under emergency. If the patient is left untreated, CO exposure may damage the neurological, cardiac, metabolic, pulmonary and renal systems of the body. Organs with a high metabolic demand for oxygen, like heart and brain, are most susceptible to injury from CO. The effects of CO poisoning, however, can be reversed if caught in time.

Specimen

Anticoagulated whole blood from vein or artery is submitted to the laboratory for determining carbon monoxide poisoning. Fluoride and oxalate are used as anticoagulants for getting the whole blood specimen. Protect the specimen from air exposure. Hence remove the syringe after drawing blood, cap tightly, and mix. Keep the tube stoppered to avoid exposure of specimen to atmosphere. Process promptly.

Principle

Whole blood is mixed with ferricyanide and lactic acid. This liberates carbon monoxide (a volatile gas) into the atmosphere of the microdiffusion chamber. This is then trapped on a palladium-chloride paper and thereby palladium ions are reduced to metallic form and appear as a black film. If the black film is visible, CO-Hb saturation greater than 30% is present.

$$CO + PdCl_2 + H_2O \rightarrow CO_2 + 2HCl + Pd$$

If a quantitative measurement is required, blood specimen is treated with palladium chloride, hydrochloric acid and chlorpromazine hydrochloride that gives a violet coloured complex whose intensity is measured at 565 nm.

$$\text{Pd + Chlorpromazine hydrochloride} \rightarrow \text{Violet coloured complex}$$

We will focus here only on the screening of CO poisoning.

Equipment

Microdiffusion dish (Figure 35.1).

Note If you have purchased a kit, follow manufacturer's instructions.

Reagent

- ***Hydrochloric acid (0.01 N):*** Dilute 0.85 mL of concentrated HCl (11.6 N) to 1000 mL with water.
- ***Palladium chloride solution:*** Dissolve 220 mg of palladium chloride in 250 mL of 0.01 N HCl. The solution is stable if protected from the carbon monoxide in the atmosphere.
- ***Potassium ferricyanide solution (3.2%):*** Dissolve $K_3Fe(CN)_6$ in 50 mL of water and make this up to 100 mL volume in a volumetric flask. It is **HIGHLY POISONOUS.**
- ***Lactic acid solution (0.1 M):*** Dissolve 90 mL of lactic acid (85% pure) in water and make up to 1000 mL.
- ***Haemolysing solution:*** Mix equal parts of potassium ferrocyanide solution with 0.1 M lactic acid solution.
- ***Sealing compound:*** Either stopcock grease or petrolatum jelly can be used for this.

Procedure

1. Apply the sealant on the rim of the outer wall of the Conway microdiffusion dish (Figure 35.1).
2. Pipette 2 mL of palladium chloride into the centre well of the dish.
3. Place 1 mL of whole blood on one side of the outer compartment. Place the cover over the dish, leaving an opening to permit the addition of haemolysing fluid opposite the blood.
4. Add 2 mL of haemolysing solution in the outer compartment (opposite the blood) and then slide the cover over the opening to seal the dish.

5. Carefully mix the contents of the outer well by swirling the Conway dish gently.
6. Allow the dish to stand at room temperature for 1 h for the diffusion to occur or as prescribed in the kit.
7. In the presence of carbon monoxide, a mirror of metallic palladium will be noted on the surface of the palladium chloride solution in the inner compartment. A bright mirror covering the entire compartment is typical of the lethal level of carbon monoxide in the blood.

Normal range

Adults: <2.3% (0.023)
Adult smokers: 2.1% – 4.2% (0.021 – 0.042)
Adult heavy smokers (more than 2 packs/day): 8% – 9% (0.08 – 0.09)
Haemolyticanaemia: Up to 4%
Newborn: 12%

Cyanide Poisoning

Cyanide poisoning frequently occurs through accidental ingestion of insecticides. Another common cause of cyanide poisoning is the inhalation of smoke from burning plastics. Identification and estimation of cyanide in blood may be carried out most simply by using the Conway microdiffusion method; however, retrospective analysis of thiocyanate in urine is a simple way of confirming cyanide intake.

Although cyanide is very toxic, levels up to 15 µg/dL in blood can be found in adults without any symptoms. In case of death due to ingestion of an overdose of a cyanide salt, levels of 1.0 mg/dL or more may be found. A lethal level is about 100 µg/dL in blood.

Inside a laboratory, there are a number of reagents prepared from cyanide salts (e.g., potassium ferricyanide). These should be clearly labelled as **POISON.** None of the cyanide solutions should be discarded in an open sink. If at all, it can be discarded in the sink which is inside the hood. Under no circumstances should you allow the cyanide reagent to get in contact with acid. If the laboratory uses a kit, follow the instructions given by the manufacturer very carefully.

Specimen

Anticoagulated whole blood and urine samples are used for determining cyanide poisoning. Blood is anticoagulated with fluoride or oxalates. Blood specimen should not be stored for a long period.

Principle

The microdiffusion method can be applied in screening as well for the quantitative analysis of cyanide in blood. Cyanide is liberated from the biological specimen by acidification. The hydrogen cyanide (HCN) evolved is trapped in dilute alkali, and converted to cyanogen chloride; in subsequent steps it is converted to a coloured compound by reacting with appropriate chemicals.

Reagents

- ***Sulphuric acid (3.6 N):*** Dilute 10 mL of concentrated sulphuric acid (36 N) to 100 mL with water.
- ***Sodium hydroxide (0.1 N):*** Dissolve 4 g of sodium hydroxide (NaOH) in water and dilute to 1000 mL.
- ***Monobasic sodium phosphate (1 M):*** Dissolve 13.8 g of monobasic sodium phosphate in water and dilute to 100 mL.
- ***Chloramine-T (0.25%):*** Dissolve 0.25 g of chloramine-T in water and dilute to 100 mL. Store in a refrigerator.
- ***Colour reagent (pyridine-barbituric acid reagent):*** Add 15 mL of pyridine to 0.3 g of barbituric acid in a 25-mL volumetric flask and mix. Add 3.0 mL concentrated HCl and

again mix. Dilute up to the mark with distilled water. Mix thoroughly since the ingredients dissolve slowly and it is a saturated solution. Let it stand for 30 min and filter if necessary. Prepare fresh solution as needed.

- ***Cyanide standard:***
  (a) ***Stock standard (100 mg/dL):*** Dissolve 250 mg of potassium cyanide in approximately 50 mL water in a 100 mL volumetric flask. Add 2.0 mL of 0.5 N sodium hydroxide (20 g/L) and dilute to 100 mL. Store in a polyethylene container and discard after three months.
  (b) ***Working standard (200 µg/dL):*** Dilute 0.1 mL of stock standard to 50 mL with water. Prepare this freshly just before use.

Procedure

1. Place 4 mL of blood or urine into the outer compartment of a Conway diffusion dish.
2. Place 2 mL of 0.1 N NaOH in the centre compartment and prepare the cover with silicone grease for a tight seal.
3. Add three drops of 3.6 N sulphuric acid in the outer compartment, seal the top quickly and swirl gently to mix. Allow diffusion to proceed for 4 h at room temperature or 3 h at 37°C.
4. After the diffusion period, transfer 1 mL of the absorbing solution (in the centre well) to a test tube. Label this as T (test).
5. Label a second tube as B (blank) and add to this 1 mL of 0.1 N NaOH.
6. To each tube add 2 mL of NaH_2PO_4 solution and 1 mL of Chloramine-T solution. Mix and wait for 3 min.
7. To each tube add 3 mL of pyridine-barbituric acid solution. Mix and allow standing for 10 min.
8. Observe the colour and make a semiquantitative report. A red colour is a positive test for cyanide in this procedure.
9. For a quantitative report, include the working standard (200 µg/dL) in the above series. Carry on the entire test procedure, including the diffusion step. Measure the absorbance of test (A_t) and standard (A_s) against the blank at 580 nm in a spectrophotometer.

Calculation

Concentration of cyanide (g/dL) = $(A_t/A_s) \times 200$

Common sources of error

Chloramine-T can oxidize certain substances like glycine to produce cyanide. Thus, care must be exercised to avoid mechanical contamination of the absorbing solution in the centre well by trace amounts of the specimen.

Acetone Poisoning

Acetone is a flammable chemical found in many household products like nail polish. Acetone poisoning can occur by accidental intake, breathing in fumes or absorbing it through the skin.

A quick spot test (Rothera test) is described here to detect the presence of ketone bodies in urine which include acetone, acetoacetic acid (diacetic acid) and beta-hydroxybutyric acid.

Specimen

Fresh urine is the specimen of choice. If it cannot be used promptly; store in the refrigerator.
Note The specimen loses acetone because it is volatile.

Reagents

- Ammonium sulphate
- Sodium nitroprusside solution: 2% in water
- Concentrated ammonia solution (specific gravity 0.91)

Procedure

1. Place a test tube (10 mL) in the test tube rack.
2. Transfer 5 mL of fresh urine specimen into the test tube.
3. Add several crystals of ammonium sulphate into the tube and mix. Keep adding more until the crystals do not dissolve any more (saturated).
4. Add 10 drops of concentrated ammonia. ***Caution*** Fumes of ammonia can be harmful. You may choose to work in a fume hood or in a well-ventilated area.
5. Stand for 15 min.

Observation (semiquantitative)

If acetone and diacetic acid are present, then a purple (permanganate calomel red) colour will form within 30–60 s. The intensity of the colour is proportional to the amount of acetone and diacetic acid present.

Additional Note

- If a false positive is suspected, heat the urine in a test tube, on the flame of a Bunsen burner for one minute. Then allow it to cool and repeat the Rothera's test. It will not show any positive reaction.
- Always check the reagent with positive control.

Methanol, Isopropanol and Formaldehyde

Methanol and isopropanol are important industrial chemicals that are also available as household items. Both these alcohols are absorbed readily following ingestion. Intoxication with methanol and isopropanol can be from accidental ingestion, industrial exposure, self-poisoning, or as substitute for ethanol. Isopropanol is metabolized to acetone which results in ketonuria. The method of choice for the identification and measurement of methanol and isopropanol is GC (gas chromatography) which is available only in reference laboratories.

From chemical structure, **formaldehyde** is closely related to methanol. It is found in automobile exhaust from cars without catalytic converters, food preservatives, cigarettes and tobacco products. Inhalation of formaldehyde invokes an inflammatory response, including a variety of allergic signs and symptoms. Therefore, formaldehyde has been considered as the most prevalent cause of **sick building syndrome,** which has become a major social problem, especially in developing urban areas.

LABORATORY INVESTIGATION OF DRUG ABUSE

Drugs frequently encountered in **overdose situations** are classified as hypnotics, analgesics, sedatives, tranquillizers, antihistamines and those used for the treatment of psychiatric disorders. Overdose may lead to coma, violent reaction or death, depending on the drug taken. Therapeutic screening is important for effective treatment.

There is another side of drug screening test for the employees. It tracks the intentional or nonintentional misuse of drugs that is prohibited by law.

- Employees are screened against the use of illegal drugs before they are employed. This helps to maintain high morale.
- Sporadic testing makes sure that the law is active. Many of the drugs can be found on the body–clothes and hair, and thus screening of employees from time to time is a good practice.

Given below is a list of drugs that can be found in urine if used by the employee:

1. Amphitamines
2. Barbiturates

3. Cocaine Methadone
4. Cannabinoids (THC)
5. Benzodiazepines
6. Buprenorphine
7. Methaqualone
8. Opioids (Codeine, Morphine, Heroin, Oxycodone, Hydrocodone, etc.)
9. Phencyclidine (PCP)
10. Propoxyphene
11. Synthetic cannabinoids (K2, Spice)
12. Antidepressants

Many of these drugs can only be detected for a few hours after use however their metabolites can be detected for several days in urine.

Specimen

Urine is the most common specimen for illegal drug testing. Other specimens include blood, hair, breath air, sweat, or saliva, to determine the presence of specified parent drugs or their metabolites. Most tests (saliva, breath air) mimics blood tests but are used to process the specimen as early as possible.

Preparation of Specimen for Drug Testing

Specimens for drug testing may be connected with police investigation and hence needs to be secured under certain circumstances. Urine and sputum are the most common specimens. They are **"as is"** for some tests, without any processing. Other tests require the drugs to be extracted from urine. Strands of hair, patches, and blood must be prepared before testing. Hair is washed in order to eliminate second-hand sources of drugs on the surface of the hair; then the keratins are broken down using enzymes. Blood plasma may need to be separated by centrifugation from blood cells prior to testing.

Urine

Urinalysis is the most common test type and used by federally mandated drug testing programs and is considered the Gold Standard of drug testing. Random "clean catch" specimen of urine is commonly submitted to the laboratory for diagnosis of drug abuse. The exception is cocaine whose reports are occasionally challenged because of different metabolites formed from it.

Blood

Anticoagulated whole blood and clotted blood are both used in illegal drug testing. They yield plasma and serum respectively. Do not use heparin or EDTA as an anticoagulant. These may give false positive results. Drugs will be found in blood before they appear in the urine.

Blood test for drug assay is tricky. Some of the drugs like marijuana are detected only until six hours after consumption. Then they decrease significantly. In addition, the protein in plasma and in serum interferes in the assay.

Saliva or oral fluid-based drug screen

Saliva or oral fluid-based drug tests can generally detect use of illicit drugs within past 12 h in case of alcohol and one day in case of Marijuana. The biggest advantage in this procedure is the collection of specimen which does not require any technical skill.

Methodologies of Illegal Drug Assay

The past decade screening for illegal drug testing is done by **enzyme immunoassay** based on the principle of **competitive binding.** Drugs which may be present in the urine specimen compete against their respective drug conjugate for binding sites on their specific antibody. A drug, if present in urine specimen below its cut-off concentration, will not saturate the binding sites of its specific antibody. The antibody will then react with the drug-protein conjugate and a visible colored line will show up in the test line region of the specific drug strip.

As a **routine procedure,** the urine specimen is given at a designated test site. The urine specimen is then split into two aliquots. One aliquot is first screened for drugs using an analyzer that performs immunoassay as the initial screen. If the urine screen is positive then another aliquot of the sample is used to confirm the findings by gas chromatography. Use of mass spectrometry (MS) is limited to only specialized laboratories.

Employment-related tests are often done on-site which is cost-efficient and reliable. Results are subjected to a medical physician review. If the result of the screen is negative, the physician informs the employer that the employee has no detectable drug in the urine. However, if the test result is positive, the employer may contact the employee for further action—treatment or discontinuation of service. The accuracy of drug tests varies with the manufacturer of the kits.

Hair analysis is also a standard procedure to detect drugs of abuse. It is now recognized by the judicial systems. Drugs of abuse that can be detected include Cannabis, Cocaine, and Amphetamines. In contrast to other drugs consumed, alcohol is deposited directly in the hair which becomes esters after reacting with fatty acids. For this reason the investigation procedure looks for direct products of ethanol metabolism (ester) on the hair. These can be used as indicators of alcohol consumption.

INVESTIGATION FOR NEW ILLEGAL DRUGS

The testing of drugs goes through **four phases**—weight testing, microscopic analysis, presumptive testing (screening) and confirmatory testing. Clinical laboratories do not provide drug monitoring services unless they are authorized and equipped with appropriate instruments.

Weight testing Volume/Weight ratio of all substances is constant. This is the simplest way of screening but not an accurate one. The microscopic analysis under experienced eye, crystalline structure of solid phase of the substance under investigation can give an idea of the possible illicit drug. If a microscope is not available, good magnifying glass may give clues of a familiar solid-phase substance.

Microscopic analysis This is suitable for plant materials. A modification of this is microcrystalline test which involves dissolving the material in small amount of solvent and then letting the materials form crystals. Substances with specific crystalline structure allow identification under polarized light.

Presumptive and confirmatory testing Wet chemistry done in routine laboratories only suggests that the drug is found in the body of an individual. The only tool it has is the colorimeter. The drug gives a colour through a series of chemical reactions. Intensity of the colour may be directly or inversely proportional to the concentration of drug in the test system. This is only a presumptive test needs to be further confirmed by specialized laboratories which are capable of identifying the drug. This will require special instrumentation beyond the scope of this book. A few are mentioned here—**Liquid chromatography, Gas chromatography, Ultraviolet spectroscopy, Infrared spectroscopy and Mass spectroscopy;** these are used to help identify the substance. In some cases further separation may be necessary and then such finer techniques are called for.

Summary

At this time, gas chromatography (GC) and mass spectrometry (MS) remain the "gold standard" for **confirmation testing.** These procedures are discussed in Chapter 31. These are highly expensive equipment and found only in special laboratories. Of the two, GC is relatively cheaper. The technique adopted by GC is able to separate the analytes in a specimen and can quantify. But the instrument is unable to determine the molecular structure of new drugs. This is done by mass spectrometry. Thus GC provides the chemical equivalent of a "fingerprint" of an analyte and its quantitative amount in the sample but its structure will be revealed only by MS.

POPULARITY OF IMMUNOASSAY

A general survey of various laboratories involved in drug assay indicates that immunoassay is most **popular** and is **cost effective.** It requires minimum laboratory facilities and the kits are easily available. One must, however, bear in mind that it is a screening test that identifies the presence of a drug and quantifies the analyte sought. These methods are usually sensitive, but may not necessarily be specific.

In a **competitive antigen-antibody reaction,** drug molecules in the sample compete with a labelled version of that drug to bind with an antibody laid on a solid surface. The technique has been discussed earlier with a number of assays. The antibody sites compete with the drug in the specimen (analyte) and another which is tagged with a colour producing enzyme. Both the antigens—**tagged and untagged**—have the equal chance to react with the antibody. After incubation and wash, colour-producing substrate is laid over and this reacts with the tagged enzyme and gives colour. Enzymes that provide colour as a result of enzymatic action are used to measure the antibody-drug reaction and thereby the concentration of the drug. More colour will indicate less amount of untagged antigen (analyte in specimen). In some cases, the assay is targeted to detect a drug metabolite rather than the parent drug.

The limitations of this method are obvious. It cannot always identify the new drugs. Lack of specificity and cross-reactivity occasionally produce **false positive results,** thus, merges the discrepancy between screening and first level confirmation testing.

Cocaine by ELISA

Cocaine is the most widely used illegal drug. The drug is generally inhaled or smoked. Urine is the easily available specimen and is ideal in many ways. There are several methods to quantitate the amount of cocaine consumed. Metabolites of cocaine appear in urine. **Direct ELISA Kit** is a single incubation assay providing reliable results against cocaine addiction. It is based upon the competitive binding of the antigen to antibody of enzyme's labelled antigen and unlabelled antigen, in proportion to their concentration in the reaction mixture. The reaction can be set up in a microtube or in wells. After incubation, the tubes/wells are washed thoroughly and a chromogenic substrate is added. The colour produced is stopped using a dilute acid stop solution and the wells are read at 450 nm. The intensity of the colour developed is inversely proportional to the concentration of drug in the sample. The technique is sensitive to 1 ng/mL.

Native (unaltered) cocaine urine concentration is far lower than that of its major metabolite **benzoylecgonine.** After intravenous administration of 100 mg of cocaine, urine concentrations range from 1.2–2.4 µg/mL as compared with concentrations ranging from 5–5.5 µg/mL for benzoylecgonine. Cocaine is **undetectable** (at a 50 ng/mL cut-off) 12 h after administration in comparison with benzoylecgonine which persists hours to days after administration.

Reagents

Prepare reagents according to the specifications given in the kit.

Reagents available in the kit:

- BE-conjugate
- Positive standard
- Negative standard
- Substrate
- Stop reagent

Additional requirement:

- Distilled or deionized water
- Precision pipettes
- Disposable pipette tips
- ELISA reader capable of reading absorbance at 450 nm
- Solid phase

Sample Preparation

- The Cocaine/Benzoylecgonine Direct ELISA Kit is to be used with human forensic samples such as whole blood, serum, and plasma. Cut-off criteria are important in deciding the sample dilution.
- Do not use sodium azide in preparing the specimens.
- Refrigerate urine samples until ready for assay.
- Repeated freezing and thawing should be avoided.
- Urine samples should be shipped refrigerated with ice packs or equivalent.

Storage/Stability

Store the kit as per manufacturer's direction. Refrigeration is recommended. Keep the well/ microtubes sealed. The reagents are stable until expiration of the kit. Do not expose reagents to heat, sun, or strong light.

Procedure

1. Follow the procedure given in the kit.
2. Add stop solution to each well, to change the colour from blue to yellow.
3. Measure the absorbance with manufacturer's specifications.
4. Wells should be read within 1 h of yellow colour development.
5. Always prepare a standard curve of benzoylecgonine (ng/mL) in order to establish the linearity. An example is presented here.

Benzoylecgonine (ng/mL)	Absorbance
0	2.20
10	0.52
25	0.33
50	0.27

The dose/response curve shown above should not be used in assay calculations. It is recommended that at least one in-house positive quality control sample be included with every assay run. A dose response curve or a cut off calibrator should be run with every plate.

LABORATORY SCREENING FOR HEAVY METAL POISONING

The heavy metals that commonly bring toxicity are mercury, bismuth, arsenic, antimony and lead. The main role of the clinical laboratory is to determine whether these substances are present or absent in the body.

Therefore, qualitative screening procedures are needed to help make this decision. We will describe the **Reinsch test** for the screening of the aforesaid heavy metals except lead. Identification procedure for mercury will also be described. The only reliable test for lead is the use of atomic absorption spectrophotometer which is expensive and beyond the reach of most laboratories of developing countries.

Specimen

Specimens submitted for detecting heavy metal poisoning are urine and gastric content.

Reinsch Screening Test

Principle

Heavy metals, such as mercury, bismuth, arsenic and antimony, react with copper in acid medium when subjected to a prolonged heating period (1 h). The surface of the copper metal takes different colours following the acid-heat treatment. The colour indicates the presence of specific heavy metal.

Reagents

- Nitric acid (concentrated)
- Alcohol (absolute)
- Ether
- Hydrochloric acid: (a) Concentrated (b) 10%, v/v
- Copper wire: From a 20-gauge copper wire make a spiral about 5 mm diameter and 1 cm long with the help of a glass tube. The wire is dipped into concentrated nitric acid for 1 s, and rinsed with demineralized water, then alcohol and finally ether; it is then allowed to dry.

Procedure

1. Place 20 mL of urine specimen or gastric contents into a 50-mL Erlenmeyer flask and add 4 mL of concentrated HCl. Mark the level of the acid and place a small funnel at the top to reduce evaporation.
2. Introduce a copper spiral and heat gently for 1 h on a steam bath.
   Note A low-temperature hot plate (surface temperature of approximately 95°C) can also be used.
3. Maintain the volume by adding 10% hydrochloric acid during the heating period.
4. After an hour, remove the copper spiral, rinse it thoroughly with water, and then dry it on a piece of absorbent filter paper.
5. Observe the colouration for presumptive identification of heavy metal:
   - ***Dull blact.*** Arsenic (sensitivity as low as 10 μg)
   - ***Shiny black:*** Bismuth (sensitivity as low as 20 μg)
   - ***Dark purple sheen:*** Antimony (sensitivity as low as 20 μg)
   - ***Silver sheet:*** Mercury (sensitivity as low as 30 μg)
6. For further identification of suspected heavy metals, perform specific tests. The specific test for mercury is described here.

Specific Tests for Mercury

Principle

Mercury held on the surface of a copper wire reacts with cuprous iodide forming the pink coloured mercuric iodide compound.

Reagents

Cuprous iodide solution: Dissolve 5 g of copper sulphate and 3 g of ferrous sulphate in 10 mL water. While stirring constantly, add a solution of 7 g potassium iodide dissolved in 50 mL water. Filter the precipitate and wash with water. Suspend the precipitated cuprous iodide with the aid of a little water and transfer to a brown bottle.

Procedure

1. Place a small piece of filter paper on a watch glass and add 2 drops of cuprous iodide suspension.
2. Place the acid-treated copper spiral on the cuprous iodide spot and cover with a second watch glass.
3. If a rose to salmon-pink colour appears as a result of formation of mercuric iodide, mercury is present.
4. Study of stained blood smear can be used for possible lead poisoning. Basophilic stippling of red cells and presence of punctuate basophils are connected to lead poisoning.

Indirect Cyanmethemoglobin Method

For **indirect cyanmethemoglobin** method, to detect mercury poisoning, a small quantity of blood is transferred onto Whatman filter-paper and dried at room temperature. After the blood has dried, the filter paper with dried blood is taken to the laboratory in a sealed plastic bag. The dried specimen can be processed up to two weeks. It is then put into Drabkin's solution for the quantitative measurement of cyanmethemoglobin which is proportional to the quantity of haemoglobin. Drabkin's reagent contains potassium ferricyanide, potassium cyanide, and $NaHCO_3$. The chemical reaction with Drabkin's solution leads to the formation of cyanmethemoglobin. Intensity of the colour is measured in a spectrophotometer at 546 nm. This is proportional to the concentration of haemoglobin ($36.77 \times A_{546}$). Mercury poisoning reduces the haemoglobin concentration.

Iron Overdose

Iron supplements in various forms of iron salts are readily available. **Ferrous sulphate,** the cheapest and most common iron salt, is involved frequently in overdose. There are several other over-the-counter iron pills, used as supplemental nutrients, but develop iron toxicity. Acute iron poisoning is particularly **common in the paediatric population,** with majority of the reported exposure occurring in children less than 6 years of age. In the worst scenario, the patient may develop systemic toxicity with cardiovascular collapse, seizure, coma and shock. Hepatic failure and GI tract obstruction alarms high level toxicity.

Serum (clotted blood) is the chosen specimen for determining iron toxicity. Both free iron and total iron binding capacity (TIBC) are determined in the chemistry laboratory. Simple colorimetric procedures are followed with the help of commercial kits. For more detail analysis, use of atomic absorption spectroscopy is recommended.

A heavy discharge of iron in **urine** is an indication of iron overdose. A new procedure is presented here which can be followed in small laboratories in order to diagnose iron toxicity.

Principle

The iron is reduced to ferrioxamine with sodium hydrosulphite (dithionite). The iron is determined after the addition of dipyridyl.

Reagents

- Sodium hydrosulphite (dithionite) powder
- Sulphuric acid (0.05 N)
- Dipyridyl solution (1% w/v in 0.05 N sulphuric acid)
- Phosphate buffer 1/15 M, pH 7
- ***Standards:*** 0, 10, 40 and 80 μg Fe/20 mL as solution of ferrous ammonium sulphate in iron-free water.

Procedure

1. Centrifuge the urine specimen.
2. Pipette 20 mL of urine into 50 mL volumetric flask.
3. Add 20 mL of phosphate buffer.
4. Add about 50 mg of dithionate with a small spatula.
5. Mix thoroughly.
6. Take 10 mL aliquots into each of two test tubes marked—T (test) and UB (urine blank).
7. Add to the first tube (marked as T), 0.2 mL of the dipyridyl solution.
8. To the other tube (marked UB) add 0.2 mL of 0.05 N sulphuric acid.
9. A reagent blank or RB (use iron-free water instead of urine) is prepared the same way as the 'Test'.
10. Allow the tubes to stand for 30 min.
11. Read colour against water (or the 0 standard) in a spectrophotometer (or colorimeter) at 510 mμ wavelength or with a suitable filter in a photometer.
12. Prepare a calibration curve with the standards (0–80 μgFe/20 mL). The calibration curve may not be linear for the entire range. Work in different ranges, 0–40 and 40–80 μgFe/20 mL.

Calculation

The optical density (OD) of the reagent blank (RB) and urine blank (UB) are subtracted from that of the sample (T). Compare it with the calibration curve and report.

POINT-OF-CARE TESTING

Point-of-care or POCT has revolutionized the laboratory assay. Here the testing is done **at the site** or on the bed side of the hospital patient, without using traditional laboratory. Typically, no training is needed in performing the test and is now been adopted for many tests and most physicians are familiar with the devices used for glucose or coagulation monitoring. The demand for POCT devices has increased since past few years as it is an alternative to collection, transport, and subsequent laboratory analysis. One should bear in mind that POCT is for screening. It does not replace the traditional laboratory confirmation tests. The staff chosen to perform the testing must be able to carry out the required steps exactly as described by the manufacturer. POCT may expedite the testing process but should not be compromised with the quality of results. Hence, when necessary, results must be compared with the confirmatory tests.

Large numbers of POCT tests are colour-reaction-based where endpoints are visually read by the operator. The "device" may be a dipstick, cup, card, or cassette. In case of urine drug test (UDT), the amount of urine needed for testing ranges from a few drops to approximately 30 mL. The simplest-to-use POCT devices combine collection and testing, but most require the operator to perform multiple steps, including sample application, timing of reaction, and reading/interpreting the endpoint. Data recording from typical POCT devices is performed manually which makes it highly subjective. These devices have a varied but limited menu in terms of drugs, drug classes, or groups of drugs detected. The antibodies used in the POCT devices target the same drug and/or metabolites detected by laboratory immunoassays, and a positive result is similarly obtained when the drug(s) or metabolite(s) of interest is present at or above a designated cutoff concentration. Border line cases are hard to interpret as the hue of the developed colour is read differently by two technicians. In short, recognition and resolution of the result rest in the hands of the POCT operator. POCT devices vary in accuracy of analytic performance, cut off concentrations, cross-reactivity, reproducibility, and ease-of-use. Use of POCT in drug monitoring is still a controversial issue.

Point-of-care for Lead Poisoning

Anodic stripping voltammetry (ASV) is a handy instrument for the determination of lead in blood. The operation is simple and can be handled by unskilled laboratory personnel. It is ready to be used in any place, school, mobile health clinics, and in any public place for epidemiological studies and in location where transport of specimen to reference laboratories is a real problem.

REVIEW QUESTIONS

1. Under what conditions specimens are referred to the toxicological laboratories? What are the commonly submitted specimens?
2. What is the role of toxicology laboratory?
3. What is microdiffusion technique? How does this technique help in the toxicological investigation?
4. Describe the screening tests for the following:
   Acetone, Alcohol, Heavy metal, Acetaminophen, and Imipramine
5. What is Reinsch test? How does this test help in diagnosing heavy metal poisoning?
6. Describe the microdiffusion test as applied in the diagnosis of toxicity due to carbon monoxide, halogenated hydrocarbons, and cyanide.
7. How would you determine the toxicity caused by methanol and formaldehyde?
8. What is the clinical significance of phenothiazine derivatives and acetaminophen? How would you recognize their overdose?