Al-Azhar University – Gaza

Faculty of Science

Biology Department

Practical Biochemistry II

Collected by:

Saleh Nazmy Mwafy

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To my parent's with everlasting love

Practical Biochemistry (II)
Course Out-line

بسم الله الرحمن الرحيم

Practical Biochemistry II
Laboratory Safety

All person must wear protective laboratory coat during practical work.

Food, drink and smoking are not allowed.

Hands should be washed immediately after each time you come in contact with blood.

Mouth pipetting is strictly not allowed.

Blood samples should be covered during centrifugation.

All used needles and contaminated sharps should be disposed.

Inflammable solvents should not be evaporated over a naked flame.

All human fluids should be used with greatest care because they considered as biohazards.

After use organic solvents, it should be disposed in special containers.

Containers, refrigerators and freezers used for blood specimens should be labled “Risk of infection” or “Biohazadous material.

Spectrophotometric methods of analysis

Quantitative analysis:

This type pf analysis determines how much of a particular substance is present in a sample.

In clinical laboratory there is a continual need for the use of quantitative technique, because the exact amount of unknown substance can be determine accurately. One of the techniques used most frequently in the clinical laboratory is photometry or absorbance spectrophotometry.

The concentration of a colored substance in a solution can be estimated by comparing visually the intensity of its color with several standard solutions of known concentration such method using the eye as detector are known as *colorimetric methods*.

Colorimetric methods:

Is analytical methods employ color and color variation to determine the concentration of substances.

Spectrophotometry or colorimetry, depend on two factors, ***the color itself*** and ***the intensity of the color***.

When using spectrophotometry as method of quantitative measurement, the unknown color substance is compared with a similar substance of known strength (a standard solution), based on the principle that the intensity of the color is directly proportional to the concentration of substance present.

In spectrophotometry sample solution in a glass cell "cuvette" is inserted into a spectrophotometer and a monochromatic light beam is directed at the cuvette containing the sample, some of the incident light (p_0) is absorbed by the chemical substance(s) in the sample, and reminder (p) is transmitted. Where the amount of light or energy absorbed is proportional to the concentration of chemical substance(s) absorbing it.

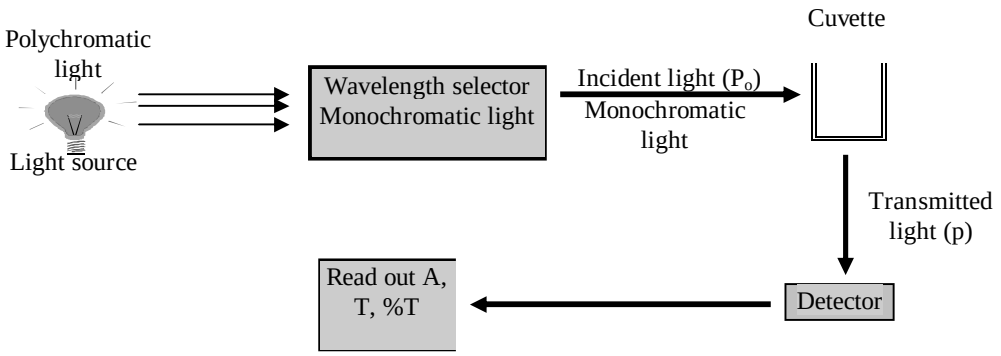
N.B:

Monochromatic light: means single color light or very narrow wavelength range light.

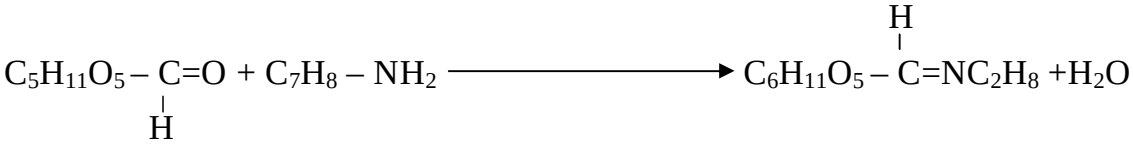
Polychromatic light: means multicolor light or very wide wavelength range light.

Light is absorbed by a chemical substance only when its wave length correspond to the energy needed to cause some changes in the electronic configuration of the species where the light pass.

Schematic diagram
For the essential components of a spectrophotometer



Any substance to be measured by spectrophotometer ***must be colored to begin with or must be capable of being colored.*** An example of substance that is colored to begin with is *Haemoglobin*, the amount of radiant energy absorbed by the Hb. ion or molecule can be determined by spectrophotometer. Sugar specifically *Glucose*, is an example of a substance that is not colored to begin with i.e. glucose ion don't absorb significant amount of radiant energy but is capable of being colored by the use of certain reagents (O-toludine) i.e. O-toludine reagent convert glucose ion to a new species that absorbs some type of radiation intensity.



Glucose (colourless) O-toludine Green product

The spectrophotometer has two forms of read out: ***Transmittance (T) or percent transmittance (%T) and absorbance (A).*** So if the incident light "monochromatic" is symbolized as (P_0) and the transmitted light is symbolized as (P), the absolute values of (P_0) and (P) can't be measured readily but it is easy to measure their ratio P/ P_0 .This is done by means of a photoelectric detector.

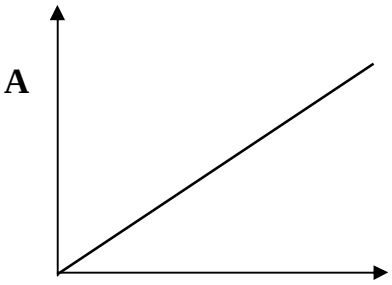
The ratio P/ P_0 is defined as Transmittance (T) and (100 x T) is defined as percent Transmittance. (T) Or (%T) is one form of read-out on the spectrophotometer.

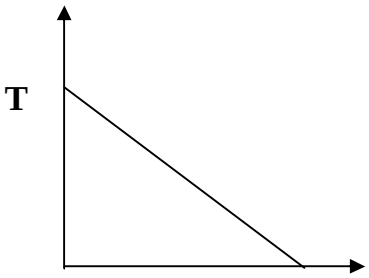
$$T=P/P_o \ , \ \%T= P/ P_0 \times 100$$

The logarithm of the inverse ratio, Log P_0 / P is defined as absorbance (A) and is the other form of spectrophotometer read-out.

$$A = \text{Log } P_0 / P = \text{Log } 1/ T = - \text{Log } T$$

Absorbance (A) is the more important read-out, because it is linearly proportional to the concentration of an absorbing chemical species.





Conc. α A **Conc. α 1/ T**

Beer's law:

The amount of light absorbed by a species in a solution will depend on:

- The number of ions or molecules of the species in the pathway "light path" of the photon beam.
- The more light will be absorbed as the concentration of the absorbing species increase, similarly longer light path will leads to more light is absorbed.
- The nature of chemical substance "absorptivity" (a).

These three factors, Conc., light bath & absorptivity, constitute the fundamental law of spectrophotometer "Beer's law" which stated as:

Abs. = a b c

a = absorptivity, a constant characteristic of a particular chemical species at particular wave length (λ).
b = is light bath through the solution "cuvette thickness in cm"
c = the conc. Of the absorbing species "mg/l or mg/dl"

Determination of unknown concentration of $KMno_4$ solution using standard curve.

An alternative method of finding concentrations doesn't require knowledge of the absorptivity value.

Standard curve: linear relationship between *absorbance* (or *Transmittance*) and the *concentration* of absorbing substance at fixed wavelength.

The readings of the instrument are plotted against the values of the concentration then the tested solutions put into the spectrophotometer and it's reading taken. This reading is converted into the corresponding value of concentration using the calibration graphs.

Standard curve is one of the most important methods used in analytical biochemistry, and there is many biochemical that depends on standard curve method, such as quantitative analysis of carbohydrates, proteins, lipids and other molecules result in metabolic pathway.

It is also used to examine the activity of certain enzymes by determining the end product conc., etc.

To have a good standard curve, the wavelength used must be in its maximum value (λ max) range.

Standard solution: Is one that contains a known, exact amount of the substance being measured in the sample.

The standard solution is usually most stable in a concentrated form, in which case it is usually referred to as **Stock Solution**.

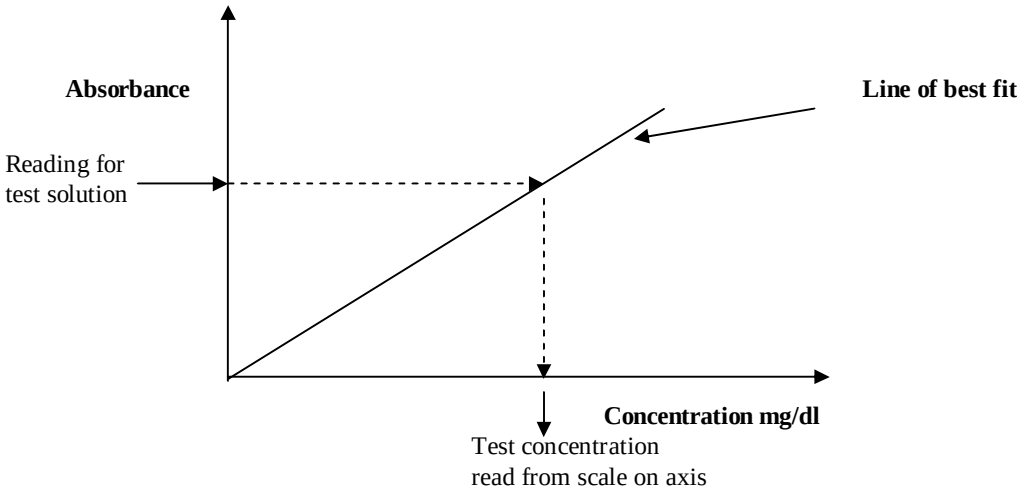
Working standards: A more diluted form of the stock solution.

Blank solution: Is a solution contains reagents used in the procedure, but it doesn't contain the substance to be measured.

♦ *How to plot the points of readings; -*

- Use special graph papers (linear graphs with squares of 1cm x 1 cm).
- Absorbance is the Y-axis, and the conc. is the X-axis.
- Readings at the spectrophotometer start from less conc. to higher one, using suitable blank.
- If use one cuvette at the experiment, it should be cleaned with the solvent after each reading.
- The line is drawn with the best points and it must pass through the origin (0.0).
- At least 3 points of absorbance readings are needed to draw a good curve.

- It is preferred to take the readings of the examined solution within the points of the standard curve. If not, dilution is needed and then multiplies the Abs. read by dilution factor.



Precautions:

Some times a non-linear plot is obtained at absorbance against concentration, and this is due to:

- Light is not a narrow wavelength beam.
- The absorbance of the reagent is not examined under wavelength with maximum absorbance (wrong λ).
- There is ionization, dissociation, association or solvation at the solute by time.
- There is evaporation at the solvent.
- Sometimes solutes are examined with other compound that may be colloidal or make interference in the absorption reading, so some times filtration is needed before Spectrophotometric reading.
- The solution is so concentrated.

The Experiment:

Materials: Potassium permenganate, Spectrophotometer, Cuvettes

Procedure:

- Prepare your stock solution of $KMno_4$ "take 1 gm of $KMno_4$ and dissolve in 1000 ml dis. H_2O".
- Prepare different concentrations of $KMno_4$, for example: 0.5 g/l, 0.25 g/l, 0.125g/l, ……etc.
- Read the absorbance of the standard solutions you prepared, against the blank solution (dis.H_2O), starting from the lower conc. at λ = 325nm.
- Read the unknown absorbance at same λ.
- Plot your standard curve, and determine the conc. of the unknown.

Obtaining blood specimens by vein puncture

All syringes, needles, lancet or other instruments used for the collection of blood specimens must be sterile.

Technique:

- Apply a tourniquet, have the patient "make a fist" and palpate the anticubital area for a suitable vein.
- After locating a prominent and firmly anchored vein clean the area with an alcohol sponge. A circular motion should be used.
- Hold the skin, tout with the left hand. Grasp the syringe with the right hand and place the needlepoint in position at the site to be penetrated. Placing the four fingers on the needle hub, Aids in controlling the needle insertion.
- Penetrate skin and move the needle forward, parallel to and along side the vein. A slide movement toward the vein should place it in the lumen. After entry to the vein blood will appear in the syringe.
- Aspiration of blood is accomplished by gently pulling upon the syringe plunger. The syringe parallel should be held steady during this process, with draw the desired quantity of blood.
- Remove the tourniquet. This must be don prior to withdrawing the needle from the vein.
- Place sterile gauze pads over the point were the needle enters the skin and deftly withdraw the needle.
- With arm still extended the patient maintains light pressure over vein puncture area.
- Removes needle from syringe, apply gentle pressure to the plunger and discharge the blood against tube margin.

Preparation of serum and plasma

Plasma: Is the fluid portion of the whole blood.

To obtain plasma, fresh blood is immediately treated with an anticoagulant, cellular elements are removed by centrifugation, and supernatant is carefully aspirated and transferred to a suitable receptacle.

N.B: sodium citrate, EDTA, heparin, etc. is used as anticoagulant.

Serum: Is the fluid portion of the whole blood from which fibrinogen and cell have been separated by allowing the blood to clot.

To obtain serum, the collecting tube containing freshly drown blood, should be plugged with sterile cotton and set side at room temperature undisturbed.

Ream of blood clot free of the tube by circling with an applicator stick before centrifuging. This facilitates the separation of cells and serum after coagulation has taken place. After centrifugation and separation of serum from cellular elements, carefully transfer the serum to a clean container.

Quantitative determination Of blood glucose in human serum

Fasting blood sugar (FBS):

Commonly used to screen for disorders of glucose metabolism, mainly *diabetes mellitus*, the fasting plasma glucose test measures plasma glucose levels following a 12 to14-hour fast. In the fasting state, blood glucose levels, stimulating release of the hormone glucagon. Glucagon then acts to raise plasma glucose by accelerating glycogenolysis, stimulating glyconeogenesis, and inhibiting glycogen synthesis. Normally, secretion of insulin checks this rise in glucose levels. In *diabetes*, however, absence or deficiency of insulin allows persistently high glucose levels.

Patient preparation:

As appropriate, explain that this test detects disorders of glucose metabolism and aids diagnosis of diabetes. Advise him to fast-taking only water-for 8 to 12 hours before the test. Tell him this requires a blood sample. Withhold drugs that affect test results. If the patient is known to have *diabetes*, blood should be drawn before administration of insulin or oral hypoglycemic drugs.

- **Clinical alert:** Alert the patient to the symptoms of hypoglycemia – weakness, restlessness, nervousness, hunger and sweating – and till him to report such symptoms immediately.

Values: Normal range for fasting blood glucose varies according to the laboratory procedure. Generally, normal values after an 8 to 12-hour fast are follows:

Fasting serum: 70 to 100 mg/dl.
Fasting whole blood: 60 to 100 mg/dl.
Non-fasting: 85 to 125 mg/dl in persons over age 50.
70 to 115 mg/dl in persons under age 50.

Implications of results:

Fasting blood glucose levels of 140 to 150mg/dl or higher, obtained on two or more occasions may be considered diagnostic of *diabetes mellitus* if possible causes of hyperglycemia have been ruled out.
Non-fasting levels that exceed 200mg/dl also suggest *diabetes.*
Note that: Depressed glucose levels can result from hyperinsulinism
"overdose of insulin is the most common cause".

Two-hour postprandial blood sugar:

The two-hour postprandial plasma glucose test reflects metabolic response to a carbohydrate challenge:
Normally, blood glucose returns to the fasting level within 2 hours. This test, performed on a blood specimen taken after a meal, is used to monitor therapy

and to confirm *diabetes* in a patient with borderline fasting blood glucose levels.

Patient preparation:

Explain to the patient that this test evaluates glucose metabolism and helps detect *diabetes*. The patient should observe an overnight fast (allowing water only) followed by a high carbohydrate breakfast (including milk, orange juice, cereal with sugar, and toast). The patient should avoid smoking and strenuous exercise after the meal. Tell the patient this test requires a blood sample, which will be drawn 2-hours after the meal.

Value: Normal glucose values are less than 120mg/dl.

Implications of results:

Values above 140mg/dl are abnormal in adults under age 50.

Values above 160mg/dl are abnormal in persons over age 60.The speed of glucose clearance declines with advancing age and levels may raise an average of 6mg/dl for each decade over age 30.

High glucose levels may also occur with *pancreatitis*, uncontrolled *diabetes mellitus*.

Depressed glucose levels occur in hyperinsulinism.

Enzymatic method for determination Of glucose in blood

There are several different methods are used to quantitatively measure the amount of glucose in blood specimen.

Glucose-oxidase procedure:

Principle:

For serum or plasma, couple assay involving both glucose oxidase and peroxidase is frequent employed.

Glucose-oxidase converts glucose to another derivative forming hydrogen peroxide as a second product in the reaction. The hydrogen peroxide then reacts further with the enzyme peroxidase to regenerate oxygen.

Interaction of the oxygen with chromogen (a material capable of forming a coloured product) produces a coloured compound, which can be detected using spectrophotometer "spectrophotometrecally".

Glucose in this method is oxidized by the enzyme glucose-oxidase in the presence of oxygen to Gluconic acid with the formation of hydrogen peroxide.

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

In the second reaction, the enzyme peroxidase catalyzes the oxidation of phenol and amino-4-antipyrine, resulting the formation of a coloured product.

The intensity of coloured product is directly proportional to glucose present initially in the first reaction

$$2H_2O_2 + \text{Phenol} + \text{Amino-4-antipyrine} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4H_2O$$

Notes:

Enzyme reagent is a mixture of the above enzymes and chemicals shown in the reaction above.

Working solution (Reagent): Dissolve the contents of one bottle R2 to the contents of one bottle buffer solution R1

This **working reagent** is stable one month at +2 to +8 ^{0}C or 7 days at RT. Avoid direct sunlight.

The colour intensity is stable tell 30min.

Linearity: This method is linear up to 500mg/dl.

If the glucose concentration is greater than 500mg/dl, dilute the sample 1:2 with saline solution and repeat the determination and multiply the result by (2).

Wavelength (λ) = 505 nm.

Procedure:

1-Marks three test tubes as **T (Test), B (Blank), S (Standard).**

2- Add the following amount as shown in the table below:

Reagent	B	S	T
Glucose standard	-	20µ	-
Sample	-	-	20µ
Working reagent	2ml	2ml	2ml

3- Mix well and incubate at 37 ^{0}C for 10min. or 30min. at RT.

"Incubation in boiling water bath"

4- Remove the tube and cool at room temp.

5- Read the Abs. of glucose standard and test solution, using B to set spectrophotometer to Zero at wavelength (λ) = 505 nm.

$$\textbf{Concentration (mg/dl)} = \frac{\textbf{Abs.}\ (T)}{\textbf{Abs. (S)}}\ \textbf{X Conc. (S)}$$

Concentration of Glucose standard: 100mg/dl

Normal range: Serum =55-110 mg/dl

Hyperglycemia: means abnormal high concentration of glucose in blood.
Hypoglycemia: means deficiency of glucose in blood.

Source of error:
Glucose solution exists on 36% alpha and 64% beta form.
Glucose oxidase is highly specific for beta form of glucose.
Other reducing substances (uric acid, ascorbic acid, bilirubin) may inhibit the reaction or make interferences in the results.

Quantitative determination of glucose in blood using O-toludine method

As we stated before glucose is a colorless compound dissolved in blood plasma, to be detected it must proceed a reaction with other chemical compound as O-toludine. The result of this reaction is a coloured complex (blue-green), which make the detection of glucose concentration easier.
The colour formed is directly proportional with the concentration of glucose in serum.

Deep colour ⟶ high concentration of glucose.
Light colour ⟶ low concentration of glucose.

Procedure:

1- Marks three test tubes as **T (Test), B (Blank), S (Standard).**

2- Add the following amount as shown in the table below:

Reagent	B	S	T
Glucose	-	0.1 ml	-
Distilled water	0.1 ml	-	-
Sample	-	-	0.1 ml
Working reagent	5ml	5ml	5ml

3- Mix well and incubate at 37 ^{0}C for 8min. exactly or 30min. at RT.

"Incubation in boiling water bath"

4- Remove the tube and cool at room temp.

5- Read the A of glucose standard and test solution, using B to set spectrophotometer to Zero at wavelength (λ) = 630 nm.

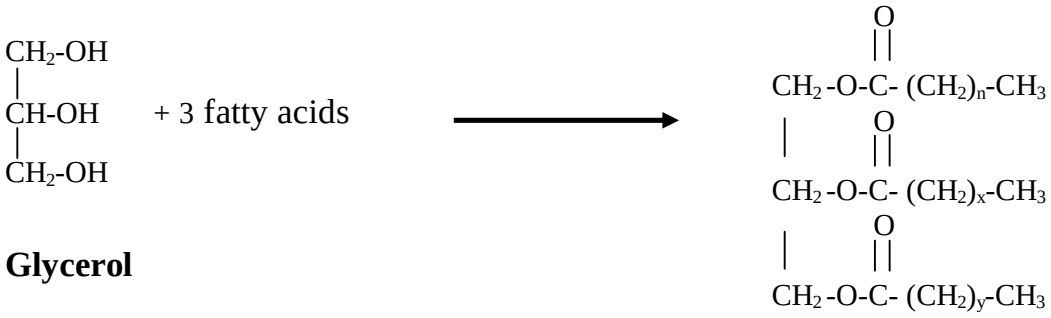
$$\textbf{Concentration (mg/dl)} = \frac{\textbf{Abs. (T)}}{\textbf{Abs. (S)}} \textbf{ X Conc. (S)}$$

Concentration of Glucose standard: 100mg/dl
Normal range: Serum =55-110 mg/dl

Quantitative determination of Triglycerides in human serum by enzymatic method

This test provides quantitative analysis of triglycerides-the main storage form of lipids-which constitute about 95% of fatty tissue. Although not in itself diagnostic, serum triglycerides analysis permits early identification of *hyperlipemia* (characteristic in nephrotic syndrome and other conditions) and the *risk of coronary artery disease (CAD).*

Triglycerids; consist of one molecule of glycerol bonded to three molecules of fatty acids (usually some combination of stearic, oleic, and palmitic). The relevant structures are:



Glycerol

Triglyceride

The three fatty acids incorporated into the triglyceride structure do not all necessarily have the same structure. In any given Triglyceride, there may be a mixture of saturated and unsaturated fatty acids.

Triglycerides are synthesized mainly in the small intestine, liver and adipose tissue (body fat). They are the major energy or components of storage or depot fat in plant and animals cells.

The degradation of triglycerides leads directly to production of fatty acid. Together with carbohydrates, these compounds furnish energy for metabolism.

Triglycerides serve important functions as apart of the cell membrane and as storage forms of lipids.

Purpose; To determine the risk of coronary artery disease.
To screen for hyperlipemia
To identify disorders associated with altered triglyceride levels.

Principle;

The triglycerides are enzymatically hydrolyzed to glycerol and free fatty acids. The glycerol librated reacts with Glycerol-kinase and Glycerol-3-phosphate oxidase yielding H_2O_2. The hydrogen peroxide (H_2O_2) concentration is determined through the Trinder's reaction.

Triglycerides + H_2O $\xrightarrow{\text{Lipase}}$ Glycerol +Fatty acids

Glycerol + ATP $\xrightarrow{\text{Glycerol-kinase}}$ Glycerol-3-phosphate + ADP.

Glycerol-3-phosphate $+O_2 \xrightarrow{\text{Glycerol-3-phosphate oxidase}}$ Dihydroxyacetone-p + H_2O_2.

$2H_2O_2$+4-aminophenazone+Parachlorophenol $\xrightarrow{\text{Peroxidase}}$ Quinoneimine + $4H_2O$.

Patient preparation: As appropriate, explain that this test helps detect disorders of fat metabolism. Advise the patient to abstain from food for 12 to14 hours before the test and from alcohol for 24 hours before the test. Tell the patient; the test requires a blood sample.
As appropriate, withhold medications that may alter test results (antilipemics, steroids, estrogen, and some diuretics).

Reagents:
Reagent 1; is a good buffer (PH 7.5), mixed with parachlorophenol.
Reagent 2; is a mixture of the above enzymes and chemicals shown in the reaction above.

Preparation of working reagent and stability;
Dissolve the contents of one bottle R2 to the contents of one bottle buffer reagent R1. This working reagent is stable 4 weeks at +2 to +8 ^{0}C or 1 week at room temperature.

Procedure:

1-Marks three test tubes as **T (Test), B (Blank), S (Standard).**

2- Add the following amount as shown in the table below;

Reagent	B	S	T
Standard	-	20micron	-
Sample	-	-	20micron
Working reagent	2ml	2ml	2ml

3- Mix well and incubate 4 min. at 37 ^{0}C or 10 min. at room temperature.

5- Measure the Absorbance of standard and test solution, against blank to set spectrophotometer to Zero at wavelength (λ) = 505 nm.

The color intensity is stable for 30 min.

Linearity; This method is linear up to 1000 mg/dl.

If the triglycerides concentration is greater than 1000 mg/dl, dilute the sample 1:2 with saline solution and repeat the determination and multiply the result by (2).

Wavelength (λ) = 505 nm. (490-550).

$$\textbf{Concentration (mg/dl)} = \frac{\textbf{Abs. (T)}}{\textbf{Abs. (S)}} \textbf{ X Conc. (S)}$$

Concentration of triglycerides standard = 200 mg/dl

Values: The triglyceride values are age-related. Some controversy exists over the most appropriate normal ranges, but the following are fairly widely accepted:

Age	Triglycerides (mg/dl)
0 to 29	10 to 140
30 to 39	10 to 150
40 to49	10 to160
50 to59	10 to 190

***Reference value** according to the kit:*

Suspected above	**150 mg/dl**
Increased above	**200 mg/dl**

Sample: Serum, heparin and EDTA-plasma.
The triglycerides in the sample material are stable for 3 days at +2 to +8 ^{o} C

Implications of results:

Increased or decreased serum triglyceride levels merely suggest a clinical abnormality, and additional tests are required for definitive diagnosis. For example, measurement of cholesterol may also be necessary, since cholesterol and triglycerides very independently. High levels of cholesterol and triglyceride reflect on exaggerated risk of atherosclerosis or coronary artery disease (CAD)

Mild-to-moderate increase in serum triglyceride levels indicates biliary obstruction, diabetes, nephrotic syndrome, endocrinopathies, or excessive consumption of alcohol. Markedly increased levels without an identifiable cause reflect congenital hyperlipoprotinemia and necessitate lipoprotein phenotyping to confirm diagnosis.

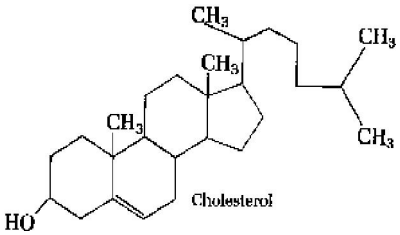
Decreased serum levels are rare, occurring primarily in malnutrition or abeta-lipoproteinemia. In the latter, serum is virtually devoid of beta-lipoproteins and triglycerides, because the body lacks the capacity to transport preformed triglycerides from the epithelial cells of the intestinal mucosa or from the liver.

Quantitative determination of Cholesterol in human serum by enzymatic method

This test, the quantitative analysis of serum cholesterol, measures the circulating levels of free cholesterol and cholesterol esters; it reflects the level of the two forms in which this biochemical compound appears in the body. Total cholesterol is the only cholesterol routinely measured.

Cholesterol, a structural component in cell membranes and plasma lipoproteins, is both absorbed from the diet and synthesized in the liver and other body tissues. It contributes to the formation of adrenocorticord steroids, bile salts, androgen, estrogens, and vitamin D.

Cholesterol is one of the more complicated lipid structures, illustrated in the following figure;



Cholesterol is hydrophobic in general, except that the (-OH) group is hydrophilic.

About two-third of plasma conc. is esterifed with fatty acids to form cholesterol esters, while the remain one-third conc. is in form of free cholesterol.

Assays in routine used, doesn't distinguished between the unesterified and esterifed forms.

Cholesterol is a waxy material, which forms plate-like crystals. This molecule plays an important role in plaque formation in the blood vessels, blocking the flow of blood and subsequently leading to heart damage.

A diet high in saturated fat raises cholesterol levels by stimulating absorption of lipids, including cholesterol, from the intestine; a diet low in saturated fat lowers them.

Elevated total serum cholesterol levels are associated with an increased risk of atherosclerotic cardiovascular disease, particularly coronary artery disease (CAD).

Sources of cholesterol: There is more than one source of cholesterol, for example:

Dietary cholesterol: This type depends on what person takes from the component of its daily diet as: egg yolk and fats or oils

Synthesized cholesterol: This type of cholesterol synthesize within the body, like the cells lining the gastrointestinal tract and the liver, which produce 1.5 to 2.0 gm per day. Almost cells can synthesize some cholesterol required for their own use.

Cholesterol can be classified into two types in human body: -

Bad cholesterol (LDL-Cholesterol); Is the main cholesterol carrier and they delivering cholesterol from liver to tissues, where hormones can be synthesized. LDL-Cholesterol enhances the deposition of cholesterol in arterial walls resulting in atherosclerosis.

Good cholesterol (HDL-Cholesterol); It serves as acceptors of cholesterol from cells and tissues.
It promotes the removal of cholesterol from various tissues and its secretion into the bile by the liver. And plays an important role in the formation of bile salts.

Purpose: To assess the risk of coronary artery disease.
To evaluate fat metabolism.
To aid diagnosis of nephrotic syndrome, pancreatitis, hepatic disease,
hypothyroidism, and hyperthyroidism.

Principle:
Cholesterol and its esters are released from lipoprotein by degredations.
Cholesterol-estrase hydrolyzes the esters and H_2O_2 is formed in subsequent enzymatic oxidation of cholesterol by cholesterol-oxidase. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminophenazone in the presence of phenol and peroxidase, according to the following equation:

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Cholesterolestrase}} \text{cholesterol} + \text{fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{cholestene-3-one} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{Peroxidase}} \text{quinoneimine} + 4\ H_2O$$

The quantity of this red dye "quinoneimine" formed is proportional to the cholesterol concentration

Patient preparation: As appropriate, explain that this test determines the body's fat metabolism. If the patient is hospitalized, impose overnight fast and abstention from alcohol for 24hours before the test. Tell the patient the test requires a blood sample. As appropriate, withhold medications that may alter test results.

Reagents:
Reagent 1; is a buffer "pipes" (PH 6.9), mixed with phenol.
Reagent 2; is a mixture of the above enzymes and chemicals shown in the reaction above.

Preparation of working reagent and stability;
Dissolve the contents of one bottle R2 to the contents of one bottle buffer reagent R1. This working reagent is stable 4 months at +2 to +8 ^{0}C or 40 days at room temperature when stored in a dark bottle.

N.B: *SOME TIMES THE REAGENT IS READY FOR USE. AND THE REAGENTS ARE STABLE UP TO THE GIVEN EXPIRY DATE, EVEN AFTER OPENING, WHEN STORED AT +2 TO +8 ^{0}C. CONTAMINATION MUST BE AVOIDED.*

Procedure:

1-Marks three test tubes as **T (Test), B (Blank), S (Standard).**

2- Add the following amount as shown in the table below;

Reagent	B	S	T
Standard	-	10µ	-
Sample	-	-	10µ
Working reagent	1ml	1ml	1ml

3- Mix well and incubate 5 min. at 37 ^{0}C or 10 min. at room temperature.

5- Measure the Absorbance of standard and test solution, against blank to set spectrophotometer to Zero at wavelength (λ) = 505 nm.

The color intensity is stable for 60 min.

Linearity; This method is linear up to 600 mg/dl. Other kits are linear up to 750 mg/dl. If the cholesterol concentration is greater than 600 mg/dl, dilute the sample 1:2 with saline solution and repeat the determination and multiply the result by (2).

Wavelength (λ) = 505 nm. (500-550).

$$\textbf{Concentration (mg/dl)} = \frac{\textbf{Abs. (T)}}{\textbf{Abs. (S)}} \textbf{ X Conc. (S)}$$

Concentration of cholesterol standard = 200 mg/dl

Values: The cholesterol concentrations vary with age and sex, and commonly range from 150 to 200 mg/dl.

***Reference value** according to the kit:*
The following limits are recommended for the recognition of hypercholesterolemia:

Suspected above	**220 mg/dl**
Increased above	**260 mg/dl**

Sample: Serum, heparin, and EDTA-plasma.

Note that:** lipemic specimens usually generate turbidity of the sample/reagent mixture, which leads to falsely elevated results. The **HUMAN cholesterol

liquicolor test *avoids these falsely elevated results through its built-in* ***lipid-clearing factor*** *(LCF). The (**LCF**) clears up totally turbidity caused by lipemic specimens.*

Implications of results:

The desirable blood cholesterol level is below 200mg/dl. Cholesterol levels of 200 to 240mg/dl are considered borderline or at high risk for coronary artery disease (CAD), depending on other concurrent risk factors.

Cholesterol levels that exceed 250mg/dl indicate high risk of cardiovascular disease and require treatment

Elevated serum cholesterol (hypercholesterolemia) may indicate incipient hepatitis, lipid disorders, bile duct blockage, nephrotic syndrome, obstructive jaundice, pancreatitis, and hypothyroidism. Hypercholesterolemia caused by high dietary intake requires modification of eating habits and, possibly, medication to retard absorption of cholesterol.

Low serum cholesterol (hyocholesterolemia) is commonly associated with malnutrition, cellular necrosis of liver, and hyperthyroidism. Abnormal cholesterol levels frequently necessitate further testing to pinpoint the disorder, depending on the type of abnormality and the presence of overt signs.

Abnormal levels associated with cardiovascular disease, for example, may necessitate lipoprotein phenotyping.

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Quantitative determination of HDL-Cholesterol in human serum by enzymatic method

Principle:

Low-density lipoproteins (LDL and VLDL) are specifically precipitated by phosphotungstic acid and magnesium ions and can then be removed by centrifugation. High-density lipoproteins (HDL) remain in the supernatant. Determination of HDL-Cholesterol is performed using the clear supernatant

Patient preparation: As appropriate, explain that this test determines the body's fat metabolism. If the patient is hospitalized, impose overnight fast and abstention from alcohol for 24hours before the test. Tell the patient; the test requires a blood sample. As appropriate, withhold medications that may alter test results.

Reagents:

Reagent A "Prep. Reagent": is a mixture of phosphotungstic acid with magnesium chloride.

Reagent B; cholesterol reagent

Preparation of working reagent and stability;

Reagent A is ready for used. Stored at +2 to +8 ^{o} C up to the expiration as specified.

Dissolve the contents of one bottle R2 to the contents of one bottle buffer reagent R1. This working reagent is stable 4 months at +2 to +8 ^{0}C or 15 days at room temperature when stored in a dark bottle.

Procedure:

***Part one**: pipette into a centrifuge tube*

Reagent	Macro-test	Semi-micro test
Serum	1ml	0.5ml
Reagent A "Prep. Reagent"	0.1ml	0.05ml

Mix well; allow standing for 10min. at room temperature.

Centrifuge at 4000 rpm for 20 min or at 12000 rpm for 2 min.

***Part two**: pipette into a reaction vessel:*

Reagent	Macro-test		Semi-micro test	
	Blank	Sample	Blank	Sample
Clear supernatant	-	40 micron	-	20 micron
Working reagent	2 ml	2 ml	1 ml	1 ml

Mix well and incubate 5 min. at 37 ^{0}C or 10 min. at room temperature.

Measure the Absorbance of sample, against blank regent to set spectrophotometer to Zero at wavelength (λ) = 505 nm.

Calculation of HDL-Cholesterol:
Conc. of HDL-Cholesterol (mg/dl)=Abs. Sample x 320 at 505 nm.

Linearity:
This method is linear up to 275mg/dl of HDL-Cholesterol.

Wavelength (λ) = 505 nm. (500-550).

***Calculation of LDL-Cholesterol*:** According to the Friedwald Formula;

$$\text{LDL-Cholesterol} = \text{Total cholesterol} - \frac{\text{Triglycerides}}{5} - \text{HDL-Cholesterol}$$

Reference value** according to the kit: **HDL-Cholesterol

Favorable prognosis:	
Women	> 65mg/dl
Men	> 55mg/dl
Standard risk:	
Women	45- 65mg/dl
Men	55- 55mg/dl
Increased risk:	
Women	< 45 mg /dl
Men	< 35 mg /dl

Reference value** according to the kit: **LDL-Cholesterol

Suspected above	150 mg/dl
Increased above	190 mg/dl

Sample: sample material is serum.
HDL-Cholesterol in serum is stable for 7 days at + 15 to + 25 ^{o}c and for 14 days at +2 to +8 ^{o}c

Quantitative determination of Total protein in human serum by enzymatic method

An adequate intake of protein is essential for higher animals since only the simple forms of life are able to synthesize their protein from other nitrogen sources. Proteins are present in all tissues of the body and make up a large part of the structure of the cell. In additional, a numbers of proteins have specialized physiological roles. The numbers and types of proteins present in living matter are vast and only plasma protein can even be briefly considered.

The plasma proteins blood plasma contains large amounts of a number of proteins, each of which has specialized biological function;
Plasma albumin, for example, acts as a store of protein, is important in the maintenance of plasma PH and osmotic pressure, and transports a variety of compounds in the blood.
The α- and β-globulins are associated with the transport of lipids and the **γ- globulin** with antibodies.

Fibrinogen is a soluble protein witch is converted into insoluble fibrin during blood clotting.

- Plasma contains a complex mixture of proteins. The conc. of total protein in human plasma is approximately 6.0 – 8.4g/dl. And comprises the major parts of the solids of the plasma. The proteins of the plasma are actually a complex mixture that includes not only simple proteins but also conjugated proteins such as; glycolipids, and various types of lipoproteins.

Purpose: To aid diagnosis of protein deficiency, hepatic disease, renal disorders,
And gastrointestinal disease.

Principle:
Proteins together with a basic copper-sulphate solution containing tartarate (Biuret reagent) form a violet blue colour complex.
In Biuret reaction occurs treatment of proteins or peptides with an alkaline solution of Cu^{+2} ions. As the copper ions are complexed by the amide nitrogen atoms of the peptide backbone. The dilute copper solution is pale blue, but the complex formed has a violet blue colour. The quantity of this colour formed is proportional to the total protein concentration.

Patient preparation: As appropriate, explain the purpose of the test. Inform the patient that restriction of food or fluid is not required before the test. Tell the patient the test requires a blood sample. As appropriate, withhold medication that may alter the test result.

Reagents:

Reagent 1**"Biuret reagent"**; is a mixture of Potassium-Sodium Tartarate, Sodium iodide, Potassium iodide, and copper sulphate.

Preparation of working reagent and stability:
The reagent is ready for use. It's stable at room temperature up to the date of expiration as specified. "Avoid direct sun light".

Procedure:

1-Marks three test tubes as **T (Test), B (Blank), S (Standard).**

2- Add the following amount as shown in the table below;

Reagent	B	S	T
Standard	-	100µ	-
Sample	-	-	100µ
Biuret reagent R1	5ml	5ml	5ml

3- Mix well and incubate 20 min. at 37 ^{0}C.

4- Wait 5 min. at room temperature.

5- Measure the Absorbance of standard and test solution, against blank to set spectrophotometer to Zero at wavelength (λ) = 540 nm.

The color intensity is stable for 60 min.

Linearity;
This method is linear up to 15g/dl.

If the concentration is greater than 15g/dl, dilute the sample 1:2 with saline solution and repeat the determination and multiply the result by (2).

Wavelength (λ) = 540 nm.

$$\textbf{Concentration (g/dl)} = \frac{\textbf{Abs. (T)}}{\textbf{Abs. (S)}} \textbf{ X Conc. (S)}$$

Concentration of standard = 7 g/dl

N.B: g/dl x 10 = g/l

Values: Normally, total serum protein levels range from 6.0 to 8.4 g/dl.

***Reference value** according to the kit:*

New born:	5.0-9.1 g/dl
Children:	5.4-8.7 g/dl
Adults:	6.7-8.7 g/dl

Sample: Serum and EDTA-plasma.

Implications of results:

Increased serum levels of total proteins may indicate pathologic states, such as; chronic inflammatory disease "such as rheumatoid arthritis", dehydration, monocytic leukemia, multiple myeloma, vomiting, diarrhea and diabetic acidosis.

Decreased serum levels of total proteins may indicate pathologic states, such as; benzene and carbon tetrachloride poisoning, blood dyscrasias, congestive heart failure, essential hypertension, gastrointestinal disease, hepatic disfunction and hemorrhage.

Quantitative determination of Albumin in human serum by enzymatic method

Albumin, it is the major protein in human serum or plasma. It makes up approximately 60% of total serum or plasma protein.
Some 40% of albumin is present in the plasma, and the other 60% is present in the extracellular spaces. Albumin is manufactured in the liver, which produces about 12 gm of albumin per day, representing about 25% of total hepatic protein synthesized and 50% of its secreted protein. It has a half-life of about 4 weeks.
Mature albumin consists of one polypeptide chain of 585 amino acids and contains 17-disulfide bond. By the use of protease, albumin can be subdivided into three domains, which have different functions.
Albumin has ellipsoidal shape, which means that it doesn't increase the viscosity of the plasma as much as elongated molecules such as fibrinogen does.

Purpose: To aid diagnosis of protein deficiency, hepatic disease,
And gastrointestinal disease.

Principle:
Serum albumin in the presence of bromocresol green at slightly acid PH, produces a colour change of the indicator from yellow-green to green-blue.
The quantity of this color formed is proportional to the albumin concentration.

Patient preparation: As appropriate, explain the purpose of the test. Inform the patient that restriction of food or fluid is not required before the test. Tell the patient the test requires a blood sample. As appropriate, withhold medication that may alter the test result.

Reagents:
Reagent 1; is a solution of bromocresol green "PH 4.2".

Preparation of working reagent and stability:
The reagent is ready for use. It's stable at room temperature up to the date of expiration as specified. "Avoid direct sun light".

Procedure:

1-Marks three test tubes as **T (Test), B (Blank), S (Standard).**

2- Add the following amount as shown in the table below;

Reagent	B	S	T
Standard	-	20µ	-
Sample	-	-	20µ
Reagent R1	4ml	4ml	4ml

3- Mix well and Wait for 10 min. at room temperature.

4- Measure the Absorbance of standard and test solution, against blank to set spectrophotometer to Zero at wavelength (λ) = 630 nm.

The color intensity is stable for 60 min.

Linearity;
This method is linear up to 6.0 g/dl.

If the concentration is greater than 6.0 g/dl, dilute the sample 1:2 with saline solution and repeat the determination and multiply the result by (2).

Wavelength (λ) = 630 nm. (600-650)

$$\textbf{Concentration (g/dl)} = \frac{\textbf{Abs. (T)}}{\textbf{Abs. (S)}} \textbf{ X Conc. (S)}$$

Concentration of cholesterol standard = 5.0 g/dl

Values: Serum 3.5 – 5.0 g/dl.

Sample: Serum and EDTA-plasma.

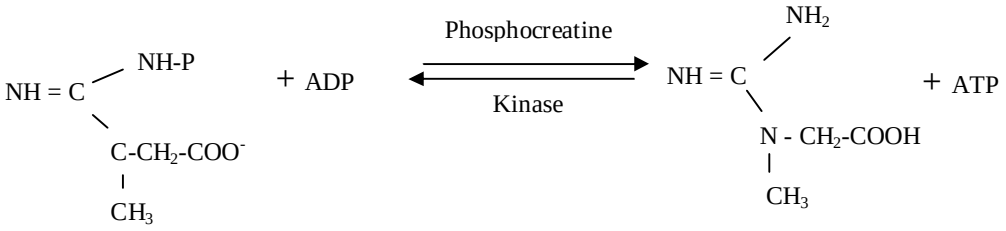
Implications of results:
Increased serum levels of albumin may indicate pathologic state, which is, multiple myeloma.
Decreased serum levels of albumin may indicate pathologic states, such as: essential hypertension, acute cholecystitis, collagen diseases, diarrhea, malnutrition and R. arthritis.

Quantitative determination of Creatinine in human serum by enzymatic method

A quantitative analysis of serum creatinine levels, this test provides a more sensitive measure of renal damage than blood urea nitrogen levels, because renal impairment is virtually the only cause of creatinine elevation. Creatinine is a non-protein end product of creatine metabolism. Similar to creatine, creatinine appears in serum in amounts proportional to the body's muscle mass, unlike creatine, it easily excreted by the kidneys with minimal or tubular reabsorption. Creatinine levels, therefore, are directly related to the glomerular filtration rate. Since creatine levels normally remain constant, elevated levels usually indicate diminished renal function. Determination of serum creatinine is commonly based on **jaffe reaction**.

Creatine: it is a substance initially synthesized in kidneys, small intestinal mucosa, pancreas, and probably, the liver, from three amino acids; Glycin, Arginine, and Methionine. Final step in creatine pathway in the body is in liver. Creatine is important in muscle metabolism in that it provides storage of high-energy phosphate through synthesis of phosphocreatine.

Phosphocreatine: it is a molecule that transfers phosphate and energy to ADP to generate ATP



Phosphocreatine (pc) Creatine

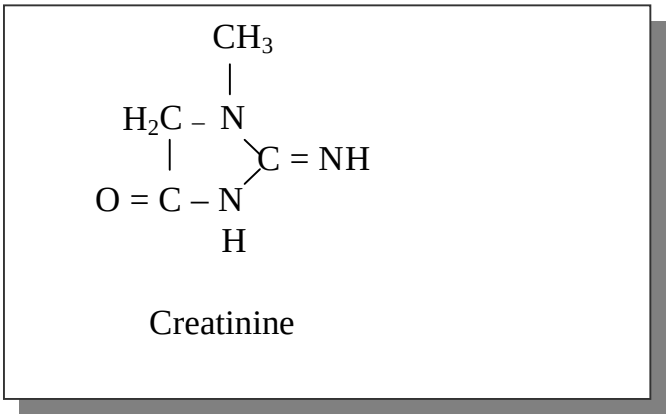
Phosphorylation occurs to the (ADP) molecule during muscle contraction activity, so the bond between phosphate group and creatine broken releasing energy similar in amount to that released from removing (P) from (ATP) molecule.

Function of phosphocreatine:

To keep the (ATP) concentration in muscle cells at constant high levels, particularly in skeletal muscle, which must perform intermittent and sometimes strenuous work at high rate. Creatine and its energy-reserve form phosphocreatine are present in muscles, brain and blood.

Creatinine: is the anhydride of creatine, which is formed through a series of enzymatic reaction in liver. Creatinine and adenosine triphosphate are involved in the contractile process in skeletal muscle, mediated by the enzyme

creatinekinase. As creatine is formed phosphocreatine, a portion of this product spontaneously cyclizes to produce the anhydride, creatine. This end product is readily filtered at the glomerulus and does not undergo any significant tubular reabsorption or secretion (although some 6-8% is eliminated by tubular secretion).
The output of creatinine can be better related to muscle mass in an individual than total body mass.
The obese individual can be expected to have a lower creatinine production rate than a non-obese individual with the same body mass.



Creatinine

Formation of creatinine is reasonably constant, and about 2% of the creatine is so transformed every 24 hours.
The 24hrs excretion of creatinine in the urine of a given subject is remarkedly constant from day to day and proportionate to muscle mass, thus, creatinine formation also has a direct relationship to muscle mass.
Free creatinine is not reutilized in the body's metabolism, and thus function solely as a waste product of creatinine
The constancy of creatinine formatted and excretion makes creatinine a useful index of renal function.

Purpose: To asses renal glomerular filtration
To screen for renal damage

Principle:
Creatinine in a basic Picrate solution forms a red-orange complex.

$$\text{Creatinine + Picric acid} \xrightarrow{\text{NaOH}} \text{creatinine-Picrate complex}$$

The absorbance of this complex is proportional to creatinine concentration in the sample.

Patient preparation:
As appropriate, explain that this test evaluates kidney function. Tell the patient the test requires a blood sample. Instruct him to restrict food and fluids for about 8hours before the test. Withhold medication that may alter the test result.

Reagents:
Reagent 1: Picric acid solution
Reagent 2: Sodium hydroxide
Preparation of working reagent and stability:
The reagents are ready for use. It's stable at room temperature up to the date of expiration as specified. "Avoid direct sun light".

Reagent mixture:
Mix proportionally **1:1** the reagents R1 and R2
Stability: 10 days at room temp.
Note that the reagent mixture is only prepared just prior to use.

Procedure:

1-Marks two test tubes as **T (Test), S (Standard).**

2- Add the following amount as shown in the table below;

Reagent	S	T
Standard	0.20ml	-
Sample: serum or diluted urine	-	0.20ml
Reagent mixture	2.00ml	2.00ml

3- Mix well and read absorbance (A1) after 30 sec., read again the absorbance (A2) after 1min. from the starting time, against air or dist. H_2O to set spectrophotometer to Zero at wavelength (λ) = 492nm.

Linearity; This method is linear up to 15 mg/dl.

If the creatinine concentration is greater than 15 mg/dl, dilute the sample 1:2 with saline solution and repeat the determination and multiply the result by (2).

Wavelength (λ) = 492 nm. (490-510)

Calculation: having calculated the **A= (A2-A1)**, the samples creatinine concentration will be obtained using the following formula.

$$\textbf{Concentration (mg/dl)} = \frac{\textbf{(A2-A1) sample}}{\textbf{(A2-A1) standard}} \textbf{X Conc. (Standard)}$$

Concentration of creatinine standard =2 mg/dl
Values:
Creatinine concentration **in males** normally range from 0.8 to 1.2 mg/dl
Creatinine concentration **in females** normally range from 0.6 to 0.9 mg/dl
Plasma creatine concentration: is more variable than creatinine and is higher in females than in males:
in males range from **0.2 to 0.6 mg/dl**
in females range from **0.6 to 1.0 mg/dl**

Increased levels in children and pregnant woman.

Reference value *according to the kit* **:**

Serum	**0.7-1.4 mg/dl.**
Urine	**1000-1500mg/24hrs**

Sample: Serum and EDTA-plasma..
Urine dilute 1:20 with dist. water

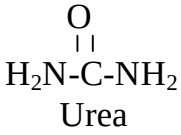
Implications of results:
Elevated serum creatinine levels generally indicate renal disease that has seriously damaged 50% or more of the nephrons.
Elevated creatinine levels may also associated with gigantism and acromegaly.

Precautions:
- Traces of creatine also normally occur in urine.
- Serum plasma must be not hemolyzed.
- The reaction is highly sensitive to temperature. The reaction temp. must be kept constant.
- Picric acid is poisonous when inhaled, swallowed, or in contact with skin.

Quantitative determination of Urea

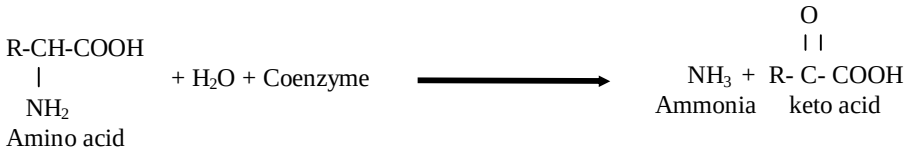
in human serum by enzymatic method

This test measures the nitrogen fraction of urea; the chief end product of protein metabolism. Formed in the liver from ammonia and excreted by the kidneys, urea constitutes 40% to 50% of the blood's non-protein nitrogen. The blood urea nitrogen (BUN) level reflects protein intake and renal excretory capacity, but is a less reliable indicator of uremic than the serum creatinine level.



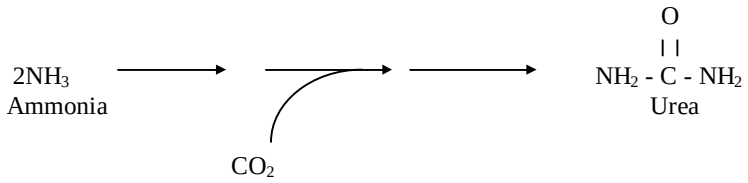
Formation of urea and urea cycle:

In cells; an oxidative determination occur for amino acids. The amino group gives rise to a molecule of ammonia (NH_3) and is replaced by an oxygen atom, derived from H_2O to form a keto acid.



NH_3 "toxic substance" will accumulate in cells, then it will pass through cell membrane and enter the blood.

From blood, NH_3 will be carried into the liver to be changed into non-toxic substance (urea) where a detoxification process occurs in liver through urea cycle.



Urea cycle it is a metabolic pathway in the liver, promotes the synthesis of urea from amino group and carbon dioxide.

Following synthesis in the liver, urea is transported by the blood plasma to be distributed to all intracellular and extracellular fluids, since urea is freely diffusible across cell membrane.

Most of urea is ultimately excreted by the kidneys after is filtered from the plasma by glomerulus's. Minimal amounts of urea are excreted through the skin with sweat and degraded by bacteria through the gastrointestinal tract.

Urea ordinary constitutes about 50% of the total urinary solids (50gm) and (80-90%) of the total urinary nitrogen.

Measurement of serum urea, often referred to as **urea nitrogen** or **blood urea nitrogen (BUN**), can give information related to the capabilities of the kidney.

Since urea nitrogen is a measure of nitrogen and not of urea one can convert (BUN) to urea by the following formula:

$$\frac{\text{Mwt. urea}}{\text{Atomic wt.no. X 2}} = \frac{60}{14x2} = 2.14$$

Conc. of urea = 2.14 X conc. of BUN (mg/dl)
Conc. of BUN = 0.466 X conc. of urea
Where 0.466, 2.14 are factors of conversion

Purpose: To evaluate renal function and aid diagnosis of renal disease.

Principle: unease splits urea into ammonia and carbon dioxide.
The free ammonia changes PH of the buffer system containing and indicator.

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2.$$

The resulting color is proportional to the urea conc. of the sample.

Patient preparation:
Tell the patient this test evaluates kidney function. Inform him he didn't restrict food or fluids before the test and that it requires a blood sample. Withhold medication that may alter test result.

Reagents:
Reagent1: is a mixture of Triethanolamin, Nacl, Phenol red, Detergent, and Stabilizer.
Reagent2: is a solution of Urease with Stabilizer.

Preparation of working solution and stability:
Reagents are ready for use and stable up to the give expiry date if contamination is avoided.
Reaction Mixture: Add reagent 1 to reagent2 in the ratio **50+1.**

Procedure:

1-Marks three test tubes as **T (Test), B (Blank), S (Standard).**

2- Add the following amount as shown in the table below;

Reagent	B	S	T
Standard	-	100µ	-
Sample	-	-	100µ
Reagent (R1)	1ml	1ml	1ml
Mix, and measure absorbance (A1) after 3 min. at 37 ^{o} C or 5 min. at 25^{0}C,Againest blank at λ=546nm.			
Reagent (R2)	-	20µ	20µ
Mix, and measure absorbance (A2) after 3 min. at 37 ^{o} C or 5 min. at 25^{0} at λ=546nm.			

Linearity; This method is linear up to 240mg/dl

If the concentration is greater than 240mg/dl, dilute the sample 1:2 with saline solution and repeat the determination and multiply the result by (2).

Wavelength (λ) = 546 nm.

Calculation:

$$\textbf{Concentration (mg/dl)} = \frac{\textbf{(A2-A1) sample}}{\textbf{(A2-A1) standard}} \textbf{ X Conc. (Standard)}$$

Concentration of urea standard =50 mg/dl

Values: Serum 10 – 50 mg/dl.

Sample: Serum and EDTA-plasma or urine

Implications of results:

Elevated BUN levels or urea levels occur in renal disease, reduced renal blood flow (caused by dehydration, for example), urinary tract obstruction, and in increased protein catabolism.

Decreased BUN levels or urea levels occur in sever hepatic damage, malnutrition, and over hydration.

Quantitative determination of Uric Acid in human serum by enzymatic method

Used primarily to detect gout. Gout is a condition related to deposition of uric acid crystals in joints. This test measures serum levels of uric acid; the major end metabolite of purine.

Large amounts of purines are present in nucleic acids and derive from dietary and endogenous sources. Uric acid clears the body by glomerular filtration and tubular secretion. However, uric acid is not very soluble at a PH of 7.4 or lower. disorders of purine metabolism, rapid destruction of nucleic acids (such as gout), excessive cellular generation and destruction (such as leukemia), and conditions marked by impaired renal excretion (such as renal failure) characteristically raise serum uric acid levels.

Purpose: To confirm diagnosis of gout.
To help detect kidney dysfunction.

Principle:

uric acid is oxidized by uricase to allantoine and hydrogen peroxide, which under

the influence of peroxidase, oxidizes DCPS and 4-aminophenazone to form a red quinoneimine compound.

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

$$2H_2O_2 + \text{4-aminophenazone} + \text{DCPS} \xrightarrow{\textbf{Peroxidase}} \text{quinoneimine} + 4\ H_2O$$

The quantity of this red dye “quinoneimine” formed is proportional to the Uric acid concentration

Patient preparation: As appropriate, explain the purpose of the test. Inform the patient that restriction of food or fluid is not required before the test. Tell the patient the test requires a blood sample. As appropriate, withhold medication that may alter the test result.

Reagents:

Mono-reagent is a mixture of pipes PH 7.5, uricase, peroxidase, and 4-aminophenazone.

Preparation of working reagent and stability;

The mono-reagent is ready to use and stable at +2 to +8 ^{o}C and keep out of light up to the date of expiration specified on the label

Procedure:

1-Marks three test tubes as **T (Test), B (Blank), S (Standard).**

2- Add the following amount as shown in the table below;

Reagent	B	S	T
Standard	-	25µ	-
Sample	-	-	25µ
Mono-reagent	1ml	1ml	1ml

3- Mix well and incubate 5 min. at 37 ^{0}C or 10 min. at room temperature.

4- Measure the Absorbance of standard and test solution, against blank to set spectrophotometer to Zero at wavelength (λ) = 520 nm.

Linearity; This method is linear up to 25 mg/dl. If the uric acid concentration is greater than 25 mg/dl, dilute the sample 1:2 with saline solution and repeat the determination and multiply the result by (2).

Wavelength (λ) = 520nm. (490-550).

$$\textbf{Concentration (mg/dl)} = \frac{\textbf{Abs. (T)}}{\textbf{Abs. (S)}} \textbf{ X Conc. (S)}$$

Concentration of cholesterol standard = 6.0 mg/dl

Values: Uric acid concentration:

Men range from **4.3 to 8 mg/dl**
Women range from **2.3 to 6 mg/dl**

Reference value *according to the kit:*
In serum or plasma

Men range from **3.4to 7 mg/dl**
Women range from **2.5 to 6 mg/dl**

In urine of 24 h.

250 to 750 mg/dl

Sample: Serum or plasma and urine.
Urine diluted 1:10 with dist. water

Notes:
if urine sample is turbid, worm up to about 60 ^{o} C for 10 min to dissolve the uric acid.
In presence of hyperlipemic or hemolyzed sample a blank with saline solution is recommended.
Uric acid in serum is stable for 3-4 days when stored at +2 to +8 ^{o} C

Implications of results:
Increased serum uric acid levels may indicate gout, although levels don't correlate with severity of disease or impaired renal function. Levels may also rise in congestive heart failure, glycogen storage disease, acute infectious disease (Such as infections mononucleosis), hemolytic or sickle cell anemia, hemoglobinopathies, polycythemia, leukemia, lymphoma, metastatic malignancy, and psoriasis.

Decreased uric acid levels may indicate defective tubular absorption, or acute hepatic atrophy

Quantitative determination of Bilirubin in human serum by enzymatic method "Total & Direct"

This test measures serum levels of bilirubin, the predominant pigment in bile.
Bilirubin; is the major product of hemoglobin catabolism, i.e. its yellow substance, formed from the breakdown of the hemoglobin molecule, and excreted in the bile as bile pigment.
After being formed in reticuloendothelial cells, bilirubin is bund to albumin and transported to the liver, where it is conjugated with glucuronic acid to form bilirubin glucuronide and bilirubin diglucuronide compounds that are then excreted in the bile. See figure bellow;

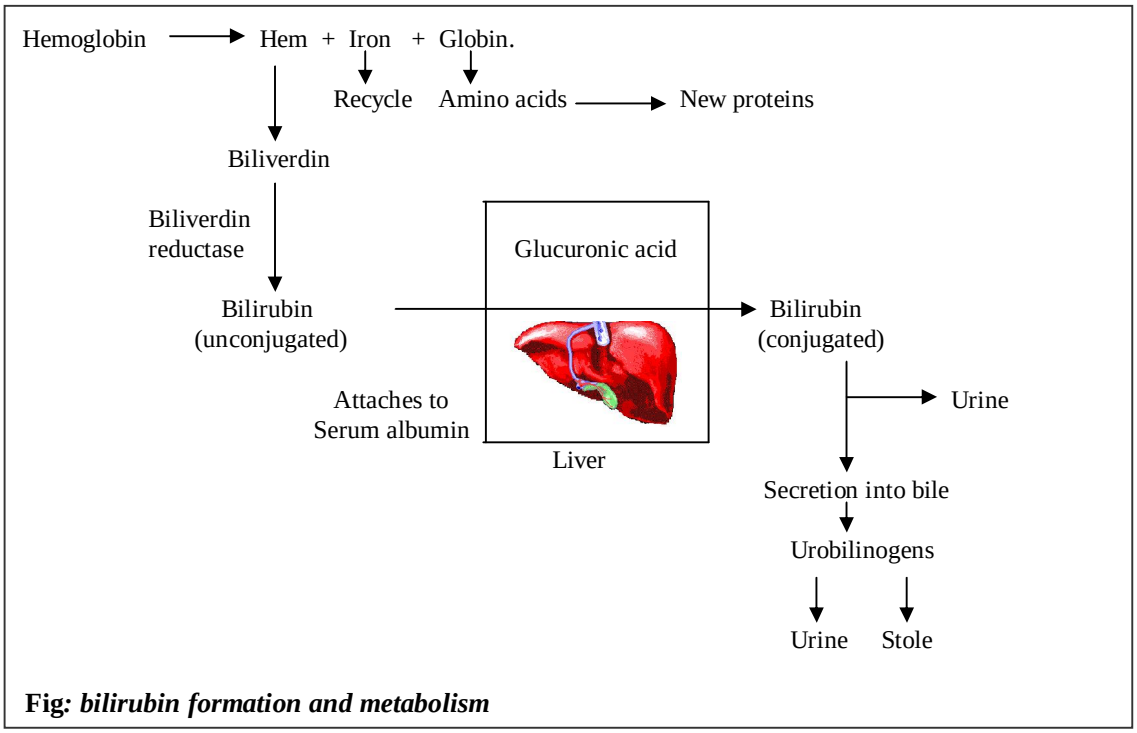
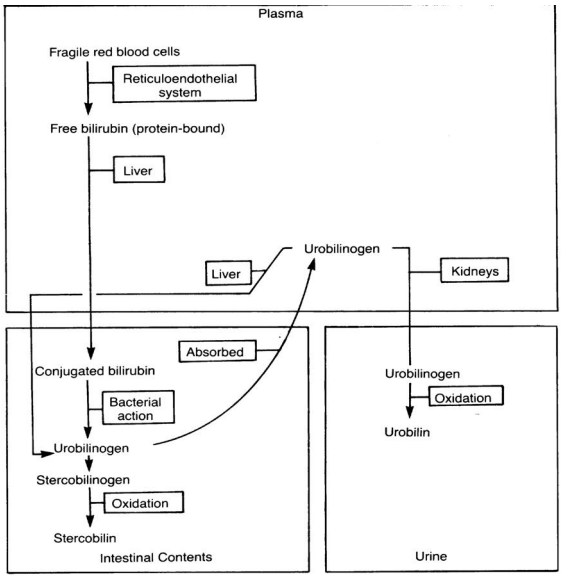


Fig*: bilirubin formation and metabolism*

- After entering the intestinal tract via bile, bilirubin is modified by bacterial enzymes to form the brown pigments that give feces their characteristic color.
- Some of the bile pigments are absorbed into the plasma during their passage through the intestinal tract and are eventually excreted in the urine, giving urine its yellow color.
- Effective conjugation and excretion of bilirubin depends on a properly functioning hepatobiliary system and a normal red blood cell turnover rate.



Therefore, measurement of unconjugated (indirect or prehepatic) bilirubin, and conjugated (direct or post hepatic)bilirubin can help evaluate hepatobiliary and erythropoietic functions.
Serum bilirubin measurements are especially significant in neonates because elevated unconjugated bilirubin can accumulate in the brain and cause irreparable tissue damage.
Elevated indirect serum bilirubin levels often indicate hepatic damage in which the parenchymal cells can no longer conjugate bilirubin with glucuronide. Consequently , indirect bilirubin reenters the blood stream.
High levels of indirect bilirubin are also likely in severe hemolytic anemia, when excessive indirect bilirubin overwhelms the liver's conjugating mechanism. If hemolysis continues, both direct and indirect bilirubin may rise.

Gallstone: when the concentration of cholesterol in the bile becomes too high, in relation to the concentration of Phospholipids and bile salts. Cholesterol will crystallized out forming the gallstone.
When the gallstone lodgers in the common bile duct it prevent bile from entering the intestine. Also the build up of pressure in a blocked bile duct inhibits further secretion of bile . then, bilirubin, which is normally secreted into the bile from the blood, accumulates in the blood and diffuses into tissues, where it produces the yellowish coloration of the skin and eyes. This condition is known as ***jaundice.***

Note that*:* Total bilirubin = ***Direct bilirubin +Indirect bilirubin***

Purpose: To evaluate liver function.
To aids differential diagnosis of jaundice .
To monitor the progression of this disorder.
To aid diagnosis of biliary obstruction and hemolytic anemia.
To determine whether a neonate requires an exchange transfusion or phototherapy because of dangerously high levels of unconjugated bilirubin.

Principle: Total bilirubin reacts with diazonium salts of 2,4-Dichloroaniline to give a red dye. The absorbance at 546nm, against a sample and a partial reagent blank, is proportional to the concentration of bilirubin. In the presence of Brij-35 the diazonium salts reacts with conjugated as well as with free bilirubin. Directly conjugated bilirubin, reacts with diazonium salt of 2,4-Dichloroaniline in aqueous solution at pH 1, the free bilirubin will be inactivated with hydrochloric acid. The distinction of direct and indirect measurable bilirubin is very difficult with values of the total bilirubin lower than 3 mg/dl.

Patient preparation: Explain this test evaluates liver function and the condition of red blood cells. If the patient is a neonate, explain the importance of this test to his parents. Advise the adult patient to fast for at least 4 hours before the test. (Fasting is not necessary for a neonate). Tell the patient the test requires a blood sample. Tell parents of a neonate that a small amount of blood

will be drown from his heel. Withhold medication that may alter bilirubin level in the serum for 24 hours before the test.

Reagents:

Reagent	Substance	
Reagent 1 "**Total**"	Detergent Nitrite	HCL Dichloroaniline Detergent Nitrite
Reagent 1 "**Direct**"	HCL Nitrite	HCL Dichloroaniline Nitrite

Reagent Preparation and stability;

- **Total bilirubin**

Blank reagent: Detergent is ready for use. The reagent is stable until the given expiry date even after opening the vial. Avoid contamination.
Reagent: Mix 2ml Nitrite solution with 1 bottle detergent. The reagent is stable for 20 days at 2-8 ^{o}C. protect reagent from direct sunlight.

- **Direct bilirubin**

Blank reagent: HCL is ready for use. The reagent is stable until the given expiry date even after opening the vial. Avoid contamination.
HCL-reagent: Mix 1ml Nitrite solution with 1 bottle HCL solution. The reagent is stable for 20 days at 2-8 ^{o}C. protect reagent from direct sunlight.

Procedure:

- ***Part I; For Total bilirubin measurement.***

1-Marks two test tubes as **S-Blank and Sample.**

2- Add the following amount as shown in the table below;

Reagent	S-Blank	Sample
Standard/ Sample	50µ	50µ
Blank solution (Total)	500µ	-
Reagent (Total)	-	500µ

3- Mix well and incubate 5 min. exactly at room temperature.

4- Measure within 30 min. the total bilirubin. the Absorbance of sample (A1) and S-Blank (A2).

5- Measure in each series 1 reagent blank (A_{RB}) for total bilirubin.

- ***Part II; For Direct bilirubin measurement.***

1-Marks two test tubes as **S-Blank and Sample.**

2- Add the following amount as shown in the table below;

Reagent	S-Blank	Sample
Standard/ Sample	50µ	50µ
Blank solution (Direct)	500µ	-
HCL-reagent (Direct)	-	500µ

3- Mix well and incubate 5 min. exactly at room temperature.

4- Measure within 5 min. the direct bilirubin. the Absorbance of sample (A1) and S-Blank (A2).

5- Measure in each series 1 reagent blank (A_{RB}) for direct bilirubin.

Linearity; This method is linear up to 40 mg/dl. If the uric acid concentration is greater than 40 mg/dl, dilute the sample 1:2 with saline solution and repeat the determination and multiply the result by (2).

Wavelength (λ) = 546nm.

Calculation :

Factor: **Conc.(mg/dl) = (A1 – A2 – A_{RB}) X 11.6**

Standard:

$$\textbf{Concentration (mg/dl)} = \frac{\textbf{(A1-A2-A}_{\textbf{RB}}\textbf{) Sample}}{\textbf{(A1-A2-A}_{\textbf{RB}}\textbf{) Standard}} \textbf{ X Conc. Standard}$$

Values: Normally in adult,
Indirect serum bilirubin measures 1.1mg/dl or less.
Direct serum bilirubin measures less than 0.5mg/dl.
Total serum bilirubin in neonate ranges from 1 to 12 mg/dl.

***Reference value** according to the kit:*

Total bilirubin: Normal value;

Men range from	0.2-1.0 mg/dl
Women range from	0.1-1.0 mg/dl
New born	
0-24hr.	< 5mg/dl
24-48hr.	< 9mg/dl
3-5days	< 12mg/dl
5weeks	< 15mg/dl

Direct bilirubin: Normal value; up to 0.25mg/dl.

Sample: Serum or plasma.

Implication of results:

Elevated serum levels of indirect bilirubin indicate hemolysis (e.g. in G-6PD deficiency, autoimmunity or transfusion), hemolytic anemia or hemorrhage, hepatocellular dysfunction or neonatal hepatic immaturity.

Elevated levels of direct conjugated bilirubin indicates biliary obstruction; in which direct bilirubin blocked from its normal pathway from the liver into the biliary tree, over flows into the blood stream.
Biliary obstruction may be resulted from (viral hepatitis, cirrhosis, gallstones, gallbladder or pancreatic carcinoma) or result from bill duct disease.

In neonates, total bilirubin levels that reach or exceed 20mg/dl indicate the need for exchange transfusion.

Quantitative determination of α-amylase in human serum by enzymatic method

Amylase, serum: Alpha amylase (amylase), synthesized primarily in the pancreas and the salivary glands, is secreted into the gastrointestinal tract. This enzyme helps digest starch and glycogen in the mouth, stomach, and intestine. In case of suspected acute pancreatic disease, measurement of serum or urine amylase is the most important laboratory test.
More thane 20 methods of measuring serum amylase exist, with different ranges of normal values. Unfortunately, values can't always be converted to a standard measurement.

Purpose: To diagnose acute pancreatitis.
To distinguish between acute pancreatitis and other causes of abdominal pain that requires immediate surgery.
To elevate possible pancreatic injury caused by abdominal trauma or surgery.

Amylase, urine: Amylase is a starch-splitting enzyme produced primarily in the pancreas and salivary glands, usually secreted into the alimentary tract, and absorbed into the blood; small amounts of amylase are also absorbed into the blood directly from these organs. Following glomerular filtration, amylase is excreted into the urine. In the presence of a adequate renal function, serum and urine levels usually rise in tandem. However, within 2 or 3 days of onset of acute pancreatitis, serum amylase persists for 7 to 10 days. One method for determining urine amylase levels is the dye-coupled starch method.

Purpose: To diagnose acute pancreatitis when serum amylase levels are normal or borderline.
To aid diagnoses of chronic pancreatitis and salivary gland disorders.

Principle:
Kinetic colorimetric determination of alpha amylase activity according to the following reaction:

$$PNPG_7 \text{ blocked} \xrightarrow{\textbf{α-amylase}} G_{2\text{-}5} + PNP\text{-}G_{2\text{-}5}$$

$$PNP\text{-}G_{2\text{-}5} \xrightarrow{\textbf{Glucoamylase}} \text{glucose} + \text{PNP-glucoside}$$

$$\text{PNP-glucoside} \xrightarrow{\textbf{α-glucosidase}} \text{glucose} + \text{PNP}$$

The rate of production of PNP is proportional to alpha amylase activity in the sample.

Patient preparation for serum amylase: As appropriate, explain that this test, which requires a blood sample, helps assess pancreatic function. Inform the

patient that he needn't fast before the test, but must abstain from alcohol. Withhold drugs that may elevate amylase levels, as appropriate.

Patient preparation for urine amylase: As appropriate, explain that this test evaluates the function of the pancreas and salivary glands. Inform the patient that the test does not require restriction of food or fluids but requires urine collection for 2, 6, 8, or 24 hours. Teach the patient how to collect a timed specimen. For example, instruct the patient not to contaminate the specimen with toilet tissue or stool. Withhold alcohol and drugs that may elevate amylase levels, as appropriate.

Reagents:

Reagent 1 Is a mixture of PIPES pH 7.20, NaCl, and CaCl.
Buffer reagent

Reagent 2 Tablets with $PNPG_7$-blocked, Glucoamylase, α-glucosidase

Preparation of working reagent and stability;
Present. 20x2ml- Dissolve one tablet with 2 ml buffer R1.
Present. 15x15ml- Dissolve one tablet with 15 ml buffer R1.
This working reagent is stable 48hours at room temperature or 15 days at 2-8 ^{o} C.

Procedure:

1-Marks two test tubes as **25-30 ^{0} C, 37 ^{0} C**

2- Add the following amount as shown in the table below;

Reagent	25-30 ^{0} C	37 ^{0} C
Working reagent	2.00ml	2.00ml
Serum sample	100µ	100µ
Or Urine	50µ	50µ

3- Mix well and wait 1 min.

4- Measure the Absorbance increase per minute for 1,2 and 3 minutes ($\triangle$E/min).against air or distilled water to set spectrophotometer to Zero at wavelength (λ) = 405 nm.

Linearity; If the mean change of absorbance increase is 0.220, repeat the test using a sample diluted 1:10 with saline solution.

Wavelength (λ) = 405nm.

Calculation: calculate the alpha amylase activity in the sample, using the following factor. For urine, multiply the result by 2 at 25-30 ^{0}C or by 2.5 at 37 ^{0}C.

At **25-30 ^{0} C** : ⧋E/min. x 2690 = U/L.

At **37 ^{0} C** : ⧋E/min. x 5125 = U/L.

Values: serum levels ranges from 140 to 130 U/L

***Reference value** according to the kit:*

	25 ^{0} C	**30 ^{0} C**	**37 ^{0} C**
Serum:	< 40 U/l	< 55 U/l	< 90 U/l
Urine:	< 190 U/l	< 240 U/l	< 490 U/l

Sample: Serum or plasma and urine.
***Note that:** Saliva and sweat contain alpha amylase. To reduce the possibility of contamination do not pipette by mouth and avoid contact of the sample and reagent with the skin.*

Implications of results:
Highest amylase levels occur 4 to 12 hours after onset of acute pancreatitis, then drop to normal in 48 to 72 hours. Determination of urine levels should follow normal serum amylase results, to rule out pancreatitis. Moderate serum elevations may accompany obstruction of the common bile duct, the pancreatic duct, or the ampulla of vater; pancreatic injury from perforated peptic ulcer; pancreatic cancer; acute salivary gland disease; ectopic pregnancy; peritonitis; and ovarian and lung cancer.Impaired renal function may raise serum levels.
Levels may be slightly elevated in a patient who is a symptomatic or who is responding usually to therapy. An amylase fractionation test helps determine the source of the amylase and aids selection of additional tests.
Depressed levels can occur in chronic pancreatitis, pancreatic cancer, cirrhosis, hepatitis, and toxemia of pregnancy.

Quantitative determination of Iron and Total iron-binding capacity

in human serum by enzymatic method

Iron is essential to the formation and function of hemoglobin, as well as many other heme and non-heme compounds. After iron is absorbed by the intestine, it's distinguished to various body compartments for synthesis, storage, and transport. Since iron appears in the plasma, bound to a glycoprotein called transferring, it is easily sampled and measured. The sample is treated with buffer and color reagents.

Serum iron assay measures the amount of iron bound to transferin; total iron-binding capacity (TIBC)measures the amount of iron that would appear in plasma if all the transferin were saturated with iron. The percentage of saturation is obtained which reveals the actual amount of saturated transferin. Normally, transferin is about 30% saturated.

Serum iron and TIBC are of greater diagnosistic usefulness when performed with the serum ferritin assay; together these tests may not accurately reflect the state of other iron compartments, such as myoglobin iron and the labile iron pool. Bone marrow or liver biopsy, and iron absorption or excretion studies may yield more information.

Purpose: To estimate total iron storage.
To aid diagnoses of hemochromatosis.
To help distinguish between iron deficiency anemia and anemia of chronic disease.
To aid evaluation of nutritional status.

Principle for iron determination:

In serum iron bound to the transferin. In weakly acidic the iron dissociates from this complex and the serum proteins remains in solution. After reduction with ascorbic, the iron is converted to a complex by specific color reagent of ferrozine.

Patient preparation: As appropriate, explain that this test evaluates the body's capacity to store iron. Inform the patient that the test requires a blood sample. Review the patient's drug history for medications that may interfere with accurate determination of test results. Withhold drugs that may elevate iron levels, as appropriate.

Reagents for iron determination:

Reagent 1	Acetone buffer pH 4.8
Reagent 2	Ascorbic acid
Redactor agent	
Reagent 3	Ferrozine
Chromogen	

Preparation of working reagent and stability for iron determination;

Add the contents of one tube R2 to the contents of one bottle R2 and dissolve completely. This working reagent is stable for 3 month at 2-8 ^{o}C or six weeks at room temperature.

Procedure for iron determination :

1-Marks four test tubes as **Reagent blank, Standard, Sample blank, Sample**

2- Add the following amount as shown in the table below;

Reagent	Reagent blank	Standard	Sample blank	Sample
Dist. water	200µ	-	-	-
Standard	-	200µ	-	-
Sample	-	-	200µ	200µ
R1 + R2	1ml	1ml	1ml	1ml
R3	50µ (1drop)	50µ (1drop)	-	50µ (1drop)

3- Mix well and wait 5 min. at 37 ^{0}C or 10 min. at 20-25 ^{0}C

4- Measure the Absorbance against reagent blank within 30 minutes.

Linearity for iron determination; This method is linear up to 1000 µg/dl. If the uric acid concentration is greater than 1000 mg/dl, dilute the sample 1:2 with saline solution and repeat the determination and multiply the result by (2).

Wavelength (λ) = 562nm (530-590).

Calculation for iron determination:

$$\textbf{Concentration (µg /dl)} = \frac{(A_{\text{Sample}} - A_{\text{Sample blank}})}{(A_{\text{Standard}})} \times \textbf{Conc. Standard}$$

Concentration of standard = 100µg/dl

Values: normal serum iron values are as follow;

Serum iron	**(mg/dl)**
Men	70 to 150
Women	80 to 150

Reference iron value *according to the kit:*

Men: 60-160 µg/dl
Women: 37-145 µg/dl

Sample: Serum. Hemolysis interferes with the test.
The iron is stable up to 7 days stored at 2-8 ^{0}C or 4 days at room temperature.

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Principle for TIBC determination:
Iron in serum is bound to the protein (siderophilin). Normally this protein is about one third satured with iron. Serum transferin is assayed by saturating with iron by absorption in magnesium carbonate powder. The iron is then assayed on the supernatant.

Patient preparation: As appropriate, explain that this test evaluates the body's capacity to store iron. Inform the patient that the test requires a blood sample. Review the patient's drug history for medications that may interfere with accurate determination of test results. Withhold drugs that may elevate iron levels, as appropriate.

Reagents for TIBC determination:

Reagent 5 Saturating solution :iron solution 500 µg/dl
Reagent 6 Precipitating agent: Magnesium carbonate (powder)

Preparation of working reagent and stability for TIBC determination;
The reagents are ready for use. All reagent is stable for date of expiration as specified when stored at 15-25 ^{o}C.

Procedure for TIBC determination : Pipette into centrifuge tube:

Reagent	Patient number
Serum sample	0.50 ml.
Saturating solution (R5)	1.50 ml
Mix and allow to stand at room temperature for 10 min.	
Precipitating agent (R6)	1 dosage
Mix and wait 10 min. at room temperature . Then centrifuge 10 min. at 3000 rpm.	

The supernatant will be processed according to the instructions of iron determination.

Linearity for TIBC determination; See linearity of iron.

Wavelength (λ) = 562nm (530-590).

Calculation for TIBC determination:

$$\textbf{Concentration (µg /dl)} = \frac{(A_{\text{Sample}})}{(A_{\text{Standard}})} \textbf{ X Conc. Standard}$$

Values: normal serum TIBC values are as follow;

Serum	**(mg/dl)**
Men	70 to 150
Women	80 to 150

Reference* TIBC *value *according to the kit:*
Serum: 200-400 µg/dl

Sample: Serum. Hemolysis interferes with the test.

Implications of results:
In iron deficiency, serum iron levels drop and TIBC increases to decrease the saturation. In cases of chronic inflammation (such as in rheumatoid arthritis), serum iron may be low in the presence of adequate body stores, but TIBC may be unchanged or may drop to preserve normal saturation. Iron overload may not alter serum levels until relatively late, but in general, serum iron increases and TIBC remains the same to increase the saturation.

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With my best wishes

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