AmbujaNeotia

THE NEOTIA UNIVERSITY

ज्ञानम् आत्म प्रदीपाय

BML- 291: CLINICAL BIOCHEMISTRY

LAB MANUAL

DEPARTMENT OF

MEDICAL LABORATORY TECHNOLOGY

SCHOOL OF HEALTH SCIENCES

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Liver Function Test

The liver is the largest organ in the human body, located in the right upper quadrant of the abdomen. The liver is of vital importance in intermediary metabolism, in the hematopoiesis and in the elimination of toxic substances.

Liver function test includes:

a. Serum bilirubin
b. SGPT
c. SGOT
d. Serum protein
e. Alkaline phosphatase
f. Urine — bile salts, bile pigments and urobilinogen

The interpretation of liver function test plays a key role to diagnose jaundice, parenchymal diseases (like hepatitis, cirrhosis, fatty liver and infiltration) chronic hepatitis, altered drug metabolism, endocrine abnormality, nutritional and metabolic abnormality, etc.

Serum Bilirubin

Bilirubin originates from the break down of haemoglobin. It is a waste product and the body eliminates this compound through the bile (from liver) into the intestine and ultimately through the stool. Only a small fraction of bilirubin metabolite is recycled through the body and a part of it is excreted through the urine.

Serum Bilirubin can be present in two forms:

1. *Conjugate bilirubin:* It is conjugated with glucuronic acid to form bilirubin glucuronide. It is water-soluble. It reacts directly in aqueous solution without alcohol. Therefore it is also called as direct bilirubin.

2. *Unconjugate bilirubin:* It is a free bilirubin formed from protoporphyrin component of heme. It is insoluble in water and carried away to liver by serum albumin. It reacts indirectly with presence of alcohol. Therefore it is also called as indirect bilirubin. *Specimen:* Serum is preferred for determination of bilirubin. It should not be haemolysed. Protect it from light. Plasma can be used with anticoagulant like heparin. Generally fasting samples are preferred.

METHODS

I. Malloy and Evelyn method
II. Jendrassik-Grof method

MALLOY AND EVELYN METHOD

Principle

Bilirubin couples with diazotized sulphanilic acid to form a purple coloured azobilirubin complex . Direct bilirubin reacts with the diazo reagent in aqueous solution to form a coloured diazo compound within 1 min. the indirect bilirubin is diazotized only in the presence of methanol. The subsequent addition of methanol accelerates the reaction of indirect bilirubin . The value of total bilirubin is obtained after letting the specimen stand for 30 min. The absorbance values of coloured solution are taken at 540 nm.

Reagents

1. Diazo reagent A and B
2. Methanol
3. Conc. HCl
4. Working bilirubin std. (10 mg %)
5. Diazo blank reagent (1.5 ml Conc. HCl diluted to make 100 ml with distilled water)

Procedure

1. Prepare Diazo mixture by adding 5 ml Diazo A and 0.15 ml of Diazo B.

2. Take four test tubes. Label them as, TT (total test),TB (total blank), DT (direct test), and DB (direct blank)
3. Add the reagents as ——
   i. 0.1ml serum and 0.9 ml distilled water in each tube.
   ii. 0.25ml Diazo blank in TB and DB
   iii. 0.25 ml Diazo reagent in TT and TB
   iv. 1.25 ml distilled water to DT and DB
   v. 1.25 ml methanol to TT and TB.
4. Mix well and read the OD of DT and DB after one min. against distilled water at 540 nm.
5. Mix well and keep the tubes TT and TB in dark at room temperature for 30 min. and read the OD against distilled water at 540 nm.
6. Read OD of bilirubin std.(reagent 4) against distilled water.

Reagent	*TT*	*TB*	*DT*	*DB*
Serum	0.1 ml	0.1 ml	0.1 ml	0.1 ml
Distilled water	0.9 ml	0.9 ml	0.9 ml	0.9 ml
Diazo blank	—	0.25 ml	—	0.25 ml
Diazo reagent	0.25 ml	—	0.25 ml	—
Distilled water	—	—	1.25 ml	1.25 ml
Methanol	1.25 ml	1.25 ml	—	—

Table showing Malloy and Evelyn method

Calculation

$$\text{Total bilirubin (A)} = \frac{\text{O.D. of TT} - \text{O.D. of TB}}{\text{O.D. of Std.}} \times 10$$

$$\text{Direct bilirubin (B)} = \frac{\text{O.D. of DT} - \text{O.D. of DB}}{\text{O.D. of Std.}} \times 10$$

Indirect bilirubin = A – B.

JENDRASSIK-GROF METHOD

Principle

Bilirubin reacts with diazotized sulphonilic acid in presence of a strong alkaline tartrate solution gives blue azobilirubin solution. This is a

reaction of direct bilirubin. Indirect bilirubin reacts with diazo reagent in presence of the accelerator caffeine benzoate. This reaction represents total bilirubin.

Reagents

1. 0.05 N HCl
2. Caffeine benzoate reagent
3. Diazo reagent
4. Ascorbic acid solution
5. Alkaline tartrate
6. Normal saline

Procedure

Part I. Direct Bilirubin

1. Dilute the specimen by mixing 1ml of specimen with 4ml of saline.
2. Take two test tubes. Label them as, DT (direct test), and DB (direct blank).
3. To the tube DT, add 0.5ml of diazo reagent and exactly after 1 min. add 1.5 ml alkaline tartrate.
4. Add 1ml of diluted serum and 2 ml of 0.05 N HCl in both the tubes. Mix and after 10 min. read the absorbance.
6. To DB, add 0.5 ml diazo A, 0.5 ml ascorbic acid and 1.5 ml alkaline tartrate. Mix and take the reading without waiting.

Reagent	*DT*	*DB*
Diazo reagent	0.5 ml	0.5 ml
Ascorbic acid	—	0.5 ml
Alkaline tartrate	1.5 ml	1.5 ml
Diluted serum	1 ml	—
0.05 N HCl	2 ml	—

Part II. Total Bilirubin

1. Take two test tubes. Label them as, TT (Total test), and TB (total blank)
2. Place 1ml of diluted serum and 2.1 ml Caffeine benzoate reagent in both the tubes.

3. Add 0.5 ml of Diazo reagent in test.
4. After 10 min. add 1.5 ml of alkaline tartrate and take the absorbance after 10 min.
5. To the tube TB, add 0.5 ml Diazo A and then 1.5 ml alkaline tartrate.

Reagent	*TT*	*TB*
Dil. Serum	1 ml	1 ml
Caffeine benzoate	2.1ml	2.1 ml
Diazo reagent	0.5ml	—
Alkaline tartrate	1.5 ml	—
Diazo reagent	—	0.5 ml
Alkaline tartrate		1.5 ml

Calculation

$$\text{Total bilirubin (A)} = \frac{\text{OD of TT}}{\text{OD of Std.}} \times \%\ \text{of Std.}$$

$$\text{Direct bilirubin (B)} = \frac{\text{OD of DT}}{\text{OD of Std.}} \times \%\ \text{of Std.}$$

Indirect bilirubin = A – B

Normal values:

Total bilirubin	Age	Value
	Newborn	up to 5.8 mg/dl
	1-2 days	up to 8.2 mg/dl
	3-5 days	up to 11.7 mg/dl
	Above one month to adult	up to 1.0 mg/dl

Direct bilirubin	0.0 to 0.2 mg /dl
Indirect bilirubin	0.4 to 0.8 mg /dl

Clinical Significance

Determination of serum bilirubin is important in diagnosis of diseases of hepatobiliary system and pancreas and other causes of jaundice.

Jaundice becomes apparent clinically when serum bilirubin level goes more than 2.5 mg/dl.

Increased direct bilirubin is seen in Hepatic cellular damage, liver diseases related to viral, toxic, alcohol or drugs, Biliary duct obstruction, Infiltrations, Space occupying lesions, live metastatic tumor, etc.

Increased indirect bilirubin is seen in hemolytic diseases, ineffective erythropoiesis, Blood transfusions, Haematomas, Hereditary disorders (e.g. Gilbert's disease)

TRANSAMINASES

Transamination is a process in which an amino group is transferred from an amino acid to an alpha keto acid. It is an important step in amino acid metabolism. The enzymes responsible for transamination are called Transaminases. Two diagnostically useful transaminases are Glutamate oxaloacetate transaminase or GOT (also called as asparate aminotransferase or AST) and Glutamate pyruvate transaminase or GPT (also called as alanine aminotransferase or ALT).

L-asparate + Oxoglutarate $\xrightarrow{\text{GOT/AST}}$ Oxaloacetate + Glutamate

L-alanine + Oxoglutarate $\xrightarrow{\text{GPT/ALT}}$ Pyruvate + Glutamate

SGPT (ALT)

Method

2- 4- DNPH Method

Specimen

Serum is required for the test. Haemolysis should be avoided.

Principle

GOT catalyses following reaction—

L-alanine + Oxoglutarate $\xrightarrow{\text{GPT / ALT}}$ Pyruvate + Glutamate

Pyruvate so formed is coupled with 2,4- Dinitrophenyl hydrazine (2,4-DNPH) to corresponding hydrazone, which gives brown colour in alkaline medium. This can be measured colorimetrically.

Reagents

1. Buffered alanine α-KG substrate at pH 7.4
2. DNPH colour reagent
3. 4N NaOH
4. Working pyruvate std. 2 mm

Procedure

Dilute 1 ml of 4N NaOH to 10 ml purified water. This makes the working solution.

Part I. Preparation of Standard Curve

Standardization is done against the standard Karmen unit Assay and this is extrapolated to different amounts of pyruvate. The standard graph of enzyme activity (in units / ml) on X-axis Vs O.D. on Y-axis is not a linear one. This shows that O.D. increases with increase in enzyme activity at a decreasing rate.

Take five test tubes, mark them as — 1, 2, 3, 4, and 5 for corresponding enzyme activity —0, 28, 57, 97 and 150. Add the reagents as follows:

Tube no.	*1*	*2*	*3*	*4*	*5*
Enzyme activity units /ml	0	28	57	97	150
Buffered alanine at pH 7.4	0.5 ml	0.45 ml	0.4 ml	0.35 ml	0.3 ml
Working pyruvate std. 2 mm	—	0.05 ml	0.1 ml	0.15 ml	0.2 ml
Purified water	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml
DNPH colour reagent	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml

Table showing SGPT procedure

Mix well and allow it to stand at RT for 20 min. Add 5 ml of diluted NaOH to each tube. Mix well by inversion. Allow it to stand at RT for 10 min measure the OD of all the five tubes against purified water on a colorimeter at 505 nm.

Part II. Preparation of Test

For test, take 0.25 ml of buffered alanine at pH 7.4 in a test tube. Incubate at 37°C for 5 min. Add 0.05 ml serum to the test tube and again incubate at 37°C for 30 min. Now add 0.25 ml DNPH colour reagent. Mix well by inversion. Allow to stand at RT for 20 min. At last add 2.5 ml diluted NaOH. Mix well and allow it to stand at RT for 10 min. Now measure the OD against purified water on a colorimeter at 505 nm.

Calculations

Mark the OD of test on Y- axis of std. curve and extrapolate it to the corresponding enzyme activity on X- axis.
Normal Values:

Age	*SGPT level*
1-3 yr	5-45 U/L
4-6 yr	10-25 U/L
7-9 yr	10-35 U/L

	Male	*Female*
10-11 yr	10-35 U/L	10-30 U/L
12-13 yr	10-55 U/L	10-30 U/L
14-15 yr	10-45 U/L	5-30 U/L
16-19 yr	10-40 U/L	5-35 U/L

Determination of SGPT level is important in differential diagnosis of diseases of hepatobiliary system and pancreas.

Increased SGPT levels are found in severe preeclampsia, rapidly progressing acute lymphoblastic leukemia, obesity, etc.

Decreased SGPT levels are found in genitourinary infection malignancy, malnutrition, pregnancy, alcoholic liver disease, etc.

SGOT (AST)

Method

2- 4- DNPH Method

Specimen

Serum is required for the test. Haemolysis should be avoided.

Principle

GOT catalyses following reaction—

$$\text{L-asparate} + \text{Oxglutarate} \xrightarrow{\text{GOT / AST}} \text{Oxoacete} + \text{Glutamate}$$

Oxalate so formed is coupled with 2,4- Dinitrophenyl hydrazine (2,4-DNPH) to corresponding hydrazone, which gives brown colour in alkaline medium. This can be measured colorimetrically.

Reagents

1. Buffered aspirate α-KG substrate at pH 7.4
2. DNPH colour reagent
3. 4N NaOH
4. Working oxalate std. 2 mm

Procedure

Dilute 1 ml of 4N NaOH to 10 ml purified water. This makes the working solution.

Part I. Preparation of Standard Curve

Standardization is done against the standard Karmen unit Assay and this is extrapolated to different amounts of oxalate. The standard graph of enzyme activity (in units/ml) on X-axis Vs OD on Y-axis is not a linear one. This shows that OD increases with increase in enzyme activity at a decreasing rate.

Take five test tubes, mark them as — 1, 2, 3, 4, and 5 for corresponding enzyme activity — 0, 24, 61, 114 and 190. Add the reagents as follows:

Tube no.	*1*	*2*	*3*	*4*	*5*
Enzyme activity units /ml	0	24	61	114	190
Buffered asparate at pH 7.4	0.5 ml	0.45 ml	0.4 ml	0.35 ml	0.3 ml
Working oxalate std. 2mm	—	0.05 ml	0.1 ml	0.15 ml	0.2 ml
Purified water	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml
DNPH colour reagent	0.5 ml	0.5 ml	0.5 ml	0.5 ml	0.5 ml

Table showing SGOT procedure

Mix well and allow it to stand at RT for 20 min. Add 5ml of diluted NaOH to each tube. Mix well by inversion. Allow it to stand at RT for 10 min. measure the OD of all the five tubes against purified water on a colorimeter at 505 nm.

Part II. Preparation of Test

For of all test, take 0.25 ml of buffered asparate at pH 7.4 in a test tube. Incubate at 37°C for 5 min. Add 0.05 ml serum to the test tube and again incubate at 37°C for 60 min. Now add 0.25 ml DNPH colour reagent. Mix well by inversion. Allow to stand at RT for 25 min. At last add 2.5 ml diluted NaOH. Mix well and allow it to stand at RT for 10 min. Now measure the OD against purified water on a colorimeter at 505 nm.

Calculations

Mark the OD of test on Y- axis of std. curve and extrapolate it to the corresponding enzyme activity on X- axis.

Normal Values:

Age	*SGOT level*
1-3 yr	20-60 U/L
4-6 yr	15-50 U/L
7-9 yr	15-40 U/L
10-11 yr	10-60 U/L

	Males	*Females*
12-15 yr	15-40 U/L	10-30 U/L
16-19 yr	15-45 U/L	5-30 U/L

Determination of SGOT level is important in differential diagnosis of diseases of hepatobiliary system and pancreas.

Increased SGOT levels are found in liver diseases like cirrhosis, hepatic ischema biliary obstruction, granulomas,etc. SGOT is also increased in — cerebral infarction, burns, intestinal injury, acute pancreatitis, etc. Marked increase, i.e. above 3000 U/L is found in-acute hypotension, toxic liver injury, liver trauma, viral hepatitis, etc.

Decreased SGOT levels are found in Azotemia, chronic renal dialysis, malnutrition, pregnancy, alcoholic liver disease, etc.

The normal ratio of SGOT/ SGPT is, 0.7 to 1.4. it is found increased in –Drug hepatotoxicity (> 2.0) , Alcoholic hepatitis (≤ 6.0), Cirrhosis (1.4 – 2.0) intrahepatic cholestasis (> 1.5) and chronic hepatitis.

SERUM PROTEINS

Serum proteins constitute of albumin and globulin. Its detection is useful in diagnosis of liver diseases.

Method

Biuret method.

Specimen

Serum. Haemolysis is strictly avoided.

Principle

Proteins react with cupric ions in alkaline medium to form a violet coloured complex. The intensity of the colour produced is directly proportional to proteins present in the specimen and can be measured at 530 nm.

Reagents

Biuret Reagent
Protein std. (6g/dl)

Procedure

Take three test tubes. Mark them as T, S and B add 5 ml of Biuret reagent in each tube. Add 0.05 ml of serum, 0.05 ml of protein std.

and 0.05 ml of distilled water in test, std. and blank respectively. Mix thoroughly and keep at room temperature for exactly 10 min. Measure the intensities of test and std. against blank at 530 nm

Reagent	*T*	*S*	*B*
Buriet reagent	5 ml	5 ml	5 ml
Serum	0.05	—	—
Protein std.	—	0.05	
Distilled water	—	—	0.05

Calculations

$$\text{Serum proteins} = \frac{\text{OD of T}}{\text{OD of S}} \times 6$$

Normal values

Age	Total proteins (gm/dl)
< 5 days	5.4 - 7.0
1 - 3 years	5.9 - 7.0
4 - 6 years	5.9 - 7.8
7 - 9 years	6.2 - 8.1
10 - 19 years	6.3 - 8.6

Clinical Significance

Determination of serum total proteins is useful in screening for nutritional deficiencies and gammopathies.

It is increased in multiple myeloma, Hypergammaglobulinemias, hypovolemic states. It is often found lower than limits in nutritional deficiency like Kwashiorkor and Marasmus. Decreased protein synthesis like in case of severe liver disease, increased protein loss like in severe skin disease, GI disease, Renal disease and blood loss. Increased catabolism like in case of fever or inflammation, malignancy etc.

SERUM ALBUMIN

Albumin is one of the important proteins, synthesized in liver.

Method

Bromocresol green method

Principle

Albumin binds specifically with Bromocresol green at pH 4.1 to form green coloured complex. Intensity of the colour is directly proportional to the amount of Albumin present in the sample. The colour is measured at 640 nm.

Specimen

Serum

Reagents

Albumin reagent
All standard (4.0 g/dl)

Procedure

Take three test tubes, Mark them as Test, Std. and Blank. Add 5ml Albumin reagent, in each tube; add 0.05ml serum, 0.05ml Albumin Std. and 0.05 ml distilled water to test, Std. and Blank respectively.

Reagent	*T*	*S*	*B*
Albumin reagent	5 ml	5 ml	5 ml
Serum	0.05	—	—
Albumin Std.	—	0.05	—
Distilled water	—	—	0.05

Mix thoroughly and keep at room temperature for exactly 10 minutes. Measure the intensity of the test and Std. against blank at 640 nm

Calculation

$$\text{Serum Albumin} = \frac{\text{OD of T}}{\text{OD of S}} \times 4$$

Normal values –

Age	Values
< 5 days	2.6 - 3.6
1 - 3 years	3.4 - 4.2
4 - 6 years	3.5 - 5.2
7 - 9 years	3.7 - 5.6

Clinical Significance

Determination of serum albumin is important in disorders of protein metabolism.

Albumin levels are high in dehydration and intravenous albumin infusions.

Albumin is found to be decreased in – malnutrition, decreased absorption, liver diseases, chronic infection, hyperthyroidism, pregnancy, burns, hemorrhage etc.

SERUM GLOBULIN

Serum Globulin can be obtained when the values of total protein and serum albumin are known.

Total Protein = Serum Albumin + Serum Globulin

∴ Serum Globulin = Total protein – Serum Albumin

Normal Values

Age	Values
< 1 year	0.4 - 3.7
1 - 3 years	1.6 - 3.5
4 - 9 years	1.9 - 3.4
10 - 49 years	1.9 - 3.5

A/G Ratio:

$$\text{A/G Ratio} = \frac{\text{Serum Albumin}}{\text{Serum Globulin}}$$

Normal Value – 1.2 : 1 to 2 : 1

Clinical Significance

Calculation of A/G ratio is also helpful in diagnostic interpretation for liver disease. An alteration in the A/G ratio and reversal may occur

due to the reduction in albumin and or elevation of globulin. The ratio is reduced and often reversed in cirrhosis with jaundice. However the ratio may be increased in some cases of xanthomatosis or billary cirrhosis.

ALKALINE PHOSPHATASE

Alkaline Phosphatase is present in most tissues but is present in high concentration in liver, bones, intestines, spleen, placenta and kidney. It is involved in transport of phosphate across cell membrane. It has hydrolytic and phosphate transferase activity.

Method

Kind and King's method.

Specimen

Serum is preferred, but heparinized plasma can also be used. Other anticoagulants inhibit the enzyme activity. Overnight fasting serum is preferred; store the serum in refrigerator if immediate analysis is not possible.

Principle

Alkaline phosphatase from serum converts phenyl phosphate to inorganic phosphate and phenol at pH 10. Phenol so formed reacts in alkaline medium with 4-Aminoantipyrine in presence of the oxidizing agent potassium ferrocyanide and forms an orange-red coloured complex which can be measured colorimetrically. The colour intensity is proportional to enzyme activity.

$$\text{Phenyl phosphate} \xrightarrow{\text{Alkaline Phosphatase}} \text{Phenol} + \text{Phosphate}$$

$$\text{Phenol} + 4 - \text{amino Antipyrine} \xrightarrow[OH^-]{\text{Pot. ferricyanide}} \text{Orange red coloured complex}$$

Reagents- Substrate – Disodium Phenyl Phosphate
Bufffer – $NaHCO_3 + Na_2CO_3$ (pH10)
Phenol Std. – 0.01mg/ml
NaOH
4- Amino Antipyrine
Potassium ferricyanide oxidizing agent.

Procedure

Take four test tubes. Mark them as –Test, Control, Std and Blank. Add the reagents according to following table.

Reagents	*T*	*C*	*S*	*B*
Buffer Substrate	1 ml	1 ml	—	—
Buffer	1 ml	1 ml	1.1 ml	1.1ml
Phenol Std.	—	—	1 ml	—
Distilled water	—	—	—	1 ml
Serum	1 ml	—	—	—
Incubate at 37°C for 15 minute				
NaOH	0.8 ml	0.8 ml	0.8 ml	0.8 ml
Serum	—	0.1 ml	—	—
4- amino antipyrine	1 ml	0.8 ml	1 ml	1 ml
Pot. ferricyanide	1 ml	0.8 ml	1 ml	1 ml

Mix after addition of each reagent and measure the OD of T,C,S and B against D/W at 540 nm after 10 minute.

Calculations

$$\text{Serum ALP In KA units} = \frac{\text{OD of Test} - \text{OD Control}}{\text{OD std.} - \text{OD Blank}} \times 0.10$$

Normal Values
1 - 3 years 145-320 U/L
4 - 6 years 150-380 U/L
7 - 9 years 175-420 U/L

	Males	*Females*
10-11 years	135-530 U/L	130-560 U/L
12-13 years	200-495 U/L	105-420 U/L
14-15 years	130-525 U/L	70-230 U/L
16-19 years	65-260 U/L	50-130 U/L

Clinical Significance

Determination of alkaline phosphatase is important in diagnosis of causes and monitoring of course of cholestasis (e.g. Neoplasm, drugs). It is also helpful in diagnosis of various bone disorders (e.g. Paget's disease)

ALP is increased in - Bone disorders like osteomalacia, Hodgkin's disease, increased deposition of calcium etc. Liver diseases like liver infiltrates, nodules in liver, hepatic congestion due to heart disease 44% of diabetic patients have 40% increase of ALP. 15 to 20 times increase in ALP is found in primary cirrhosis and liver cancer.

ALP is decreased in – Excess vitamin D ingestion, celiac disease, malnutrition, scurvy, zinc deficiency, Mg. deficiency, and hypothyroidism. In one-third patients of pernicious anemia, ALP level is decreased.

URINE ANALYSIS – BILE SALT, BILE PIGMENT AND UROBILINOGEN

INTRODUCTION

Bile is a yellow-green fluid that is made by the liver. It is stored in the gallbladder and passes through the common bile duct into the duodenum where it helps to digest fat. The principal components of bile are cholesterol, bile salts, and the pigment bilirubin.

Conjugated bilirubin is excreted into the biliary canaliculi and then through the bile duct, it passes to the intestine. In large intestine, it is reduced by bacterial action to a group of colourless chromogen including urobilinogen. A small fraction of urobilinogen is absorbed into the portal circulation. Partly it is re-excreted in bile while kidneys excrete the remainder. Most of the urobilinogen is excreted in the feces where it is oxidized by air to the pinkish brown urobilin.

Bile Salts (Hay's test)

Bile salts consist of glycocholic acid and taurocholic acid. They lower the surface tension of the fluid and thus cause sulphur particles to sink.

Take about 3 to 5 inch column of urine in a small beaker or in a test tube. Sprinkle finely powered dry sulfur over the surface from a height of about half-inch. If bile salts are present, the sulfur powder will sink at bottom.

The presence of bile salts indicates obstructive jaundice.

Bile Pigments

It is present in urine in obstructive jaundice and hepatocellular jaundice. The detection test includes:

a. *Fouchet's test:* This is the most sensitive test. If the urine is alkaline or neutral, acidify it with few drops of 2% acetic acid. To about 10 ml of acidic urine add about 5 ml of 10% barium chloride solution. Mix well and filter. To the residue on the filter paper add a drop of Fouchets reagent. A green or blue colour indicates presence of bile pigment's, i.e. biliverdin and bilirubin respectively.
b. *Smith's test:* Take 3 ml of urine in a test tube, over lay with equal amount of diluted tincture iodine (equal volume of distilled water and tincture iodine.) A bright ring develops at the junction of the fluids if bile pigment is present.

Urobilinogen

If the urine sample contains bile pigment it should be removed by addition of 1 part of 10% aqueous solution of calcium chloride to 4 parts of urine and filtering it.

To 10 ml of fresh urine, add 1 ml of Ehrlich Aldehyde Regent. Allow it to stand for 3 min. If red / Cherry colour is obtained, it indicates presence of urobilinogen in urine.

Normal Value < 4.0 mg/dl 24 m.

Clinical Significance

Bile in urine implies increased serum direct bilirubin. It often precedes clinical icterus. May occur without jaundice in early hepatitis, early obstruction or liver metastases.

Complete absence of urine urobilinogen strongly suggests complete bile duct obstruction. Level is normal in incomplete obstruction. Decreased in some phases of hepatic jaundice, Increased in haemolytic jaundice and subsiding hepatitis. Increase may indicate hepatic damage even without clinical jaundice.

For example, some patients with cirrhosis metastatic liver disease, congestive heart failure, presence in viral hepatitis depends on phase of disease.

Renal Function Test

The important role of kidney in the body metabolism is formation of urine. The kidney not only excretes waste product from the blood but also preserves essential high threshold substances. It also regulates hydration and electrolytic balance.

Any change in the normal constituent of urine reflects improper functioning of kidneys.

The important blood and urine parameters that are measure of kidney functions are urea, uric acid and creatinine.

SERUM UREA

Urea is the major end product of protein metabolism in human body. Urea is synthesized in the liver by urea cycle and is excreted by the kidney. Urea constitutes the major non-protein nitrogen (NPN) of the blood. It represents 45-50% NPN of the blood. It is also the major NPN substance excreted in the urine.

In some countries blood urea is represented as blood urea nitrogen (BUN).

When urea standard is used, the value comes out is blood urea and if urea nitrogen standard is used the value is in terms of BUN. The values are converted to vice versa by:

BUN = mg % Urea × 0.467

and mg % Urea = BUN × 2.14

Urea can be estimated by following methods:

I. Rate of reaction method – UV Kinetic
II. Berthelot reaction method (end point reaction)
III. Diacetylmonoxime method (DAM method)

RATE OF REACTION METHOD – UV KINETIC

Principle

Urea is hydrolysed to ammonia and carbon dioxide by urease. In presence of ammonia, α- ketoglutarate and glutamate dehydrogenase (GLDH), NADH is reduced to NAD^+.

The rate of decrease in OD is measured at the interval of 30 seconds upto 3 minutes at 340 nm. This is directly proportional to the urea concentration in the specimen

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

$$NH_3 + \alpha\text{- Ketoglutarate} + NADH + H^+ \xrightarrow[\text{GLDH}]{} \text{Glutamate} + NAD^+$$

Specimen–Serum

Reagent

1. Enzyme vials
2. Diluent (at pH8)
3. Urea nitrogen Std.

Procedure

Prepare working reagent by mixing contents of one enzyme vial with 20 ml of the diluent. It is stable at 2-4°C for 20 days. Take 1ml of working reagent in a cuvette, add 0.01ml serum, mix well and note the change in OD/min (Δ AT) after every 30 second up to 3 min. Then take 1 ml of working reagent in a cuvette and add 0.01 ml of urea nitrogen std. (20mg/dl) mix well and note the change in OD/min (Δ AS) after 30 seconds up to 3 min.

$$\text{BUN} = \frac{\Delta AT}{\Delta AS} \times 20$$

BERTHELOT REACTION METHOD (END POINT REACTION)

Principle

The principle is based on the Berthelot reaction. Urease splits urea into ammonia and carbon dioxide. The ammonia reacts with phenol in

the presence of hypochlorite to form indophenol, which with alkali gives a blue coloured compound. The intensity of coloured compound can be measured at 546 nm.

Specimen

Serum or heparinized plasma

Reagents

1. Urease/Buffer reagent
2. Phenol reagent
3. Hypochlorite reagent
4. Std. urea nitrogen (20 mg/dl)

Procedure

Take three test tubes. Label them as T, S, and B. Add 0.5 ml of urease reagent in each tube. Add 0.02 ml of serum in test and 0.02 ml of standard in std. Mix well and keep at 37°C for 10 minutes. Add 1 ml of phenol reagent and 1 ml of hypochlorite reagent in each tube. Mix well and keep at 37°C for 10 minutes. Now add 5 ml of distilled water in each tube. Mix thoroughly and read optical densities of test and standard against blank at 546 nm.

Reagent	*Test*	*Std.*	*Blank*
Urease/Buffer reagent	0.5 ml	0.5 ml	0.5 ml
Serum/Plasma	0.02 ml	—	—
Std. Urea nitrogen (20 mg/dl)	—	0.02 ml	—
Mix well and keep at 37 °C for 10 minutes			
Phenol reagent	1 ml	1 ml	1 ml
Hypochlorite reagent	1 ml	1 ml	1 ml
Mix well and keep at 37 °C for 10 minutes			
Distilled water	5 ml	5 ml	5 ml

Calculations

$$\text{Serum urea nitrogen} = \frac{\text{OD of T}}{\text{OD of S}} \times 20$$

DIACETYLMONOXIME (DAM) METHOD

Principle

Urea reacts with diacetylmonoxime in hot medium and in the presence of thiosemicarbazide and ferric ions to form a pink coloured compound. The colour intensity is directly proportional to amount of urea in specimen. It is measured at 520 nm.

Specimen

Serum is preferred, however heparinized plasma or fluoride plasma can be used.

Reagents

1. DAM – TSC Reagent (diacetylmonoxime thiosemicarbazide)
2. Uric Acid Reagent
3. Stock Urea Std. 1g%
4. Working Std. (50 mg%)

Procedure

Take 3 test tubes, mark them as T, S and B, for Test, Std. and Blank respectively. Add the reagents as per following table.

Reagent	*Blank*	*Std.*	*Test*
Distilled water	3.6 ml	3.5 ml	3.5 ml
Blood	—	—	0.1 ml
Working Std. (50 mg %)	—	0.1 ml	—
10 % sodium tungstate	0.2 ml	0.2 ml	0.2 ml
2/3 N sulphuric acid	0.2 ml	0.2 ml	0.2 ml

Mix well and allow to stand for 5 min; centrifuge into 3 test tubes pipette out as follows:

	Blank	Std.	Test
Supernatant	2 ml	2 ml	2 ml
DAM-TSC Reagent	3 ml	3 ml	3 ml
Urea acid Reagent	3 ml	3 ml	3 ml

Mix and plug with cotton and place it in a boiling water bath for exactly 15 min. cool and take the reading at 520 nm.

Calculation

$$\text{Serum Urea Nitrogen} = \frac{\text{OD of Test} - \text{OD of Blank}}{\text{OD of Std.} - \text{OD of Blank}} \times 50$$

Blood Urea Nitrogen

Normal Value

Age	Values
1 – 3 years	5 – 17 mg/dl
4 – 13 years	7 – 17 mg/dl
14 – 19 years	8 – 21 mg/dl

Clinical Significance

Determination of BUN is used in diagnosis of renal insufficiency. A BUN of 50 –150 mg/dl implies serious impairment of renal function. Markedly increased BUN (150-250 mg/dl) indicates severely impaired glomerular function. BUN is also increased in Hemorrhage to GI tract, stress, shock, congestive heart failure, acute myocardial infarction, vomiting-diarrhea etc. BUN is found to be lowered in over hydration, severe liver damage, increased utilization of proteins for synthesis, malnutrition, low protein diet, poisoning, hepatitis etc.

SERUM CREATININE

Creatine is a substance that forms when food is converted into energy through a process called metabolism. It is present in muscle, brain and blood in free form as well as in the form of creatine phosphate. Creatinine is largely formed in muscle by irreversible and non-enzymatic removal of water from creatine phosphate. Creatinine (cree-AT-ih-nin) is a compound that is excreted from the body in urine. It is a waste product creatinine levels is measured to monitor kidney function.

It is filtered out of the blood by the kidneys and then passed out of the body in urine. It is filtered at the glomeruli and secreted by the tubules. Creatinine is produced at a steady rate and is affected very little by diet or normal physical activities. If the kidneys are damaged and cannot function normally, the amount of creatinine in the urine decreases while the amount of creatinine in the blood increases. Thus, estimation of creatinine directly reflects the kidney function.

Method

Alkaline-picrate method (Jaffe reaction)

Principle

Creatinine reacts with picric acid in alkaline medium to form a reddish yellow complex. Intensity of which is directly proportional to the concentration of creatinine in the specimen and can be measured at 520 nm.

Specimen

Serum or plasma

Reagents

1. 0.04 M picric acid reagent
2. 10g/dl sodium hydroxide
3. Working creatinine std. 1mg/dl

Prepare alkaline picrate reagent by mixing 4 parts of reagent 1 and 1 part of reagent 2. This working reagent is to be freshly prepared whenever needed.

Procedure

Part I. Preparation of Protein Free Filtrate

Take two test tubes. Mark them as test and std add the reagents as follows:

Reagent	*Test*	*Std.*
Distilled water	3 ml	4 ml
Serum	1ml	—
Standard (1mg/dl)	—	1ml
2/3 N sulfuric acid	0.5ml	—
10g/dl Sodium tungstate	0.5ml	—

Centrifuge the content in the test and get clear filtrate.

Part II. Formation of Colour

Take three test tubes and label it as T, B, and S. Add the reagents as per following table.

Reagent	*Test*	*Std.*	*Blank*
Distilled water	3 ml	3 ml	3 ml
Filtrate	2ml	—	—
Diluted Std.(part I)	—	2ml	—
Alkaline picrate reagent	1ml	1ml	1ml

Mix well and keep at room temperature for 20 min. Read the intensities of test and std. at 520 nm against blank.

Calculations

$$\text{Serum creatinine mg/dl} = \frac{\text{OD of T}}{\text{OD of S}} \times 1$$

Normal Value – S. creatinine :
Fetal – 0.4–0.9
Infant – 0.3–0.7
Male – 0.7–1.4
Female – 0.6–1.1

Serum creatinine determination is useful in the diagnosis of renal insufficiency. Serum creatinine is more specific and sensitive indication of renal disease than BUN.

Serum creatinine levels are high in ingestion of creatinine (roast meat), Muscle disease like – Gigantism and acromegaly, prerenal azotemia and postrenal azotemia. 50 percent loss of renal function is

needed to increase serum creatinine from 1.0 to 2.0 mg/dl. Therefore, it is not sensitive for mild to moderate renal injury. In pregnancy serum creatinine value is found to be decreased.

URIC ACID

Uric acid is the end product of purine metabolism. The first step in the catabolism of purines (adenine and guanine) is their hydrolytic deamination to form xanthine and hypoxanthine. These are then oxidized to uric acid. Uric acid is filtered in the glomeruli and partially reabsorbed by the tubules and then it is excreted in urine.

Serum

Uric Acid-Determined by two methods.

Method

I. Henry-Caraway method
II. Enzymatic method.

I. Henry-Caraway Method

Specimen–Serum

Principle

Uric acid in protein free filtrate reacts with phosphotungstic acid reagent in alkaline medium to form a blue coloured complex. The intensity of colour is measured at 660 nm.

Reagents

1. Deproteinizing reagent
2. Sodium carbonate (10 g/d W/V)
3. Stock phosphotungstic acid reagent
4. Stock uric acid Std (100 mg/dl)

Procedure

Dilute stock phosphotungstic acid to 1:10 and stock uric acid std. to 1:200. In a centrifuge tube, take 5.4 ml of Deproteinizing reagent.

Add 0.6 ml of serum. Mix well and centrifuge at 3000 RPM for 10 minutes. Now take 3 test tubes, labelled them as Test, Std. and Blank. Take 3 ml of filtrate, 3 ml of diluted std. and 3 ml of D/W in T, S, and B tubes respectively. Add 1ml of sodium carbonate and 1ml of diluted phosphotungstic acid in each tube. Mix well and keep in dark for exactly 10 minute. Read OD of test and Std. at 660 nm against blank.

Reagent	*T*	*S*	*B*
Filtrate	3 ml	—	—
Dil. Std	—	3 ml	—
Distilled water	—	—	3 ml
Sodium Carbonate	1 ml	1 ml	1 ml
Dil. Phosphotungstic Acid 1 ml	1 ml	1 ml	1 ml

Calculation

$$\text{Serum Uric Acid} = \frac{\text{OD of T}}{\text{OD of S}} \times 5$$

II. Enzymatic Method

Principle

Enzyme uricase converts uric acid to allantonin and hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide reacts with phenolic chromogens to form red coloured compound. The intensity of red colour is proportional to the amount of uric acid in the sample.

Reaction

$$\text{Uric Acid} + H_2O \xrightarrow{\text{Uricase}} \text{Allantonin} + H_2O_2$$

$$H_2O_2 + \text{phenolic chromogens} \xrightarrow{\text{peroxidase}} \text{Red Colour Compound}$$

Reagents

Uric Acid reagent
Uric Acid Std. (5 mg/dl)

Specimen

Serum

Procedure

Take 3 test tubes, mark them as T, S and B. Take 0.025ml of serum, 0.025 ml of std. and 0.025 ml of distilled water in tubes T, S and B respectively. To each tube add 1ml of uric acid reagent.

Reagent	*T*	*S*	*B*
Serum	0.025 ml	—	—
Std.	—	0.025 ml	—
Distilled water	—	—	0.025 ml
Uric Acid Reagent	1ml	1ml	1 ml

Allow the tubes to stand for 10 minutes at room temperature. Measure the OD at 590 nm against blank.

Calculation

$$\text{Uric Acid} = \frac{\text{O.D. of T}}{\text{O.D. of S}} \times 5$$

Normal value:

1 – 3 yr	1.8 – 5 mg/dl
4 – 13 yr	2.2 – 4.7 mg/dl
14 – 19 yr	2 – 5 mg/dl

	Males	Females
10 – 11 yr	2.3 – 5.4 mg/dl	3 – 4.7 mg/dl
12 – 13 yr	2.7 – 6.7 mg/dl	3 – 5.8 mg/dl
14 – 15 yr	2.4 – 7.8 mg/dl	3 – 5.8 mg/dl
16 – 19 yr	4 – 8.6 mg/dl	3 – 5.9 mg/dl

Clinical Significance

Uric acid levels are very liable and show day-to-day and seasonal variation in some person. It is also increased by emotional stress, total fasting, increased body weight, and renal failure. It is found to be increased in gout, leukemia, polycythemia, anaemia, psoriasis, hypo

and hyperparathyroidism. It is increased with high protein, weight reduction diet, alcohol consumption, arteriosclerosis and hypertension. Serum uric acid is increased in 80% patients with elevated serum triglycerides.

Serum uric acid levels are decreased in—Wilson's disease, Fanconi's syndrome, carcinomas, Hodgkin's disease, 5 percent patients of postoperative state (GI surgery, coronary artery bypass) diabetes mellitus. It is also low in healthy adults with isolated defect in tubular transport of uric acid.

URINE UREA

This test is mainly used to assess the protein balance and the amount of dietary protein needed by severely ill patients. Urine urea serves this purpose, as it is a measure of protein breakdown in the body.

Urea is excreted by the kidneys, so excretion of urea can reflect kidney function. The urine urea excretion can be measured to obtain a ratio between the plasma (blood) urea and the urine urea. This ratio (U/P urea) is an indicator of how well the kidneys are able to filter and excrete urea from the bloodstream.

For the determination of urine urea 24 hrs of urine sample diluted to 1:20 is used. Diaectylmonoxime method (serum urea) can be applied.

Urine Urea Nitrogen

$$= \frac{\text{OD of test} - \text{OD of Blank}}{\text{OD of Std.} - \text{OD of Blank}} \times 200 \times \text{dilution of urine}$$

Normal Values

Normal values range from 6 to 17 gm/24 hours or (60 to 90 mg/dl).

Abnormal results are indicated as follows:

Low levels usually indicate:
Malnutrition (inadequate protein in diet), Kidney dysfunction and increased re-absorption.

High levels usually indicate:
Excessive protein intake and increased protein breakdown in the body.

UREA CLEARANCE

Urea is filtered at glomerulus, but it is so diffusible that some is reabsorbed in the tubule. The urea clearance is therefore lower than the glomerular filtration rate (GFR), but can still be a test of some usefulness.

Urea clearance can be defined as the ml of blood (plasma/serum), which contain the urea excreted in a minute by the kidneys.OR The volume of the blood cleared of urea per minute by either renal clearance or hemodialysis.

When there is a good flow of urine (more than 2ml per min.) the proportion of urea, which is reabsorbed, is fairly constant and the clearance is calculated by the usual formula. The value obtained is then called the maximum urea clearance (C_m)

$$C_m = \frac{UV}{P}$$

Where,
V = Urine volume in ml per min.
U = Urine urea conc. in mg /100 ml
P = Plasma urea conc. in mg/ 100 ml
With maximum clearance, normal is 64-99 ml/min.

If urine flow is less than 2 ml per min, the high concentration of urea in the renal tubules causes increased re-absorption. To compensate for the lower clearances observe with the reduced urine flow with a different formula is applied.

$$C_s = \frac{U\sqrt{V}}{P}$$

With standard clearance, normal value is 40-68 ml/min

Clinical Significance

The urea clearance values fall progressively with increasing renal failure. If the clearance falls below 20% it is considered to be severe renal failure. Below 5 percent uremic comas may be present.

URINE CREATININE

For the determination of urine creatinine, 24 hrs of urine sample diluted to 1:10 is used. Alkaline picrate method (serum creatinine) can be applied. If proteins are present, deproteinization of urine is must.

Calculations

$$\text{Urine creatinine mg/dl} = \frac{\text{OD of T}}{\text{OD of S}} \times 100$$

For the determination of 24hr creatinine excretion, measure the urine volume, and calculate the result as follows:

$$\text{Creatinine excretion, mg/24hr} = \frac{\text{urine creatinine mg/dl} \times \text{vol. of 24 hrs urine}}{100}$$

Normal Value :
Males — 19-26 mg/kg of body weight/24 hr.
Females — 14-21 mg/kg of body weight/24 hr.

Clinical Significance

The amount of creatinine excreted varies with the muscle mass and is nearly constant for individual. Increased excretion of creatinine occurs in tissue metabolism i.e. in fever. The excretion rate decreases in all kinds of renal diseases and also in postrenal conditions. Decreased excretion of creatinine also occurs in starvation, muscle atrophy and muscular weakness.

CREATININE CLEARANCE TEST

A creatinine clearance test measures how well creatinine is removed from blood by the kidneys. Compared to a blood creatinine level, a creatinine clearance test provides a more precise measure of how well the kidneys are working. A creatinine clearance test is performed both on a blood sample and on a sample of urine collected over 24 hours (24-hour urine sample).

Creatinine clearance is defined as the amount of plasma in ml, which would have to be completely cleared of the creatinine, each minute by both the kidneys in order to account for its rate of excretion.

The specimen is 24-hour urine sample. The actual period of urine collection must be accurately timed. The starting and finishing of the test of the patient with an empty bladder. The urine volume is measured and minute volume V- is calculated.

$$V = \frac{\text{Total volume of urine in ml}}{\text{Time of collection in minute}}$$

The creatinine concentration of urine (U) and plasma (P) are determined by above methods. In case of any delay in performing the test, specimen should be stored in refrigerator.

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

Normal Value:

Clearances vary with body weight. It is generally expressed as $1.73m^2$ of the body surface area.

Males — 95 - 140 ml/min.

Females — 85 - 125 ml/min.

Clinical Significance

Clearance values are decreased in impaired renal function and so provide a rough impression of glomerular damage. It decreases in renal failure with values below 10 ml per minute in severe cases. In less severe failure cases the creatinine clearance will have fallen to about half before a raise in blood creatinine is detectable. Clearance tests are therefore of most value in the diagnosis of early renal disease They are also useful in monitoring progress, although once the serum creatinine is raised, this estimation alone will be sufficient.

URINE URIC ACID

The urine uric acid is made up of an exogenous part that is formed from purine rich diet and an endogenous part that is formed from the breakdown of nucleoproteins.

For the determination of urine uric acid, 24 hr of urine sample diluted to 1:200 is used. Henry – Caraway Method (serum uric acid) can be applied. If proteins are present, deproteinization of urine is must.

Calculations

$$\text{Urine uric acid mg/dl} = \frac{\text{O.D. of T}}{\text{O.D. of S}} \times 100$$

For the determination of 24 hr uric acid excretion, measure the urine volume, and calculate the result as follows:

$$\text{uric acid excretion,mg/24 hr} = \frac{\text{urine uric acid mg/dl} \times \text{vol. of 24 hrs. urine}}{100}$$

Normal Value: $\leq$ 750 mg/24 hr.

Clinical Significance

A consistently high uric acid excretion is found in gout and leukemia. The uric acid determination is important to find out the possibility of urinary calculi (of uric acid type). The uric acid creatinine ratio is > 1.0 in most patients with acute renal failure due to hyperuricemia but lower in other causes of acute renal failure.

Cardiac Function Test

The cardiac function test estimates all the lipid contents of the body. The important lipid profile tests include:

Total lipids

- Serum total cholesterol
- Serum HDL cholesterol
- Serum triglycerides
- LDL and VLDL

SERUM TOTAL CHOLESTEROL

Cholesterol is a lipid and is classified as sterol. It is widely distributed in various animal tissues and vegetable oils and consumed with food. It can also be synthesized in the liver. It is a normal constituent of bile, and is principal constituent of most gallstones. It is important in metabolism serving as precursor of various steroid hormones, e.g. sex hormones and adrenal corticoids.

Serum total cholesterol includes esterified cholesterol as well as non-esterified cholesterol.

Method

I. Watson method, and
II. Enzymatic method

I. Watson Method

Specimen: Serum is preferred. Plasma can also be used. Fluoride, oxalate, or EDTA can also be used for this method.

Principle

Cholesterol reacts with acetic anhydride in the presence of glacial acetic acid and conc. sulfuric acid to form green coloured complex. Intensity of the colour is proportional to the cholesterol concentration. It is measured at 520–580 nm.

Reagent

1. Cholesterol reagent (this should be stored in amber coloured bottle at room temperature)
2. Conc. sulfuric acid
3. Cholesterol std. (200 mg/dl in glacial acetic acid)

Procedure

Cholesterol reagent is highly corrosive so, it should not be pipetted by mouth. Take three test tubes and mark them as T, S, and B. Add 2.5 ml cholesterol reagent in each tube. Add 0.1 ml of serum, 0.1 ml of cholesterol std. and 0.1 ml of distilled water in test, std. and blank respectively. This is an exothermic reaction. So, cool the tubes to room temperature by placing in water bath. Now carefully add 0.5ml of sulfuric acid to each tube mix thoroughly and keep in water bath at room temperature for 10 min. Read the absorbance of test and std. against blank at 575nm.

Reagent	*T*	*S*	*B*
Cholesterol reagent	2.5 ml	2.5 ml	2.5 ml
Serum	0.1 ml	—	—
Cholesterol std.	—	0.1 ml	—
Distilled water	—	—	0.1 ml
Cool to room temperature			
Conc. Sulfuric acid	0.5ml	0.5ml	0.5ml

Calculations

$$\text{Serum cholesterol mg/dl} = \frac{\text{OD of T}}{\text{OD of S}} \times 200$$

II. Enzymatic Method

Specimen

Serum or heparinised plasma

Principle

Cholesterol esterase hydrolyses cholesterol ester into free cholesterol and fatty acids. In the second reaction, cholesterol oxidase converts free cholesterol to cholest-4en-3one and hydrogen peroxide. In presence of peroxidase, hydrogen peroxide reacts with 4- amino antipyrine and phenol to produce red colour. The intensity of red colour is directly proportional to the amount of cholesterol present in sample.

$$\text{Cholesterol ester} \xrightarrow{\text{cholesterol esterase}} \text{Free cholesterol + Fatty acids}$$

$$\text{Free cholesterol} \xrightarrow{\text{cholesterol oxidase}} \text{Cholest- 4en-3one + } H_2O_2$$

$$H_2O_2 + \text{4- amino antipyrine + Phenol} \xrightarrow{\text{peroxidase}} \text{Red colour}$$

Reagent

1. Buffer/Enzymes/Chromogen
2. Phenol (30 mg/dl)
3. Cholesterol std. (200 mg/dl)

Procedure

Prepare working reagent freshly by mixing 10 ml of reagent 1. and 5ml of reagent 2. i.e, phenol. Take three test tubes and mark them as T, S, and B. Add one ml of freshly prepared working reagent in each tube. Add 0.1 ml of serum, 0.1 ml of cholesterol std. and 0.1 ml of distilled water in test, std. and blank respectively. Mix well and keep at 37°C for 20 min. Read the absorbance of test and std against blank at 530 nm.

Reagent	*T*	*S*	*B*
Working reagent	1ml	1ml	1ml
Serum	0.1 ml	—	—
Cholesterol std.	—	0.1ml	—
Distilled water	—	—	0.1 ml

Calculations

$$\text{Serum cholesterol mg/dl} = \frac{\text{OD of T}}{\text{OD of S}} \times 200$$

Normal Value:

Neonates	45 – 100 mg %
1 – 19 yrs	120 – 240 mg %
20 – 29 yrs	144 – 275 mg %
30 – 39 yrs	165 – 295 mg %
40 – 49 yrs	177 – 350 mg %
50 – 59 yrs	160 – 330 mg %
> 69 yrs	170 – 300 mg %

Clinical Significance

Total cholesterol estimation is useful in monitoring for increased risk factor for coronary artery disease, screening and monitoring for hyperlipidemias.

It is high in hyperlipoproteinaemias, hypothyroidism, nephrosis, pancreatic disease like diabetes mellitus, chronic pancreatitis, biliary obstruction like stone, carcinoma, biliary cirrhosis, it is also increased in cholesterol ester storage disease and Von Gierke's disease. Usage of some drugs like birth control pills, amiodarone, or vitamins can also show increased cholesterol value.

The serum total cholesterol levels are decreased in severe liver cell damage, hyperthyroidism, chronic anaemia, cortisone and ACTH therapy. The levels of total cholesterol are also low in Tangier disease and in some infections.

HIGH DENSITY LIPOPROTEINS (HDL)

HDL is a lipoprotein found in the blood. It is called "good" cholesterol because it removes excess cholesterol from the blood and takes it to

the liver. A high HDL level is related to lower risk of heart and blood vessel disease.

Method

Watson Method

Specimen

Serum (fasting)

Principle

In the presence of phosphotungstic acid and magnesium chloride, LDL, VLDL and chylomicrons are precipitated. Centrifugation leaves only HDL in supernatant. Cholesterol in HDL fraction can be tested by usual method.

Reagent

1. Cholesterol reagent
2. Conc. sulfuric acid
3. PTA phosphotungstic acid reagent
4. Magnesium chloride reagent
5. Cholesterol std. (100 mg/dl)

Procedure

Part I. Precipitation of LDL and VLDL

Take 0.5 ml of serum. add 0.05 ml of PTA reagent and 0.02 ml of magnesium chloride reagent. Mix well and centrifuge at 3000 RPM for 20 min. to obtain a clear supernatant. LDL and VLDL will form precipitate. Only HDL will remain in the supernatant.

Part II. Estimation of HDL

Separate the supernatant by using Pasture pipette. Take three test tubes and mark them as T, S, and B. Add 2.5 ml cholesterol reagent in each tube. Add 0.1 ml of supernatant, 0.1 ml of cholesterol std. and 0.1 ml

of distilled water in test, std. and blank respectively. This is an exothermic reaction. So, cool the tubes to room temperature by placing in water bath. Now carefully add 0.5ml of sulfuric acid to each tube. Mix thoroughly and keep in water bath at room temperature for 10 min. Read the absorbance of test and std. against blank at 520 nm

Reagent	*T*	*S*	*B*
Cholesterol reagent	2.5 ml	2.5 ml	2.5 ml
Supernatant	0.1 ml	—	—
Cholesterol std.	—	0.1 ml	—
Distilled water	—	—	0.1 ml
	Cool to room temperature		
Conc. sulfuric acid	0.5 ml	0.5 ml	0.5 ml

Calculations

$$\text{Serum HDL cholesterol mg/dl} = \frac{\text{OD of T}}{\text{OD of S}} \times 114$$

Clinical Significance

Estimation of HDL is useful in diagnosis of various lipoproteinemias and to assessment the risk for CAD(coronary heart disease). HDL is inversely proportional to CAD.

It is increased in — increased clearance of triglyceride(VLDL), insulin treatment, vigorous exercise, moderate consumption of alcohol, oral esterogen use, familial lipid disorders with the protection against arteriosclerosis, in hyperalphalipoproteinaemias and hypobetalipoproteinaemias. HDL also increases in 1 in 20 adults with mild increase in total cholesterol.

There are various secondary causes for decrease in HDL level, e.g. obesity, smoking, diabetes mellitus, stress and recent illness, lack of exercise, hypo- and hyperthyroidism, starvation (non- fasting sample is 10-15% lower). As stated earlier, HDL is inversely proportional to CAD. For every 1 mg/dl decrease in HDL, risk for CAD increases by 2- 3%.

TOTAL CHOLESTEROL/HDL RATIO

The risk associated with total cholesterol/HDL ratio is expressed as follows:

Low risk	3.3 – 4.4
Average risk	4.4 – 7.1
Moderate risk	7.1 – 11.0
High risk	> 11

VLDL AND LDL

i. VLDL: It is a major carrier of triglyceride (60 – 70 percent triglyceride, 10-15 cholesterol) circulating fatty acids are vitalized by the liver to form triglycerides that are packaged with apoprotein and cholesterol and exported into blood as very low density lipoproteins.

Normal Value = 25 – 50 %

VLDL can be estimated by Friedewald equation

$$\text{VLDL} = \frac{\text{Triglycerides}}{5}$$

This formula is applicable only when the triglycerides level is below 400 g/dl.

ii. LDL –Low Density Lipoproteins: Degradation of VLDL leads to major source of LDL. LDLs are cholesterol rich remnants of the VLDL. LDL is a lipoprotein found in the blood. It is called "bad" cholesterol because it picks up cholesterol from the blood and takes it to the cells. A high LDL level is related to a higher risk of heart and blood vessel disease. LDL is more prevalent in blood. It is finally catabolised in the liver and possibly in non-hepatic cells as well.

Normal Value: 62- 185 mg/dl

LDL is measured by ultracentrifugation and by analysis after antibody separation from HDL and VLDL. LDL can be estimated by following formula. (Friedewald equation)

LDL = Total cholesterol – (HDL cholesterol) – (VLDL)

Clinical Significance

Determination of LDL helps in assessment of risk and decide treatment for CAD.

Increase in LDL is directly related to risk of CAD. The LDL is estimated high in–Chronic renal failure, hypothyroidism, diabetes mellitus, nephritic syndrome, Wolman's disease, etc.

LDL is low in severe illness, abetalipoproteinemia and oral estrogen use.

TOTAL LIPIDS

Lipids are usually defined as those components that are soluble in organic solvents (such as ether, hexane or chloroform), but are insoluble in water. This group of substances includes triacylglycerides, cholesterol, LDL and VLDL, HDL lipoproteins, free fatty acids, phospholipids, sterols, and carotenoids.

Method

Sulfo-phoshovanillin

Specimen

Serum (fasting)

Principle

Lipids react with vanillin in the presence of sulfuric acid and phosphoric acid to form a pink coloured complex. This is measured at 546 nm.

Reagent

1. Total lipid std. (1000mg/dl)
2. Phoshovanillin (colour reagent)
3. Conc. sulfuric acid

Reagent: 1. and 2. should be kept in refrigerator in amber colour bottle.

Procedure

Take two test tubes. Add the reagents as follows:

Reagent	*T*	*S*
Total lipid std.	—	0.05ml
Serum	0.05ml	—
Conc. sulfuric acid	2.0 ml	2.0 ml

Mix thoroughly. Cotton plug it. Keep in a boiling water bath for 10 min. Then cool the tubes and pipette into dry test tubes as follows.

Reagent	*T*	*S*	*B*
From above solution	0.10 ml	0.10 ml	—
Conc. sulfuric acid	—	—	0.10 ml
Colour reagent	2.5 ml	2.5 ml	2.5 ml

Mix thoroughly and keep at room temperature for 15 min. Read the absorbance of test and std. against blank at 546 nm.

Calculations

$$\text{Serum Total lipids mg/dl} = \frac{\text{OD of T}}{\text{OD of S}} \times 1000$$

Normal value : 400 – 1000 mg/dl

SERUM TRIGLYCERIDES

Triglycerides are a type of fat found in the blood. The blood level of this type of fat is most affected by the foods one eat (such as sugar, fat or alcohol) but can also be high due to being overweight, having thyroid or liver disease and genetic conditions. High levels of triglycerides are related to a higher risk of heart and blood vessel disease.

Method

GPO POD / Enzymatic method

Specimen

Serum /plasma (heparin or EDTA). Preferably fasting

Principle

Triglycerides are hydrolysed by lipase to glycerol and fatty acids. Glycerol is phosphorylated by ATP in the presence of glycerol kinase (GK) to Glycerol-3-phosphate(G-3-P) which is oxidized by enzyme Glycerol-3-phosphate oxidase (G-P-O) producing hydrogen peroxide. Hydrogen peroxide so formed reacts with 4-amino antipyrine and P- chlorophenol in presence of enzyme peroxidase (POD) to produce a red quinoneimine dye.The intensity of red colour is directly proportional to the amount of cholesterol present in sample.

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

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{G-3-P} + \text{ADP}$$

$$\text{G-3-P} + O_2 \longrightarrow H_2O_2 + \text{Dihydroxyacetone phosphate}$$

$$H_2O_2 + \text{4- amino antipyrine} + \text{P- chloro phenol} \xrightarrow{\text{perioxidase}} \text{Red colour} + H_2O$$

Reagent

1. Buffer/Enzymes/Chromogen
2. P-chlorophenol
3. Triglyceride std (100 mg/dl)

Procedure

Prepare working reagent freshly by mixing 10 ml of reagent 1. and 5ml of reagent 2. i.e, P- chlorophenol. Take three test tubes and mark them as T, S, and B. Add one ml of freshly prepared working reagent in each tube. Add 0.1 ml of serum, 0.1 ml of triglyceride std. and 0.1 ml of distilled water in test, std. and blank respectively. Mix well and keep at 37°C for 20 min. Read the absorbance of test and std. against blank at 530 nm.

Reagent	*T*	*S*	*B*
Working reagent	1 ml	1 ml	1 ml
Serum	0.1 ml	—	—
Triglyceride std.	—	0.1 ml	—
Distilled water	—	—	0.1 ml

Calculations

$$\text{Serum Triglyceride mg/dl} = \frac{\text{OD of T}}{\text{OD of S}} \times 100$$

Normal value : 80 – 150 mg/dl

Electrolytes

SODIUM

Sodium is a major extracellular cation (Na^+) of the body. Sodium salts are necessary to preserve a balance between $Ca^{++}$and K^+ 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 blood sodium is found in the plasma. There is very little in the red cells.

Method

Modified Maruna and Trinder's method.

Specimen

Serum

Principle

Sodium from the specimen is quantitatively precipitated as the triple salt uryl magnesium sodium acetate and the excess of uryl salt reacts with potassium ferrocyanide to produce brown colour. The intensity of brown colour produced is inversely proportional to the sodium conc. of the specimen.

Reagent

1. Std. sodium chloride solution (equivalent to 300 mg of Na)
2. Uranlymagnesium acetate solution
3. Acetic acid 1 % aq. solution
4. Potassium ferrocyanide 20% solution

Procedure

Part I. Precipitation Step

Take three test tubes and mark them as T, S, and B. Add five ml of Uranylmagnesium acetate solution in each tube. Add 0.1 ml of serum, 0.1 ml of sodium std. and 0.1 ml of distilled water in test, std. and blank respectively. Mix well and allow to stand for 5 min. Centrifuge for one min. at 3000 rpm. to get clear supernatant.

Reagent	*T*	*S*	*B*
Uranlymagnesium acetate solution	5 ml	5 ml	5 ml
Serum	0.1 ml	—	—
Sodium std.	—	0.1 ml	—
Distilled water	—	—	0.1 ml

Part II. Colour Formation

Take three test tubes and mark them as T, S, and B. Add 0.2 ml of supernatant from Part I. in respective tubes. Add 8 ml of acetic acid in each tube. Now add 0.2 ml of potassium ferrocyanide in each tube. Now make up the volume to 10 ml by acetic acid. Read the absorbance of test and std. against blank at 480 nm. Be sure that the readings are taken within 10 min of last step.

Reagent	*T*	*S*	*B*
Supernatant from Part I (T)	0.2 ml	—	—
Supernatant from Part I (S)	—	0.2 ml	—
Supernatant from Part I (B)	—	—	0.2 ml
Acetic acid	8 ml	8 ml	8 ml
Potassium ferrocyanide	0.2 ml	0.2 ml	0.2 ml
Acetic acid	1.6 ml	1.6 ml	1.6 ml

Calculations

$$\text{Serum sodium mg/dl} = \frac{\text{OD of T}}{\text{OD of S}} \times 300$$

SERUM POTASSIUM

Unlike sodium, potassium is the major intracellular cation of the body. Within the cells it plays important role in maintenance of acid-base

balance, osmotic pressure and water retention. Intracellular potassium is essential for several important metabolic reactions catalyzed by enzymes. It is also very important constituent of the extracellular fluid because it influences muscle activity notably the cardiac muscle.

Method

Turbidometric method:

Specimen

Serum

Principle

Potassium ions from specimen react with sodium tetraphenyl boron resulting in a turbid suspension. The extent of turbidity is measured photometrically at 620 nm is proportional to the potassium concentration.

Reagent

1. Potassium reagent
2. Potassium Std. (5 mol / lit.)

Procedure

Take two test tubes and mark them as T, and S. Add three ml of potassium reagent in each tube. Add 0.1 ml of serum, and 0.1 ml of potassium std. in test, and std. respectively. Mix well and allow to stand for 5 min at room temperature. Read the absorbance of T and S against reagent at 620 nm.

Reagent	*T*	*S*
Potassium reagent	3 ml	3 ml
Serum	0.1 ml	—
Sodium std.	—	0.1 ml

Another popular method for determination of sodium and potassium is flame photometry—

Reagent

1. Stock standard for sodium (1000 mEq/l)
2. Stock standard for potassium (100 mEq/l)

Mixed working standards are prepared as follows:

1. Sodium/potassium (120/2.0 mEq/l): It contains 120 mEq/l of sodium and 2.0 mEq of potassium in one liter distilled water. It is prepared by mixing 12 ml Stock standard for sodium and 2 ml Stock standard for potassium in 86 ml of distilled water.
2. Sodium/potassium (140/4.0 mEq/l): It contains 140 mEq/l of sodium and 4.0 mEq of potassium in one liter distilled water. It is prepared by mixing 14 ml stock standard for sodium and 4 ml stock standard for potassium in 82 ml of distilled water.
3. Sodium/potassium (160/6.0 mEq/l): It contains 160 mEq/l of sodium and 6.0 mEq of potassium in one liter distilled water. It is prepared by mixing 16 ml stock standard for sodium and 6 ml Stock standard for potassium in 78 ml of distilled water.

Note: mEq/day = milliequivalents per day

Specimen

Serum or heparinised plasma

FLAME PHOTOMETER

It is a spectrophotometer in which a spray of metallic salts in solution is vaporized in a very hot flame and subjected to quantitative analysis by measuring the intensities of the spectral lines of the metals present.

1. Main unit and
2. Compressor unit are the important components of the equipment.

1. Main Unit

It consists of —a. An atomizer b. Mixing chamber c. Burner d. Optical filters e. Photo detectors f. Two digital displays g. Air regulator h. Gas regulator i. Gas pressure gauge.

2. Compressor Unit

It delivers oil free compressed air to the atomizer.

The atomizer and flame are the most important components in the flame photometer. The function of atomizer is to break up the solution into fine droplets so that the atoms will absorb heat energy from the flame and becomes excited.

The gases used for the flame photometer are 1. a mixture of hydrogen and oxygen 2. natural gas 3. acetylene and propane with air or oxygen 4. LPG (liquid petroleum gas).

Principle

The solution under test is passed carefully, under controlled conditions as a very fine spray in the air supply to non-luminous flame. In the flame the solution evaporates and the salt dissociates to give natural ions, which emit light of the characteristic wavelength. The flame is simultaneously monitored by both the channel consists of a detector which views the flame through a narrow band optical filter. The photo detector out puts are connected to two independent digital displays, which are calibrated for direct concentration readouts. Initial calibration is done by using at least three standards of different concentrations.

Procedure

Take four test tubes. Mark them as Test, std.1, std.2, and std.3. to each tube add 10 ml of distilled water. Add 0.1 ml of serum to test. Add 0.1 ml of std. (120/2.0 mEq/ l), 0.1 ml of std. (140/4.0 mEq/ l), 0.1 ml of std. (160/6.0 mEq/ l), to std.1, std.2, and std.3 respectively.

Reagent	*Test*	*Std.1*	*Std.2*	*Std.3*
Distilled water	10 ml	10 ml	10 ml	10 ml
Serum	0.1 ml	—	—	—
Std. (120 / 2.0 mEq/ l)	—	0.1 ml	—	—
Std. (140 / 4.0 mEq/ l)	—	—	0.1 ml	—
Std. (160 / 6.0 mEq/ l)	—	—	—	0.1 ml

Put on the main switch. Put on air compressor and adjust the required air pressure, by adjusting the knob meant for air. Introduce the distilled water through atomizer. Put on gas and control the flame by adjusting the knob meant for gas till the flame is divided into five

sharp cones. Adjust the proper filters for the simultaneous determination of sodium and potassium. Make zero adjustment by using distilled water. Introduce the std. 120 /2.0 mEq/l and by using the knob meant for sodium the digits 120.0 and by using the knob meant for potassium the digits 2.0. are adjusted. Introduce the std. 140/4.0 mEq/l. If the standards are accurately prepared the digital display will indicate exact concentration for both sodium and potassium. Introduce the std. 160/6.0 mEq/l and confirm the accuracy of the standard. Now introduce the test and record the readings for sodium and potassium.

Normal value:

Solium (Na) — 135 - 145 mEq/ l

Potassium (K) — 1 - 15 yr —— 3.7 - 5.0 mEq/ l

16 - 59 yr —— 3.6 - 4.8 mEq/ l

≥ 60 yr —— 3.9 - 5.3 mEq/ l

Clinical Significance

Estimation of serum sodium is useful in diagnosis and treatment of dehydration and over hydration. Changes in sodium more often reflect changes in water balance. Increased sodium values (hypernatremia) are observed in conditions such as —

1. Severe dehydration
2. Diabetes insipidus
3. Salt poisoning
4. Cushing's syndrome
5. In certain post-renal conditions like enlarged prostate leading to obstruction of urine flow.

Decreased sodium values (hyponatremia) are observed in conditions such as —

1. Severe prolonged diarrhoea and vomiting
2. Salt losing nephritis, and
3. Addison's disease.

Estimation of serum potassium is very useful in paralysis, severe fluid and electrolyte loss, diabetic coma, renal failure, etc. Increased potassium values (hyperkalemia) are observed in conditions such as

1. Addison's disease
2. Renal glomerular disease

3. In anuria and oliguria
4. Familial hyperkalemic paralysis
5. Acute acidosis
6. Decreased insulin, and
7. Intravascular haemolysis.

Decreased potassium values (hypokalemia) are observed in conditions such as —

1. Cushing's syndrome
2. Renal tubular damage
3. Metabolic alkalosis, and
4. Malnutrition.

SERUM CHLORIDE

Chloride is the major extracellular anion of the body. Its primary role in the body is to maintain proper water distribution, osmotic pressure and normal anion-cation balance in the plasma. In gastric juice, chloride also plays important role in the production of HCl. The chloride ions are ingested through the food (regular salt) and filtered or reabsorbed by the kidney as per the body need.

Method

Modified Schoenfeld and Lewellen's method

Specimen

Serum or heparinised plasma

Principle

Chloride ions reacts with mercuric thiocynate to form mercuric chloride, an undissociated salt to liberate thiocynate ions. These thiocynate ions reacts with the ferric ions to form ferric thiocynate, which is coloured compound. The colour formed is proportional to the chloride content of the specimen. The absorbance can be read at 520 nm. The final colour is stable for half an hour:

$$Hg(SCN)_2 + 2Cl^- \longrightarrow HgCl_2 + 2SCN^-$$

$$3\,SCN + Fe^{3+} \longrightarrow Fe(SCN)_3 \text{ (coloured compound.)}$$

Reagent

1. Chloride reagent
2. Chloride std (100 mEq/l)

Procedure

Take three test tubes and mark them as T, S, and B. Add two ml of chloride reagent in each tube. Add 0.1 ml of serum, 0.1 ml of chloride std. and 0.1 ml of distilled water in test, std. and blank respectively. Mix well and keep at room temperature for 2 min. Read the absorbance of test and std. against blank at 505 nm.

Reagent	*T*	*S*	*B*
Chloride reagent	1 ml	1 ml	1 ml
Serum	0.1 ml	—	—
Chloride std.	—	0.1 ml	—
Distilled water	—	—	0.1 ml

Calculations

$$\text{Serum chloride mEq/dl} = \frac{\text{OD of T}}{\text{OD of S}} \times 100$$

Normal Value: 96 – 109 mEq/dl

Clinical Significance

Serum chloride is very useful to assess electrolyte, acid-base and water balance. Serum chloride is increased in metabolic acidosis associated with prolonged diarrhoea, renal tubular diseases, respiratory alkalosis, some cases of hyperparathyroidism, diabetes insipidus, dehydration, and in conditions causing decreased renal blood flow, i.e. congestive heart failure.

Serum chloride levels are decreased in prolonged vomiting (loss of HCl), salt losing renal diseases, chronic respiratory acidosis, burns, and effect of certain drugs like-corticosteroids, bicarbonates, etc.

SERUM PHOSPHORUS

Most of the phosphorus in the blood exists as inorganic phosphate. About 80 percent of the total phosphorus is combined with calcium in bones and teeth. It is found in every cell of the body. About 10 percent is combined with proteins, lipids and carbohydrate and other compounds in blood and muscle. The remaining 10 percent is widely distributed in various chemical compounds.

Method

Gomorri's method.

Clinical Significance

Decreased serum phosphorus values are observed in preliminary hyperparathyroidism, rickets (vitamin D deficiency) and in Fanconi's syndrome (defect in re-absorption of phosphorus). Increased serum phosphorus levels are found in hypervitaminosis-D, hypoparathyroidism and in renal failure.

Principle

Protein in serum is first removed by treating with TCA . Protein-free filtrate is then treated with an acid molybdate, which reacts with inorganic phosphate to form phosphomolybdic acid. The colour reagent, metol reduces phosphomolybdic acid to give a blue coloured compound. The intensity of the colour is measured at 660 nm.

Reagents

1. Trichloroacetic acid (10 g/dl)
2. Molybdate reagent
3. Colour reagent, Metol
4. Phosphorus std. (5 mg/dl)

Procedure

Take two centrifuge tubes. Mark them as test and diluted std. add 4.5 ml TCA reagent in each tube. Add 0.5 ml of serum in test and 0.5 ml of std. in diluted std. tubes. Mix and centrifuge to get clear filtrate. Pipette in the tubes as follows.

	Test	*Std.*	*Blank*
Filtrate	2.5 ml	—	—
Diluted std	—	2.5 ml	—
Distilled water	—	—	2.5 ml
Molybdate reagent	0.5 ml	0.5 ml	0.5 ml
Colour reagent	0.5 ml	0.5 ml	0.5 ml

Mix thoroughly and keep in the dark for 10 min. Read the intensities at 660 nm.

Calculation

$$\text{Serum inorganic phosphorus (mg/dl)} = \frac{\text{OD of T}}{\text{OD of S}} \times 5$$

Normal Value:

Neonates	45 – 100 mg %
1 – 19 yr	120 – 240 mg %
20 – 29 yr	144 – 275 mg %

SERUM CALCIUM

Calcium is the major constituent of bone. Calcium in serum is present in ionized form or as a complex with protein or other inorganic substances like citrate, phosphate and others. Calcium plays many important roles in physiology of the body like it activates many enzymes and plays a key role in blood coagulation.

Method

OCPC method

Specimen

Serum or heparinised plasma. It should be separated as soon as possible.

Principle

Calcium in an alkaline medium reacts with O-cresolphthalein complexone (OCPC) to form an intense chromophore, which is of purple colour. Read the absorbance at 575 nm.

Reagent

1. O-cresolphthalein complexone reagent
2. Buffer solution
3. Calcium std. (10 mg/dl)

Procedure

First of all prepare working solution by mixing equal amounts of reagent 1. and reagent 2. This is to be freshly prepared as it is stable only for one day. Take three test tubes and mark them as T, S, and B. Add six ml of freshly prepared working reagent in each tube. Add 0.05 ml of serum, 0.05 ml of calcium std. and 0.05 ml of distilled water in test, std. and blank respectively. Mix well and keep at room temperature for exactly 10 min. Read the absorbance of test and std. against blank at 575 nm.

Reagent	*T*	*S*	*B*
Working reagent	6 ml	6 ml	6 ml
Serum	0.05 ml	—	—
Calcium std.	—	0.05 ml	—
Distilled water	—	—	0.05 ml

Calculations

$$\text{Serum calcium mg/dl} = \frac{\text{OD of T}}{\text{OD of S}} \times 10$$

Normal value:

1-3 yr	8.7-9.8 mg/dl
4-11 yr	8.8-10.1 mg/dl
12-13 yr	8.8-10.6 mg/dl
14-15 yr	9.2-10.7 mg/dl
> 16 yr	8.9-10.7 mg/dl

Clinical Significance

Determination of serum calcium level is useful in diagnosis of parathyroid dysfunction, hypercalcemia of malignancy, 90 percent of cases of hypercalcemia are due to hyperparathyroidism, neoplasms or

granulomatous diseases. Hypercalcemia of sarcoidosis adrenal insufficiency and hyperthyroidism tend to be found in clinically evident disease. Blood calcium should be monitored in renal disease, effects of various drugs, acute pancreatitis, postoperative thyroidectomy, and parathyroidectomy.

Calcium levels are found to be low in hypoparathyroidism, malabsorption of calcium and vitamin D, chronic renal disease with uremia, bone disease, late pregnancy, asphyxia, infants of diabetic mothers, cerebral injuries, malignant disease, etc.

Enzyme Assay

AMYLASE

Amylase is a hydrolytic enzyme that splits complex carbohydrates such as starch and glycogen into simpler molecules of sugars. (e.g. glucose, maltose). Serum amylase is composed of pancreatic and salivary type of isoamylases.

$$\text{Starch} + \text{Water} \xrightarrow{\text{Amylase}} \text{Sugar(s)}$$

The amylases normally occurring in human plasma are small molecules. Amylase is the only plasma enzyme normally found in the urine.

Method

I. Iodometric method
II. Visible kinetic method

I. Iodometric Method

Specimen

Unhaemolysed serum/diluted urine (1: 100)

Principle

The enzyme amylase degrades starch into dextrins and maltose. This reaction should be carried out under defined conditions. When end products are treated with iodine (colour reagent) decrease in blue colour is observed, comparing to that produced with blank. The disappearance of blue colour is directly proportional to the amylase concentration in the specimen.

Reagent

1. Buffered substrate
2. Stock colour reagent

Procedure

Dilute stock colour reagent to 1:10 with distilled water to get working colour reagent. Take two test tubes. Label it as test and control. Add 2.5 ml of buffered substrate in each tube . Keep at 37°C for 5 min. Add 0.1ml serum in test. Mix and incubate it for 7 min. Add 2.5 ml of working colour reagent in each tube. Now add 0.1 ml serum in control. At last add 20 ml of distilled water in both the tubes. Mix thoroughly and read the absorbance of test and std. against distilled water at 660 nm.

Reagent	*T*	*C*
Buffered substrate	2.5 ml	2.5 ml
Keep at 37°C for 5 min		
Serum	0.1 ml	—
Mix and incubate it for 7 min		
Working colour reagent	2.5 ml	2.5 ml
Serum	—	0.1 ml
Distilled water	20 ml	20 ml

Calculation

The serum amylase is expressed in Caraway units. One Caraway unit is the amount of enzyme that will hydrolyse 10 mg of starch in 30 min to a colourless stage.

$$\text{Serum amylase in Caraway unit} = \frac{\text{OD of C} - \text{OD of T}}{\text{OD of C}} \times 400$$

II. Visible Kinetic Method

Principle

Alpha-amylase hydrolyzes p-nitrophenyl-alpha-D-maltoheptoside ($PNPG_7$) to p-nitrophenylmaltotriose ($PNPG_3$) and maltotertrose. Glucoamylase hydrolyses $PNPG_3$ to p-nitrophenylglycoside ($PNPG_1$) and glucose $PNPG_1$ is hydrolyzed by α- glucosidase to glucose and

p-nitrophenol, which produces yellow colour. The rate of increase in yellow colour is proportional to α- amylase activity in the sample and is measured at 405 nm.

$$PNPG_7 \xrightarrow{\alpha\text{ - amylase}} PNPG_3 + \text{maltotertrose.}$$

$$PNPG_3 \xrightarrow{\text{Glucoamylase}} PNPG_1 + \text{glucose}$$

$$PNPG_1 \xrightarrow{\alpha\text{- glucosidase}} \text{p-nitrophenol} + \text{glucose}$$

Reagent

Amylase reagent — the lyophilized reagent is reconstituted with volume of distill water stated on the vial label. This working reagent is stable for one month in refrigerator.

Specimen

Unhaemolysed serum/diluted urine (1: 100)

Procedure

Take one ml of working amylase reagent in a test tube. Add 0.02 ml of serum and mix well. Read absorbance every 30 seconds for 2 min. Determine the absorbance difference (ΔA)/min.

Calculations

Serum amylase, IU = $\Delta A \times 7123$

Normal value:
In urine — 50 – 300 IU / hr. for 24 hr.
In serum — Below 18 yr 0 – 260 U/L
18 and above 18 yr 35 – 115 U/L

The serum amylase levels are high in acute pancreatitis, obstruction of pancreatic duct by – stone/carcinoma, biliary tract disease, acute cholecystitis complications of pancreatitis, pancreatic trauma, altered GI tract permeability, acute alcohol ingestion. Serum amylase level is also increased in salivary gland disease (like mumps), malignant tumors

specially of pancreas, lung, ovary, oesophagus, breast and colon, advanced renal insufficiency. The other causes include – chronic liver disease, ovarian cyst, diabetic ketoacidosis, splenic rupture. Increased serum amylase with low urine amylase may be seen in renal insufficiency and macroamylasemia.

Decreased serum amylase levels are clinically significant in marked destruction of pancreas, severe liver damage like in hepatitis, severe burns etc.

SERUM LIPASE

Lipases are the enzymes that hydrolyze glycerol esters of long chain fatty acids. It is fully active in presence of bile salts and a cofactor, called colipase. Most of the lipase in the serum is produced in the pancrease, but some is also secreted by the lingual glands, and gastric, pulmonary, and intestinal mucosa. Lipase activity is also seen in leucocytes, adipose tissue cells and milk. Lipase is filtered at the glomeruli but completely reabsorbed by the tubules. Therefore, it is not normally detected in urine.

Method

Colorimetric

Principle

Serum pancreatic lipase acts on a natural type of substrate, 1,2-diglyceride to liberate 2-monoglyceride. The 2-monoglyceride is hydrolyzed by monoglyceride lipase (MGLP) to produce glycerol and fatty acid. Glycerol kinase (GK) then acts on the glycerol to produce glycerol-3-phosphatase which is converted to dihydroxyacetone phosphate and hydrogen peroxide in a reaction catalyzed by glycerol-3-phosphate oxidase (GPO). The hydrogen peroxide then reacts with 4-aminoantipyrine and N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine sodium salt (TOOS) in a reaction catalyzed by peroxidase (POD) to yield a quinone dye. The rate of increase in absorbance at 550 nm is directly proportional to the lipase activity of the sample.

1, 2-diglyceride + H_2O —— Pancreatic Lipase ⟶ 2-monoglyceride + fatty acid

2-monoglyceride + H_2O —— MGLP ——→ glycerol + fatty acid

glycerol + ATP —— GK ——→ glycerol-3-phosphatase + ADP

glycerol-3-phosphatase + O_2 —— GPO ——→ dihydroxyacetone phosphate + H_2O_2

$2H_2O_2$ + 4-aminoantipyrine + TOOS —— POD ——→ quinone dye + 4 H_2O_2

Reagent

1. Lipase substrate
2. Lipase substrate buffer
3. Lipase activator

Reagent Preparation

Reconstitute the lipase substrate vial with the amount of lipase substrate buffer indicated on the vial label. Swirl to dissolve. Reconstitute the lipase standard with 3 ml of distilled water.

Specimen

Fasting, non-hemolyzed serum is the preferred.

Procedure

Take four test tubes. Label them as "Blank", "Standard", "Control", "Test", etc. Pipette 3 ml of reconstituted lipase substrate reagent to all tubes.Pipette 0.05 ml of distilled water to the blank tube and 0.05 ml of the appropriate sample to the tubes labeled "Standard", "Control", etc. Mix each tube well and incubate for 3-5 minutes at 37°C.

After the pre-incubation, add 1ml of Lipase activator to the blank tube. Mix well and incubate for 3 minutes at 37°C. Then measure the rate of increase in absorbance per minute at 550 nm (540-560 nm). Repeat this step for all tubes.

Reagent	*T*	*S*	*B*	*C*
Lipase substrate	3 ml	3 ml	3 ml	3 ml
Distilled water	—	—	0.05 ml	—
Serum	0.05 ml	—	—	—
Std.	—	0.05 ml	—	—
Control serum	—	—	—	0.05 ml
Mix each tube well and incubate for 3-5 minutes at 37°C.				
Lipase activator	1 ml	1 ml	1 ml	1 ml

Calculation

$$\text{Serum Lipase} = \frac{\Delta A \text{ Sample} - \Delta A \text{ Blank}}{\Delta A \text{ Standard} - \Delta A \text{ Blank}} \times \text{Conc of std.}$$

Normal values: 56 – 200 IU

Clinical Significance

Serum lipase is recognized as an important indicator for the diagnosis, and therapeutic monitoring, of pancreatic diseases. The lipase test is most often used in evaluating inflammation of the pancreas (pancreatitis), but it is also useful in diagnosing kidney failure, intestinal obstruction, mumps, and peptic ulcers. Doctors often order amylase and lipase tests at the same time to help distinguish pancreatitis from ulcers and other disorders in the abdomen. If the patient has acute (sudden onset) pancreatitis, the lipase level usually rises somewhat later than the amylase level—about 24–48 hours after onset of symptoms—and remains abnormally high for 5–7 days. It is also increased in obstruction of pancreatic duct by stone, intestinal infarction, after organ transplant, chronic liver diseases. 2–3 times increase in serum lipase is found in 80 percent patients with acute and chronic renal failure. Alcoholism, diabetic ketoacidosis, increases in lipase.

No clinically significant role has been known in which serum lipase activity is lower.

SERUM LACTATE DEHYDROGENASE (LDH)

Lactate dehydrogenase activity is present in all cells of the body. It is found in the cytoplasm of the cell. It is present in high concentration

in heart, kidney, erythrocytes and skeletal muscle. Many of the tissues show different isoenzymes composition. LDH catalyses following reaction.

$$\text{Lactate} + \text{NAD} \xrightarrow{\text{LDH}} \text{Pyruvate} + \text{NADH} + \text{H}^+$$

The optimum reaction conditions are at 37°C temperature and 8.8 – 9.8 pH.

Method

King's method

Specimen

Strictly unhaemolysed serum since, RBCs are rich in LDH .

Principle

LDH catalyses following reaction.

$$\text{Lactate} + \text{NAD} \xrightarrow{\text{LDH}} \text{Pyruvate} + \text{NADH} + \text{H}^+$$

The products formed react with 2, 4-dinitrophenyl hydrazine (DNPH) to give corresponding hydrazone. Hydrazone gives brown colour in alkaline medium, which is measure of LDH.

Reagent

1. Glycine reagent
2. Buffered substrate
3. NAD solution
4. NADH solution
5. DNPH reagent
6. 0.4 N NaOH

Procedure

Part I. Preparation of Standard Curve

Take seven test tubes. Mark them as Blank, 1, 2, 3, 4, 5, and 6 for

enzyme activity—0, 167, 333, 500, 667, 883, and 1000 respectively. Add the reagent as follows:

Reagent	*Blank*	*1*	*2*	*3*	*4*	*5*	*6*
S. LDH activity, IU	0	167	333	500	667	883	1000
NADH solution	0 ml	0.05 ml	0.10 ml	0.15 ml	0.20 ml	0.25 ml	0.30 ml
Pyruvate	0 ml	0.05 ml	0.10 ml	0.15 ml	0.20 ml	0.25 ml	0.30 ml
Buffered substrate	1.0 ml	0.9 ml	0.8 ml	0.7 ml	0.6 ml	0.5 ml	0.4 ml
NAD solution	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml
Distilled water	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml
DNPH reagent	1.0 ml	1.0 ml	1.0 ml	1.0 ml	1.0 ml	1.0 ml	1.0 ml
Mix each tube well and keep at room temperature for 15 minutes.							
0.4 N NaOH	10 ml	10 ml	10 ml	10 ml	10 ml	10 ml	10 ml

Mix well and keep at room temperature for exactly 10 min. measure the intensities of all the tubes setting against blank at 445nm. Draw a graph by plotting OD on Y-axis and IU on X-axis.

Part II. Preparation of Test

For sample, take two test tubes - label it as test and control. Add the reagent as follows.

Reagent	*T*	*C*
Buffered substrate incubate for 5 minutes at 37°C	1.0 ml	1.0 ml
NAD solution	0.2 ml	0.2 ml
Serum	0.02 ml	—
Mix well and incubate for 15 minutes at 37°C.		
DNPH reagent	1.0 ml	1.0 ml
Serum	—	0.02 ml
Mix well and keep at room temperature for 15 minutes		
0.4 N NaOH	10 ml	10 ml

Calculation

Net OD of test = OD of T – OD of C

From the standard graph calculate the serum LDH value.

Normal value:

Newborn	—	160 – 1500 U/L
Infant	—	150 – 360 U/L
Child	—	150 – 300 U/L
Adult	—	100 – 250 U/L

Clinical Significance

SLDH is very non-specific test. It can be used as marker of haemolysis in anemia.

It is increased in congestive heart failure, acute myocarditis, rheumatic fever, in liver diseases like cirrhosis, obstructive jaundice, hepatitis. In lung disease like pulmonary embolus and infarction. SLDH is increased in 50 percent of patients with carcinomas specially in advanced stages. In muscle disease, in renal diseases like in nephritic syndrome, in acute pancreatitis, hypothyroidism. LDH is increased in various infections and parasitic diseases.

The only condition in which LDH is known to be decrease is X-ray irradiation.

PHOSPHATES

Phosphates belong to the class of enzymes called hydrolyses.

Phosphates are able to hydrolyze a large variety of organic phosphate esters with the formation of an alcohol and phosphate ion.

Phosphates of diagnostic importance are of two kinds- alkaline phosphates and acid phosphates. These are differentiated by their reaction in alkaline and acidic medium. The pH for measuring the alkaline phosphates activity is 10 and for acid phosphates it is 5.

Specimen

Serum is preferred, but heparinized plasma can also be used other anticoagulants inhibit the enzyme activity. Overnight fasting serum is preferred; store the serum in refrigerator if immediate analysis is not possible.

i. Alkaline Phosphates : As is described earlier .

ii. Acid Phosphatase:

A total acid phosphatase includes all phosphatase with optimum activity in the range 4 to 6. ACP is present in lysosomes of all the cells, except erythrocytes. Extralysosomal ACPs are also present in many cells. ACP activity is mainly observed in bone marrow, liver spleen, milk, platelets and highest in prostate glands.

The ACPs are unstable above pH 7.0 and temperature 37°C. More than 50 percent of ACP activity is lost in 1hour at room temperature. Acidification of the serum specimen to a pH below 6.5 aids in stabilizing the enzyme.

Method

Gutman and Gutman.

Principle

Acid phosphatase from serum converts phenyl phosphatase to inorganic PO_4 and phenol at pH 4.9. Phenol in alkaline medium reacts with 4-aminoantipyrine in presence of potassium ferricyanide forms orange red coloured complex. The colour intensity is directly proportional to enzyme activity.

$$\text{Phenyl phosphatase} + \text{Acid phosphatase} \longrightarrow \text{Phenol} + \text{inorganic } PO_4$$

$$\text{Phenol} + \text{4-aminoantipyrine} \xrightarrow[OH^-]{\text{K ferricyanide}} \text{Orange Red Complex}$$

Since tartarate inhibits prostatic fraction of enzyme, the difference in acid phosphatase activity with and without tartarate represents activity and prostratic fraction.

Reagents

1. Buffered substrate at pH4.9
2. 0.5N NaOH

3. 0.5N $NaHCO_3$
4. 4-aminoantipyrine
5. Potassium ferricyanide
6. 1M tartarate
7. Phenol std.

Procedure

Take 5 test tubes, mark them as blank, std, control, test and tartarate stable.
Add the reagents as follows

Reagents	*B*	*S*	*C*	*T*	*Ts*
Buffered Substrate	—	—	1 ml	1 ml	1 ml
Distilled water	2.2 ml	1.2 ml	1 ml	1 ml	1 ml
Mix well and incubate for 3 min. at 37°C					
Working Std.	—	1 ml	—	—	—
1 M tartarate	—	—	—	—	0.05 ml
Serum	—	—	—	0.2ml	0.2 ml
Mix well and incubate for 1hr. at 37°C					
0.5 N NaOH	1 ml	1 ml	1 ml	1 ml	1 ml
Serum	—	—	0.2 ml	—	—
0.5 N $NaHCO_3$	1 ml	1 ml	1 ml	1 ml	1 ml
4- aminoantipyrine	1 ml	1 ml	1 ml	1 ml	1 ml
Pot. Ferricyanide	1 ml	1 ml	1 ml	1 ml	1 ml

Mix well after addition of each reagent and measure the OD of blank, std., test, control and tartarate stable against distilled water at 510 nm.

Calculations

$$\text{Serum acid phosphatase activit in KA units} = \frac{\text{OD of Test} - \text{OD of control}}{\text{OD of std.} - \text{OD of blank}} \times 5$$

$$\text{Prostatic fraction in KA units} = \frac{\text{OD of Test} - \text{OD of Ts}}{\text{OD of std.} - \text{OD of B}} \times 5$$

Normal values	*Males*	*Females*
Total	2.5 to 11 U/L	0.3 to 9U/L
Prostatic	0.2 to 3.5 U/L	0.0 to 0.8 U/L

Clinical Significance

Determination of serum ACP is important in detecting and monitoring carcinoma of the prostate, in prostatic carcinoma with metastasis, total ACP activity may reach 40 to 50 times the upper limit. Moderate elevations in total ACP activity often occur in Paget's disease, hyperparthyroidism and breast cancer. Increased serum ACP levels are also seen Gaucher's disease and in myelocytic leukemia. As it is present in high concentration in semen, it is utilized in forensic medicine in the investigation of rape offences.

Diabetic Profile

Diabetes is a disease in which blood glucose levels are above normal. People with diabetes have problems converting food to energy. After a meal, food is broken down into a sugar called glucose, which is carried by the blood to cells throughout the body. Cells use insulin, a hormone made in the pancreas, to help them convert blood glucose into energy.

People develop diabetes because, the pancreas does not make enough insulin or because, the cells in the muscles, liver, and fat do not use insulin properly, or both. As a result, the amount of glucose in the blood increases while the cells are starved of energy. Over the years, high blood glucose, also called hyperglycemia, damages nerves and blood vessels, which can lead to complications such as heart disease and stroke, kidney disease, blindness, nerve problems, gum infections, and amputation.

TYPES OF DIABETES

The three main types of diabetes are type 1, type 2, and gestational diabetes.

Type 1/Juvenile Diabetes/Insulin-dependent Diabetes Mellitus/IDDM

- Type 1 diabetes, formerly called juvenile diabetes, is usually first diagnosed in children, teenagers, or young adults. In this form of diabetes, the beta cells of the pancreas no longer make insulin because the body's immune system has attacked and destroyed them.

Type 2 / Adult-onset Diabetes/Noninsulin-dependent Diabetes Mellitus/NIDDM

- Type 2 diabetes, formerly called adult-onset diabetes, is the most common form. People can develop it at any age, even during childhood. This form of diabetes usually begins with insulin resistance, a condition in which muscle, liver, and fat cells do not use insulin properly. At first, the pancreas keeps up with the added demand by producing more insulin. In time, however, it loses the ability to secrete enough insulin in response to meals.
- Gestational diabetes, develops in some women during the late stages of pregnancy. Although this form of diabetes usually goes away after the baby is born, a woman who has had it is more likely to develop type 2 diabetes later in life. Gestational diabetes is caused by the hormones of pregnancy or by a shortage of insulin.
- Pre-diabetes, in pre-diabetes, blood glucose levels are higher than normal but not high enough to be characterized as diabetes. However, many people with pre-diabetes develop type 2 diabetes within 10 years. Pre-diabetes also increases the risk of heart disease and stroke. With modest weight loss and moderate physical activity, people with pre-diabetes can delay or prevent type 2 diabetes.

DIAGNOSIS OF DIABETES

The following tests are used for diagnosis:

- *A fasting plasma glucose test* measures the blood glucose after at least 8 hours of fasting. This test is used to detect diabetes or pre-diabetes.
- *A post meal plasma glucose test* measures the blood glucose after usual adequate meals. Patient must complete meal within 15-20 min. Specimen is collected at 2 hr from beginning of meal.
- *An oral glucose tolerance test* measures the blood glucose after at least 8 hours of fasting and 2 hours after drinking a glucose-containing beverage. This test can be used to diagnose diabetes or pre-diabetes.
- *In a random plasma glucose test,* blood glucose can be checked without regard to when one ate the last meal. This test, along with an assessment of symptoms, is used to diagnose diabetes but not pre-diabetes.

Positive test results should be confirmed by repeating the fasting plasma glucose test or the oral glucose tolerance test on a different day.

Blood Glucose

Blood glucose can be determined at different times. As stated above, it may be fasting, post meal or random.

Specimen: Sreum, plasma, or whole blood can be used. Serum should be separated from blood clot within half hr of blood collection. For plasma preparation, fluoride with oxalate is used as an anticoagulant.

There are three important methods to determine plasma glucose level.

I. Folin-Wu method
II. O-Toludine method
III. Glucose oxidase method

Folin-Wu Method

It is economical, simple and convenient method.

Principle

Glucose on boiling with alkaline copper solution reduces copper from the cupric to cuprous state. The cuprous oxide so formed reduces phosphomolybdic acid to blue coloured molybdenum blue, which is measured colorimetrically. The intensity of blue colour is proportional to glucose concentration.

Reagents

1. 10 percent Sodium tungstate
2. 0.66 N sulphuric acid
3. Alkaline copper sulphate solution
4. Phosphomolybdate solution
5. Stock glucose standard
6. Working standard—dilute 1ml of glucose stock standard with 9 ml of distill water to give 100 mg percent working standard. Prepare this everyday.

Procedure

Part I: Preparation of Protein Free Filtrate

In a 10 ml test tube take 3.5 ml distill water and 0.1 ml of plasma / serum, followed by 0.2 ml of 10% sodium tungstate. Mix and add slowly 0.2ml of 2 / 3 N sulfuric acid. Mix well stand for 5 min. Filter or centrifuge.

Part II: Colour Development

Folin blood sugar test tubes are recommended for this test. The tubes are specially designed to prevent contact of atmospheric oxygen with the reaction mixture, which affects the result.

Fig. 15.1: Folin Wu's blood sugar tube

1. Take four Folin-Wu tubes (Fig. 15.1) and label it as blank (B), Standard (S), Control (C) and Test (T). Add 1ml of distilled water, glucose standard, protein free filtrate of control serum and test serum in respective tubes.
2. Add 1ml of alkaline copper reagent to each tube.
3. Transfer the tubes to boiling water bath for 10 min.
4. Cool the tubes for 2-3 min. under running tap water without shaking
5. After cooling add 2 ml phosphomolybdate reagent to each tube.
6. Mix well by inversion and dilute the contents of each tube upto 12.5 ml with distilled water.
7. Set the wavelength of photometer to 620 nm and measure the O.D.

Reagent	*Blank*	*Standard*	*Control*	*Test*
Distilled water	1ml	—	—	—
Glucose std.	—	1ml	—	—
Filtrate of 'C'	—	—	1ml	
Filtrate of 'S'	—	—	—	1ml
Alk. Cu reagent	1ml	1ml	1ml	1ml
	Boiling water bath for 10 min.			
Phosphomolybdate	2 ml	2 ml	2 ml	2 ml

Table showing Folin-Wu procedure

O- TOLUIDINE METHOD

Principle

The aldehyde group of glucose condenses with O- toluidine in glacial acetic acid, which on heating gives an emerald-blue green colour which is measured photometrically. The intensity of colour is directly proportional to the glucose concentration.

Reagents

1. O-toluidine reagent
   Orthotoluidine 60 ml
   Thiourea 1.5 gm
   Glacial acetic acid 1000 ml
2. Glucose stock standard (200 mg/dl in 0.2% benzoic acid solution)
3. Glucose working solution.

Procedure

1. Take three test tubes and label them as blank standard and test.
2. Transfer 1ml distilled water, 1ml working standard, and 1ml test serum to respective tube.
3. To each tube, add 0.9 ml of distilled water and 7 ml of O-toluidine reagent. Cover the tubes with loose cap or aluminium foil.
4. Place in a boiling water bath for 10 min. work inside the hood as it gives strong smell, which is injurious.
5. Cool the tubes for 2-3 min under running tap water.
6. Read the absorbance within 30 min of cooling at 630 nm.

Reagent	*Blank*	*Standard*	*Test*
Distilled water	1 ml	—	—
Glucose std.	—	1 ml	—
Test serum	—	—	1 ml
Distilled water	0.9 ml	0.9 ml	0.9 ml
O-toluidine reagent	7 ml	7 ml	7 ml

Table showing O- Toluidine procedure

GLUCOSE OXIDASE METHOD

Principle

Glucose oxidase (GOD) oxidizes glucose to gluconic acid. Hydrogen peroxide is produced in this reaction. In presence of peroxide hydrogen peroxidase (POD) reacts with 4-aminoantipyrine and phenol to form red coloured quinoneimine dye. The intensity of colour is directly proportional to the glucose concentration. This method is highly specific for glucose and does not involve any other sugar.

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

$$H_2O_2 + \text{phenol} + \text{4-aminoantipyrine} \xrightarrow{\text{POD}} \text{Quinoneimine dye}$$

Reagents

1. Glucose oxidase reagent
2. Phenol solution
3. Glucose stock standard (200 mg/dl in 0.2% benzoic acid solution)
4. Glucose working solution.

Procedure

1. Take three test tubes and label them as blank standard and test.
2. Add 2 ml of glucose oxidase reagent into the three test tubes.
3. Add 0.5 ml of distilled water; 0.5ml of ten times diluted serum, 0.5ml of working standard in blank, test and serum respectively.
4. Add 2 ml of phenol reagent into the three test tubes.
5. Shake well and allow it to stand for 30 min. at room temperature or 15 min. at 37°C.
6. Read the absorbance at 515 nm.

Reagent	*Blank*	*Standard*	*Test*
Glucose oxidase	2 ml	2 ml	2 ml
Glucose std.	—	0.5 ml	—
Diluted test serum	—	—	0.5 ml
Distilled water	0.5 ml	—	—
Phenol reagent	2 ml	2 ml	2 ml

Table showing glucose oxidase procedure

Calculation

$$\text{Glucose mg/100 ml} = \frac{\text{O.D. of Test}}{\text{O.D. of Std.}} \times \text{Conc. of Std.}$$

Normal Values

- Fasting 65 — 110 mg/dl
- Post meal 120 —140 mg/dl
- Random 70 — 140 mg/dl

Clinical Significance

Blood glucose level is mainly determined to diagnose diabetes mellitus. It provides the valuable information about the course, severity and therapeutic control of diabetes mellitus.

With the FPG test, a fasting blood glucose level between 100 and 125 mg/dl signals pre-diabetes. A person with a fasting blood glucose level of 126 mg/dl or higher has diabetes. The early symptoms of untreated diabetes mellitus are related to the elevated blood glucose levels. Excess glucose in the blood ultimately results in high levels of glucose being present in the urine (glucosuria). This increases the urine output, which leads to dehydration and increased thirst. Other symptoms include extreme tiredness, weight loss, blurred vision, itchy skin and repeated minor infections such as thrush and boils.

Beside the diabetes, blood sugar level is also increased in —

With increased circulatory epinephrine, pancreatitis, some CNS lesions, or effects of drugs like alcohol, phenytoin, etc

Decreases in—Extrapancreatic tumors, hepatic disease, endocrine disorders, pediatric abnormalities, enzyme diseases and malnutrition.

ADVANCES IN MONITORING DIABETES

1. Hb A1c Test
2. Glucometer

HbA1c Test

(Also called as Glycosylated hemoglobin; Hemoglobin-glycosylated; A1c; GHb; Glycohemoglobin; Diabetic control index)

Definition

HbA1c is a test that measures the amount of glycosylated hemoglobin in the blood. The test gives a good estimate of how well diabetes is being managed over time.

Normally, only a small percentage of the hemoglobin (Hb) molecules in red blood cells become glycosylated (that is, chemically linked to glucose). The percent of glycosylation increases over time, and is higher if there is more glucose in the blood. Therefore, older red blood cells will have a greater percent of glycosylated hemoglobin, and diabetics whose blood glucose has been too high will have a greater percent of glycosylated hemoglobin.

This test measures blood sugar control over an extended period in people with diabetes. In general, the higher the HbA1c value, the higher the risk that a person will develop problems such as eye disease, kidney disease, nerve damage, heart disease, and stroke. This is especially true if the HbA1c remains elevated for more than one occasion.

Normal Values

HbA1c is normal if it is 5 percent or less. The test can show that the blood glucose levels have not been well-regulated over a period of weeks to months. If the HbA1c value is above 7%, it means diabetes is poorly controlled. High values indicates greater risk of diabetic complications. This test is recommended usually every 3 or 6 months.

Glucometer

A glucose meter (or glucometer) is a medical device for determining the approximate amount of glucose in a drop of blood obtained by pricking the skin with a lancet. Glucose meters are portable and designed for use by laypersons, including those with diabetes.

The glucose meter is a key element of *home blood glucose monitoring* by people with diabetes mellitus or with proneness to hypoglycemia.

There are now dozens of models of glucose meters are available. Typical features common to most are:

- The average size is now approximately the size of the palm of the hand. They are battery powered.
- A consumable element containing chemicals, which react with glucose in the drop of blood, is used for each measurement. For most models this element is a plastic *test strip* with a small spot impregnated with glucose oxidase and other components. Each strip can only be used once and is then discarded.
- The glucose value in mg/dl or mmol/l displayed in a small window.
- Glucose levels in plasma are generally 10-15% higher than glucose measurements in whole blood (and even more after eating). This is important because home blood glucose meters measure the glucose in whole blood while most lab tests measure the glucose in plasma.
- Current "count times" range from 5 to 60 seconds for different models.
- The size of the drop of blood needed by different models currently varies from 0.3 to 10 μl.
- All meters now include a *clock,* which must be set for date and time, and a *memory* for past test results. The memory is an important aspect of diabetes care, as it enables the person with diabetes to keep a record of management and look for trends and patterns in blood glucose levels over days. Most memory chips can display an average of recent glucose readings.
- Many meters have now had more sophisticated data handing capabilities. Many can be downloaded by a cable or infrared to a computer which has software to display the test results in a variety of formats. Some meters allow entry of additional data throughout the day, such as insulin dose, amounts of carbohydrates eaten, or exercise.
- A number of meters have been combined with other devices, such as insulin injection devices, PDAs. A radio link to an insulin pump allows automatic transfer of glucose readings to a calculator that

assists the wearer in deciding on an appropriate insulin dose. One model also measures beta-hydroxybutarate in the blood to detect ketoacidosis.

- Special glucose meters for multi-patient hospital use are now used. These provide more elaborate quality control records, and the data handling capabilities are designed to transfer glucoses into electronic medical records and the laboratory computer systems for billing purposes.

The cost of daily testing is one of the most expensive aspects of diabetes care. In 2006, the consumer cost of each glucose strip ranges from about Rs. 17 to 50.

Accuracy of glucose meters is a common topic of clinical concern. Nearly all of the meters have similar accuracy (±10-15%) when used optimally. However, a variety of factors can affect the accuracy of a test. Factors affecting accuracy of various meters have included calibration of meter, ambient temperature, pressure use to wipe off strip, size of blood sample, high levels of certain drugs in blood, hematocrit, dirt on meter, humidity, and aging of test strips.

GLUCOSE TOLERANCE TEST (GTT)

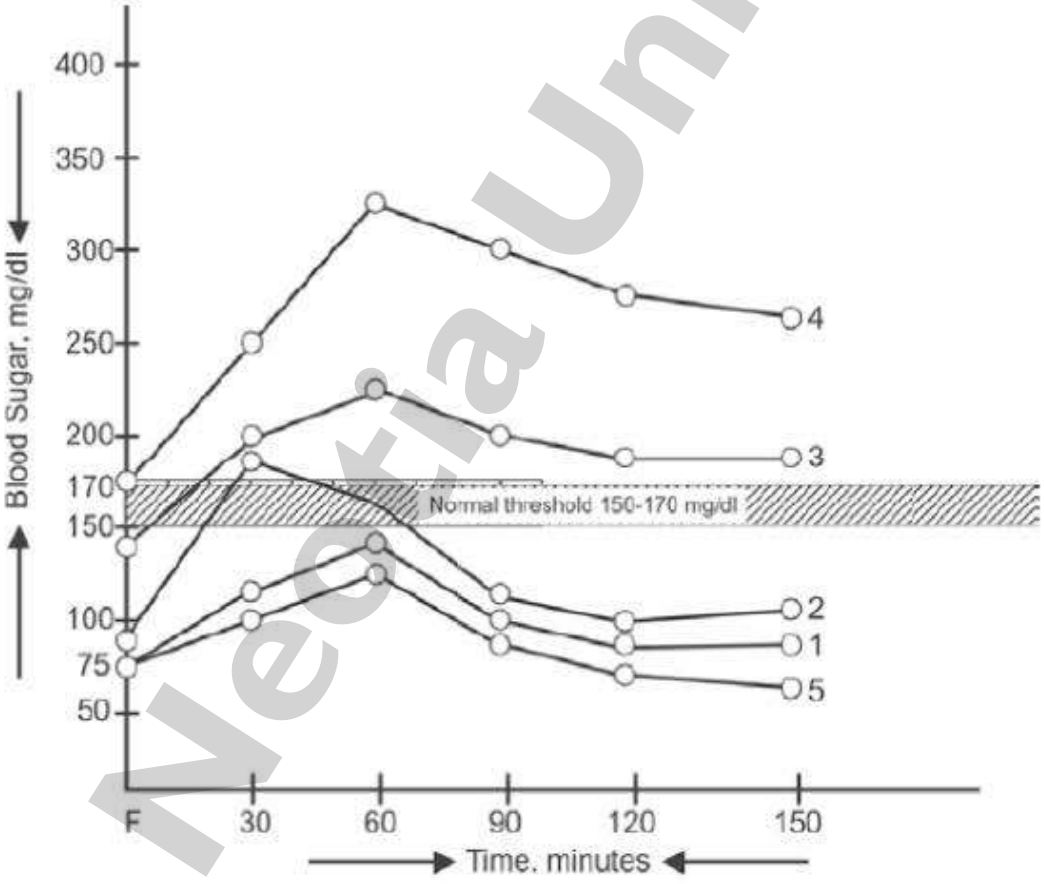
Glucose tolerance test means ability of the body to utilize glucose in blood circulation. Glucose tolerance decreases in certain diseases like diabetes mellitus and endocrine disorders.

Blood glucose in case of normal individual remains fairly constant throughout the day. Except, after meals, there is temporary rise, which comes to normal within 2-3 hr. of meal. In decreased tolerance level however, the blood sugar level does not return to normal within 2-3 hrs. of meal. Glucose tolerance test determines the degree and duration of hyperglycemia after an oral intake of known quantity of glucose.

The patient is prepared for the test by being kept on a diet containing 300 g of carbohydrate a day for three days before the test. Patient should come to lab in the morning after overnight fasting of 12-16 hr. Patient is not allowed to have tea/coffee/medicine/smoke/tobacco chew.

Procedure

1. First, collect the fasting urine and blood sample. If glucose is present in urine, do not perform GTT, instead take a post meal sample.
2. If glucose is absent, give patient 75 to 100 gm glucose (1.50 gm/ kg body weight) dissolved in water. Note the time.
3. Thereafter the blood and urine samples are collected for every 30 min for two and half hr.
4. Determine blood and urine sugar.
5. Prepare a Glucose tolerance curve by plotting time on X- axis and glucose values on Y-axis (Fig. 15.2).



(1) Normal (2) Lag type (3) Mild diabetic (4) Severe diabetic

Fig. 15.2: GTT curves

INTERPRETATION

1. *Glucose tolerance curve No.1:* This is a normal type of curve. It shows fasting glucose within normal limits. Maximum blood glucose level is reached either half or one hour after taking glucose. Within 2 hrs it comes rapidly to normal limits. Glucose should not present in any of urine sample.
2. *Glucose tolerance curve No.2:* This type of curve is termed as Lag curve. Here the peak of blood glucose level may be higher than normal but the 2 hr value is within normal limits or often low. The increase in blood glucose level is due to delay in insulin mechanism coming into action. If the blood glucose level at peak of the curve is above the renal threshold level, glucose appears in next urine sample. Such a curve is seen in—— Normal individuals, after gastrectomy and in severe liver disease.
3. *Glucose tolerance curve No.3 and 4:* This is present when ability of body to utilize glucose decreases. The rise in blood glucose is greater than in normal persons and the return of blood glucose to normal fasting level is delayed. GTC No. 3 indicates mild diabetes and GTC No. 4 indicates severe diabetes. This type of curve may be seen in hyperactivity of hormones, very severe liver disease and severe infection.
4. *Glucose tolerance curve No.5:* This curve indicates increased glucose tolerance i.e., ability of the body to utilize more glucose. The fasting blood sugar may be below usual limits and only a small rise in blood glucose is observed. This type of curve may be observed with hypoactivity of endocrine gland, in patients with idiopathic steatorrhea and sprue.

URINE GLUCOSE DETERMINATION

When blood sugar level exceeds 170 mg/ dl, glucose appears in urine. This condition is called as glycosuria. Increased concentration of glucose in urine indicate proportionate increase in blood sugar level.

Qualitative Test for Sugar Determination Test for Glucose (or Reducing Substances Like Fructose, Lactose, Galactose, Pentose)

- *Benedict's test (Qualitative):* Take 5 ml of Benedicts qualitative reagent in test tube and add 0.5 ml of urine. Boil the content of

tube. Let it stand on the rack for 5 to 10 min. The appearance of a yellow or red deposits indicates the presence of reducing substances i.e. sugar. Cupric sulphate is reduced to cuprous oxide by boiling with reducing agents.

Report: A slight green colour, light turbidity or a bluish white ppt or no change is reported as negative. A greenish colour with a little yellow deposit is reported as a trace (+), green yellowish (++), orange (+++) and brick red (++++).

Nowadays, paper strips are available commercially which are dipped in urine as directed and the colour produced is matched against the colour chart supplied.

Quantitative Test for Sugar Determination

Reducing sugars (glucose) reacts with Benedict's quantitative reagent. Here glucose reduces cupric ions to cuprous ions which reacts with potassium thionate in the reagent to form white ppt. This is very sharp reaction and easy to detect.

Procedure

1. Pipette 5 ml of the Benedict's quantitative reagent in a porcelain dish.
2. Add 2-3 gm anhydrous sodium carbonate and mix well.
3. Heat the mixture to the boiling point.
4. Add urine dropwise with constant stirring with glass rod till the blue colour of the reagent disappears and white ppt. is formed.
5. Note the titration reading.

Calculations

$$\text{Urinary glucose mg/ dl} = \frac{10 \times 10}{\text{Titration reading (ml)}}$$

Normal Value

0 to 0.3 gm /24 hrs.

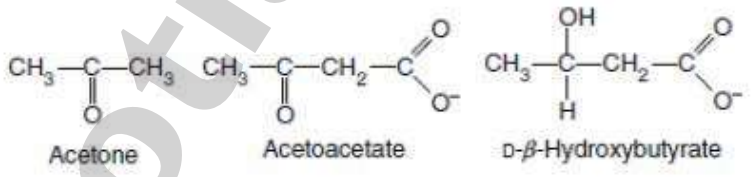
Causes of Glycosuria

Diabetes mellitus, non-diabetic glycosuria includes emotional disturbances, hyperthyroidism, pregnancy, after ingestion of considerable carbohydrates, either anaesthesia, in some infections, like pneumococcal pneumonia, etc.

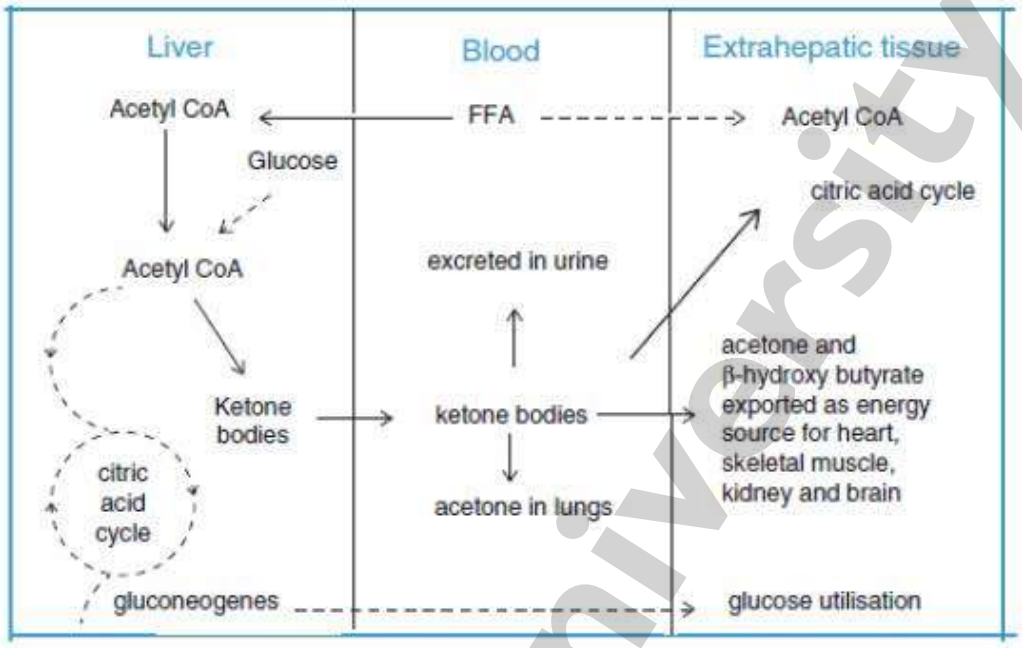
Qualitative Analysis of Ketone Bodies in Urine

Theory

The three compounds, namely, acetone, acetoacetic acid, and β-hydroxybutyrate, are called ketone bodies. Ketone bodies are water-soluble and energy-yielding substances. The synthesis of ketone bodies is called ketogenesis. The ketogenesis process occurs in the liver particularly during periods of low food intake, carbohydrate-restrictive diets, starvation, or in untreated type 1 diabetes mellitus. Ketone bodies are transported from the liver to the extrahepatic tissues and converted into acetyl-CoA which then enters the citric acid cycle and is utilized for energy production (Fig. 30.1). The liver, however, is unable to metabolize ketone bodies. The tissues which lack mitochondria, i.e., erythrocytes, also cannot utilize ketone bodies. Ketone bodies are major fuel source for the brain during starvation and can meet 50–70% of total energy needs. The relative proportion of ketone bodies present in blood may vary from 78% (β-hydroxybutyric acid) to 20% (acetoacetic acid) and 2% (acetone). Tests for ketone bodies should be done on fresh urine sample.



Ketone bodies



Utilization of ketone bodies

Rothera's Test for Acetoacetic Acid and Acetone

Principle

Alkaline nitroprusside reacts with keto-group of acetone and acetoacetic acid to form a purple-colored complex.

Reagents

(a) **Rothera's reagent: dry mixture**

Take ammonium sulfate and sodium nitroprusside in 100:1 ratio. Grind well to mix powder of salts.

(b) **Conc. ammonia** in liquid form

Procedure

5 ml of urine is saturated with Rothera's reagent in a test tube. Then 0.5–1.0 ml of conc. ammonia is added through the sides of the tube in such a way that it layers on top of the urine. Any change in color is observed within 30–60 s.

Result

Appearance of a purple/red ring at the junction of the two layers within 30–60 s indicates the presence of acetone and diacetic acid. Based on the intensity of the color formed, the result can be represented as grade from trace to 3^+.

The presence of L-dopa and phenyl pyruvic acid in urine may produce weak false positive reactions. In case of suspicion of a false positive test, the urine sample is heated in a test tube for 1 min, allowed to cool, and Rothera's test is repeated. The heated urine will not produce a positive Rothera's test due to ketone bodies.

Gerhardt's $FeCl_3$ Test for Acetoacetic Acid

This test is based on the reaction of $FeCl_3$ with acetoacetate producing a red wine color. The test is non-specific, and antipyrine gives similar color.

Reagent

10% aqueous solution of $FeCl_3$.

Procedure

Take 5 ml of urine and add 10% $FeCl_3$ solution drop by drop. Red wine color (purple) indicates positive test.

Ketostix Test for Acetone and Acetoacetate

Ketostix are plastic strips, one end of which is impregnated with sodium nitroprusside and glycine. When these strips are immersed in urine, sodium nitroprusside and glycine react with acetone and acetoacetate to give lavender or purple color.

Procedure

Dip the test end of strip in fresh specimen of urine, and remove immediately, briefly touching the tip on side of container to remove excess liquid. Compare color with chart. Ketostix is sensitive to 10 mg/dl acetoacetic acid and 25 mg/dl acetone.

Detection of β-Hydroxybutyrate

Principle

β-hydroxybutyrate forms acetoacetate on oxidation with H_2O_2 which can be detected with Rothera's test.

Procedure

Add few drops of acetic acid in about 10 ml of urine diluted 1:1 with distilled water. Boil for few min to remove acetone and acetoacetic acid. Add about 1 ml of H_2O_2, warm gently, and apply Rothera's test.

Note If Rothera's test is positive before oxidation with H_2O_2 (with fresh urine sample), then acetone and acetoacetic acid ketone bodies are present. If Rothera's test give positive result after oxidation with H_2O_2, then *β*-hydroxybutyrate is present in urine.

Clinical Significance

In normal conditions, the ketone bodies are produced constantly by the liver and utilized by peripheral tissues. The normal concentration of ketone bodies in blood is about 1–3 mg/dl, and their excretion in urine is negligible, that is, undetectable by routine urine tests. The concentration of ketone bodies increases in the blood when the rate of synthesis exceeds the rate of utilization, a condition named ketonemia. The higher increase of ketone bodies in blood leads to their excretion in urine. The excretion of ketone bodies in urine is called ketonuria. In untreated diabetes patient and starving patient, the serum ketone body levels may reach 90 mg or above per 100 ml of serum, and urinary excretion may reach 5000 mg/24 h urine. In type I diabetes, low insulin levels impair carbohydrate metabolism that leads to accumulation of acetyl CoA and its conversion to ketone bodies. During starvation also increased lipolysis causes overproduction of acetyl CoA which is diverted for overproduction of ketone bodies. Both these conditions result in very high levels of ketone bodies, lowering the blood pH, and kidneys excrete very acidic urine.

To Measure Activity of Creatine Kinase in Serum

Theory

Creatine kinase (CK), also called creatine phosphokinase, exists as three isoenzymes, i.e., CK_1, CK_2, and CK_3. Each isoenzyme is dimeric enzyme composed of subunits M (muscle) or B (brain). The isoenzyme CK_1 contains subunit BB and occurs primarily in the brain. CK_2 possess subunit MB and are found in the heart. Skeletal muscles primarily contain the MM isoform (CK_3). Healthy individuals typically contain the MM isoform and a small amount of the MB isoform in their serum. The enzyme is not found in the liver, kidney, and blood cells. Various conditions including skeletal muscle injury and myocardial damage release CK-MB into the bloodstream. In myocardial infarction, levels of both CK-total and CK-MB increase significantly, but CK-MB is considered a specific cardiac marker.

Sample Requirement

Serum or plasma (heparinized/EDTA) is used. Protect from light. Loss of activity occurs within 7 days at 4°C or within 24 h at 25°C.

Principle

Creatine kinase enzyme hydrolyzes creatine phosphate to liberate creatine and ATP at pH 7.4. Enzyme hexokinase converts glucose to glucose-6-phosphate in presence of ATP. Glucose-6-phosphate is converted to 6-phosphogluconolactone. The change in absorbance is monitored at 30 s intervals for 3 min at 340 nm. The enzyme in serum is relatively unstable and loses its activity due to sulfhydryl group oxidation at the active site of the enzyme. The enzyme activity is partially restored by incubating the enzyme reaction with sulfhydryl containing compounds such as

N-acetylcysteine, thioglycerol, dithiothreitol, cysteine, etc. The reaction sample is also added with an antibody specific to CK-M subunit which inhibits CK-M monomer.

$$\text{Creatine phosphate} + \text{ADP} \overset{\text{CK}}{\rightleftarrows} \text{Creatine} + \text{ATP}$$

$$\textit{Glucose} + \text{ATP} \overset{\text{Hexokinase}}{\rightleftarrows} \text{Glucose-6-Phosphate} + \text{ADP}$$

$$\text{Glucose-6-Phosphate} + \text{NADP} \overset{\text{G-6-P dehydrogenase}}{\rightarrow} \text{6-phosphogluconolactone} + \text{NADPH}_2$$

Enzyme Reagents

Imidazole buffer (10 mM, pH 7.7)
Phosphocreatine (30 mM)
N-acetylcysteine (20 mM)
Magnesium acetate (10 mM)
Glucose (20 mM)
Glucose-6-phosphate dehydrogenase $\geq$ 1.5 KU/L
Hexokinase $\geq$ 2.5 KU/L
EDTA, ADP, and NADP (2 mM each)
AMP (5 mM)

Procedure

Keep enzyme reagent at 37°C before use. Take 1 ml of this enzyme reagent in a thermostated cuvette; add 0.05 ml of diluted serum/plasma, mix thoroughly, and incubate exactly for 5 min at 37°C and then read absorbance at 30 s intervals for further 3 min.

Calculation

CK-MB activity/L of serum (IU/L)

$$= \Delta \text{OD/min} \times 6752 \times \text{dilution factor}$$

where, factor 6752 is obtained as:

$$= \frac{\text{total volume in cuvette}}{(\text{molar extinction coefficient of NADPH}) \times \text{volume of serum used (ml)}}$$

$$= \frac{1.05}{6.22 \times 10^{-3} \times 0.05}$$

$$= 3376$$

Note If we use an antibody against CK-M, we get value of only CK-B from this enzyme. So to get activity of CK-MB, multiply value 3376 with factor 2 (i.e., $3376 \times 2 = 6752$). If we follow the above process without CK-M antibody, then value obtained will be for total CK activity. Calculation of CK-MB % is used to predict the occurrence of myocardial infarction. CK-MB % is calculated as:

$$\text{CK-MB}\% = \frac{\text{CK-MB IU/L}}{\text{Total CK IU/L}} \times 100$$

Clinical Significance

Normal range of CK-MB activity is 24–195 IU/L in males and 24–170 IU/L in females. The total CK activity range is 60–400 U/L in males and 40–150 U/L in females. The increase in CK activity is mainly caused by skeletal muscle disease, i.e., muscular dystrophy, myocardial infarction, and cerebrovascular damages. Other factors like polymyositis, viral myositis, and hypothyroidism also increase CK levels. The increase in the CK activity (particularly CK_2 or MB isoenzyme) is observed within 6–8 h after onset of myocardial infarction. The activity reaches to maximum levels after 12–24 h and comes to normal after 3–4 days. Increase in levels of both total CK activity and CK-MB activity can be observed in the serum of patients suffering myocardial infarction or after physical exercise. The increase is not observed in heart failure and coronary insufficiency. The CK-MB % less than 5.5% indicates probability of no myocardial infarction or myocardial infarction occurred in less than 4–6 h prior to sample collection. The CK-MB % between 5.5 and 20% indicates most probable cause of myocardial infarction or myocardial damage due to cardiac catheterization.