M.Sc. MEDICAL BIOCHEMISTRY
LAB MANUAL
2nd Year

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Medical Microbiology

MIDNAPORE CITY COLLEGE

PG MLT BIOCHEMISTRY
PAPER 1 PRACTICAL
CLINICAL BIOCHEMISTRY LAB MANUAL

Folin-Wu Method For Estimation of Blood Glucose

Folin-Wu method is one of the oldest methods for the estimation of blood sugar. However, it is almost obsolete for now but is in use in countries where enzyme preparations are not easy to obtain. This method is old and not specific for glucose determination since other substances (e.g. fructose, lactose, and glutathione) also bring about a reduction. The blood glucose level when estimated by the Folin-Wu method is higher than true glucose.

Principle

Proteins from the blood are removed by 10% sodium tungstate and 2/3N sulphuric acid. The glucose present in the protein-free filtrate on boiling in an alkaline medium will be changed to enediol form. This enediol will reduce cupric ions to the precipitate of cuprous oxide. This oxide is dissolved and reacted by phosphomolybdic acid to form phosphomolybdenum blue which is blue in color. Constricted tubes (Folin-Wu tubes) are used to avoid reoxidation of cuprous oxide by atmospheric oxygen. The final blue color is measured at 680 nm which is proportional to the amount of glucose present in the specimen.

Requirements

1. Folin Wu tubes
2. Colorimeter
3. Reagents:
    1. **2/3 N H2SO4:** Add 2ml H2SO4 to about 50ml of D/W and dilute up to 100ml.
    2. **10% Sodium Tungstate:** Dissolve 10 gm in 100ml of D/W.
    3. **Alkaline Copper tartarate:**
       A) Dissolve 40gm sodium carbonate and 7.5 gm tartaric acid in about 400ml of D/W.
       B) Dissolve 4.5 gm copper sulphate in about 100ml D/W.
       Mix A and B and make volume up to 1000ml with D/W.
    4. **Phosphomolybdic acid:** Dissolve 35 gm molybdic acid and 5 gm sodium tungstate in 200 ml 10% NaOH. Add it to 200ml D/W and boil for 45 minutes to remove ammonia. Cool and add slowly 125 ml of 89% phosphoric acid. Make up the volume to 500ml with D/W.
    5. Distilled water
    6. **Glucose standard**
       Stock (1g/dl): Dissolve 1 gm of glucose in 100 ml saturated benzoic acid (0.3%).
       Working standard (10mg/dl): Dilute stock 1:100 with saturated benzoic acid.

Procedure

Step 1: Preparation of protein-free filtrate:

1. Add 1 ml of blood to 7 ml of distilled water and mix.
2. Add 1 ml of 10% sodium tungstate.
3. Add 1 ml of 2/3N H_2SO_4 and mix. Allow standing for 5 minutes.
4. Centrifuge or filter using Whatmann number 1 filter paper.

Step 2: Testing

1. Set up 3 Folin-Wu tubes as follows:

	Blank	Standard	Test
Distilled water	1 ml	–	–
Working glucose standard	–	1 ml	–
Protein-free filtrate	–	–	1 ml
Alkaline copper tartarate	1 ml	1 ml	1 ml

2. Place the tubes in a boiling water bath for 10 minutes.
3. Cool and add 1ml phosphomolybdic acid reagent to each tube.
4. Shake the tubes to get rid of air bubbles. Add distilled water up to 12.5 ml mark.
5. Mix and read the absorbance at 680 or red filter. Set the zero using the blank.

Calculations

Calculate the concentration of glucose in the blood specimen using the following

formula:

$$\text{Glucose (mg/dl)} = \frac{\text{OD of Test}}{\text{OD of Standard}} \times \text{Conc. of Standard} \times \frac{100}{\text{Amount of Sample Taken}}$$

$$= \frac{\text{OD of Test}}{\text{OD of Standard}} \times 0.1 \times \frac{100}{0.1}$$

$$= \frac{\text{OD of Test}}{\text{OD of Standard}} \times 100$$

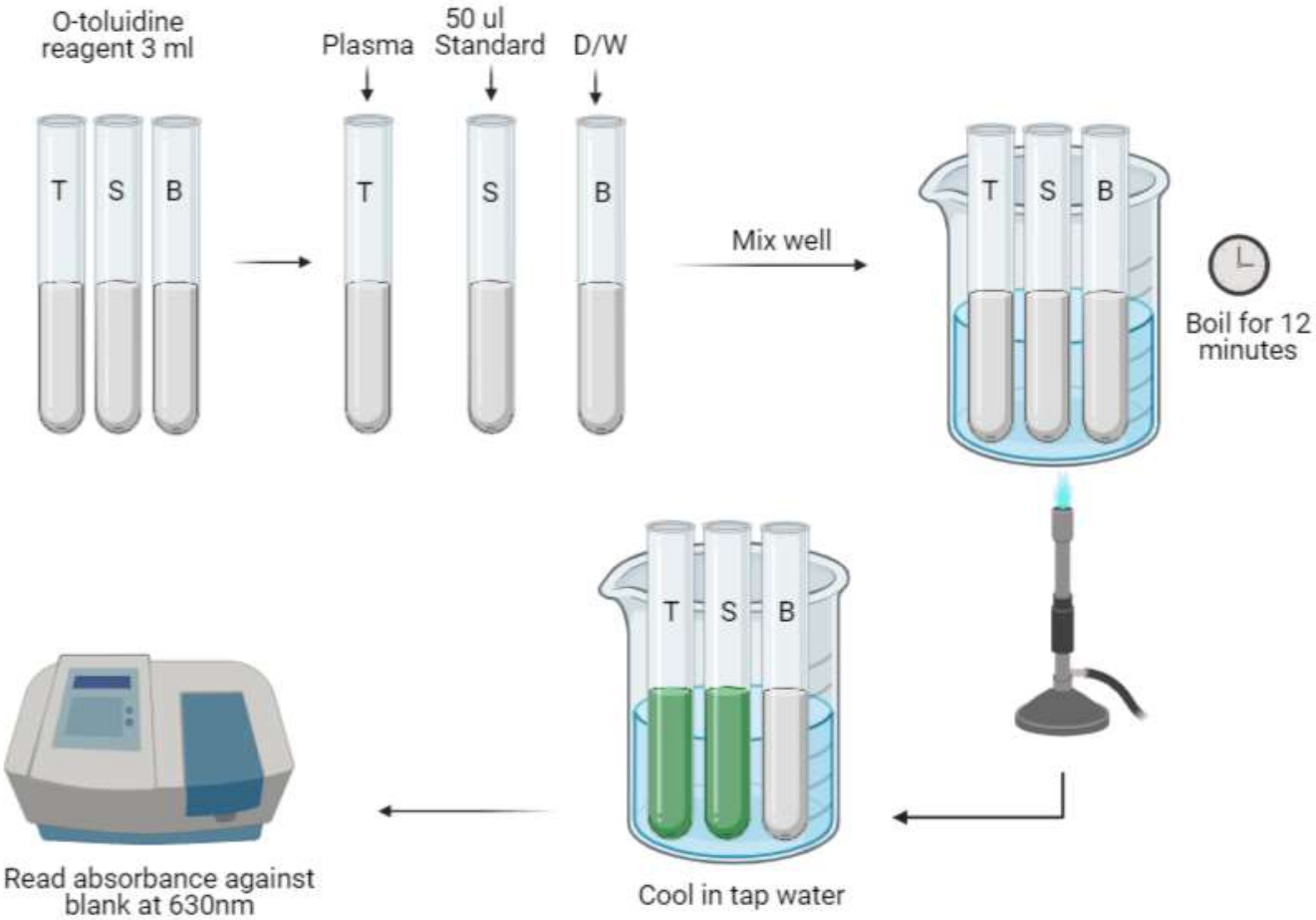
Note: *1 ml of blood was diluted 1:10 for protein precipitation. 1 ml of the diluted blood was then used for test. Therefore, the actual volume of blood used for the test is 0.1 ml.*
Also, 1 ml of the working standard (10 mg/dl) contains 0.1 mg of glucose.

O-Toluidine Method for Estimation of Blood Glucose

The **O-toluidine** method is an older method of blood glucose estimation. This method is no longer used today because O-toluidine is believed to be a carcinogen and is replaced by enzymatic methods. This method is still popular because of its simplicity, sensitivity and accuracy.

Principle

The proteins are first precipitated by tricholoroacetic acid. The glucose present in a protein free filtrate react with O-toluidine (primary aromatic amine) in a hot acidic medium to form a stable green colored complex, namely N-glycosamine. The presence of thiourea stabilizes the o-toluidine reaction. The intensity of the color developed is measured photometrically at 630nm, which is directly proportional to the concentration of the glucose present in the fluid.



Requirements

- **Apparatus:**
  Graduated pipettes
  Test tubes
  Micropipettes
  Heating Bath, 100C
  Spectrophotometer, wavelength 630nm
- **Reagents:**
  Benzoic acid
  D-glucose
  Glacial acetic acid
  O-toluidine
  Thiourea
  Trichloroacetic acid
- **Specimen:**
  Collect 2-3 ml of blood in a fluoride tube. Use plasma for testing. Serum/Whole blood can also be used. Prepare protein-free filtrate if the sample is grossly hemolyzed or icteric. Add 100 ul specimen to 900 ul of 5gm/dl TCA, mix and centrifuge to get the filtrate.

Preparation of Regaents

1. **Benzoic acid solution 1 g/l**:
   Dissolve 1 gm of benzoic acid in water and make upto 1 liter. Prepare the benzoic acid at least 24 hours before use.
2. **Stock glucose solution 100 mg/dl**:
   Dissolve 1 gm of glucose in 1 liter of benzoic acid solution (1 g/l).
3. **O-toluidine reagent**:
   Dissolve 1.5 gm thiourea in 940 ml of glacial acetic acid. When completely dissolved, add 60ml of O-toluidine. Mix well and store in amber bottle.

Procedure

1. Take three (or more if needed) large test-tubes and label as follows:
   Blank tube (B)
   Standard tube (S)
   Test tube (T)
2. Pipette into each tube as follows:

	Test (T)	Standard (S)	Blank (B)
O-toluidine reagent	3 ml	3 ml	3 ml
Serum/Plasma	50 µl	–	–
Glucose Standard 100 mg/dl	–	50 µl	–
Distilled water	–	–	50 µl

3. ***Note:*** *If protein free filtrate is used instead of serum/plasma, pipette 500 ul of protein free filtrate instead of 50 µl serum/plasma.*
4. Mix the contents of each tube. Place all the tubes in the water-bath at 100°C for exactly 12 minutes.
5. Remove the tubes and allow them to cool in a beaker of cold water for 5 minutes.
6. Measure the colour produced in a colorimeter at a wavelength of 630nm.

Calculations

Calculate the concentration of glucose in the blood specimen using the following formula:

$$\text{Concentration of glucose in the specimen, mg/dl} = \frac{\text{O.D of Test}}{\text{O.D of Standard}} \times 100$$

GOD-POD Method For The Estimation Of Blood Glucose

Glucose is the major carbohydrate present in the blood. It serves as a principal fuel for all the body tissues including the brain. It undergoes a series of chemical reactions to produce energy. Accurate and precise measurement of blood glucose level is of great importance in the diagnosis and management of diabetes and other disorders of carbohydrate metabolism, hyperglycemia, and hypoglycemia.

Different methods based on different properties of glucose are described for blood glucose estimation. They are:

1. Reduction Methods:

- **Ferric reduction methods**
  1. Hagedorn-Jensen ferric reduction method
  2. Hoffman's method
- **Cupric reduction methods**
  1. Somogyi-Nelsen method
  2. Neocuproine method
  3. Shaffer-Hartmann method
  4. **Folin-Wu method**
  5. Benedict's method

2. Aromatic amine condensation methods:

1. **O-toluidine method**

3. Enzymatic Methods:

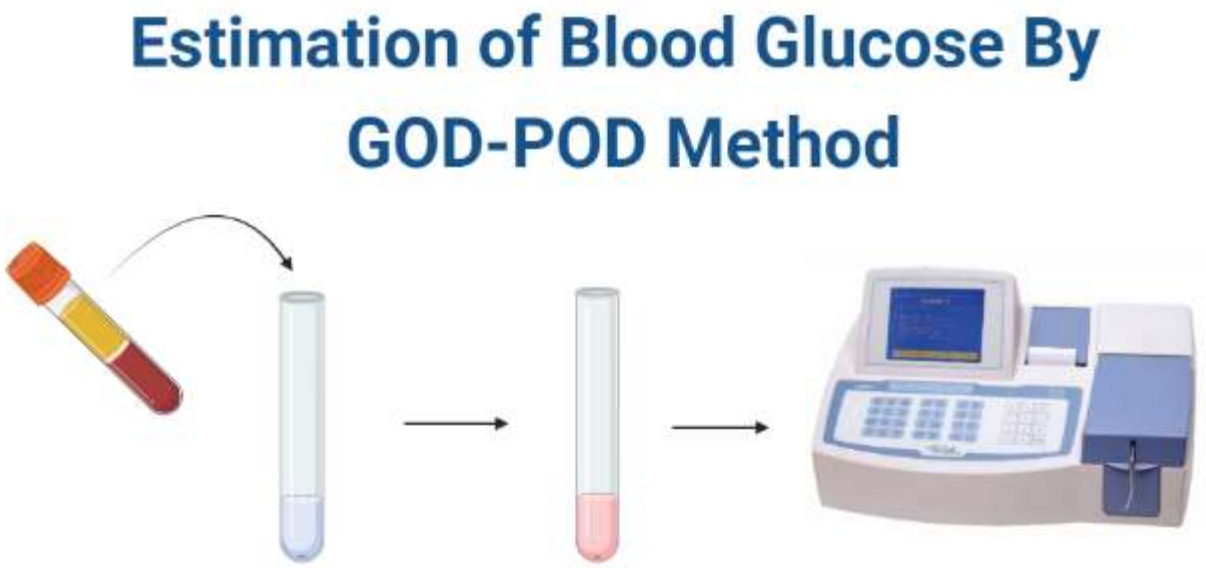
1. Glucose-oxidase Peroxidase (GOD POD) method (Trinder method)
2. Hexokinase method
3. Glucose dehydrogenase (GDH) method
4. Kinetic method
5. Polarographic method

4. Electrochemical methods:

1. Glucometer

GOD-POD Method for Glucose Estimation

Being more specific, easier, and more accurate, enzymatic methods are preferred these days. Among them, the GOD-POD method is the most common method of glucose estimation.



Principle:

In the presence of atmospheric oxygen, glucose present in the specimen is oxidized by the enzyme glucose oxidase (GOD) to gluconic acid and hydrogen peroxide (H_2O_2).

Thus formed H_2O_2 oxidatively couples with 4-aminoantipyrine and phenol in presence of peroxidase (POD) to form red-colored **quinoneimine dye**, which is measured colorimetrically at 540nm. The intensity of the color is directly proportional to the concentration of glucose present in the specimen.

$$\text{Glucose} + H_2O + O_2 \xrightarrow{\text{Glucose Oxidase (GOD)}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{Phenol} + \text{4-aminoantipyrine} \xrightarrow{\text{Peroxidase (POD)}} \text{Red quinoneimine dye} + H_2O$$

Requirements:

Specimen:

Serum, or plasma free of hemolysis. Sodium fluoride is preferred as an anticoagulant due to its antiglycolytic activity.

Reagents:

1. Glucose standard (100 mg/dl)
2. **GOD-POD reagent**: Enzyme reagent mixture containing glucose oxidase (GOD), peroxidase (POD), 4-aminoantipyrine, phenol, and phosphate buffer (pH≈7.0), some stabilizers and activators.

Instruments:

1. Test tubes
2. Pipettes, disposable tips, rack
3. Water bath
4. Colorimeter

Procedure:

1. Label three clean, dry test tubes as **Blank (B)**, **Standard (S)**, and **Test (T)**.
2. Pipette as follows:

	Blank	Standard	Test
GOD-POD Reagent	1 ml	1 ml	1 ml
Distilled water	10 µl	–	–
Glucose standard	–	10 µl	–
Sample	–	–	10 µl

3. Mix well and incubate at 37^0C for 10 minutes. Or, at room temperature (25^0C) for 30 minutes.
4. Measure the absorbance of the standard and test sample at 540nm (green filter) against blank within 60 minutes.

Calculation:

Calculate the concentration of blood glucose in the specimen using the following formula:

$$\text{Conc. of Glucose in the specimen (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times 100$$

Interpretation - ……

Estimation of protein by biuret method

CLINICAL SIGNIFICANCE- Total protein is useful for monitoring gross changes in protein levels caused by various disease states. It is usually performed in conjunction with other tests such as serum albumin, liver function tests or protein electrophoresis. An albumin/globulin ratio is often calculated to obtain additional information. Increased levels of serum protein are observed in dehydration, multiple myeloma and chronic liver disease. Decreased levels are encountered in renal diseases and terminal liver failure

PRINCIPLE - Biuret method. The peptide bonds of protein react with copper II ions in alkaline solution to form a blue-violet ion complex, (the so called biuret reaction), each copper ion complexing with 5 or 6 peptide bonds. Tartrate is added as a stabiliser whilst iodide is used to prevent auto-reduction of the alkaline copper complex. The colour formed is proportional to the protein concentration and is measured at 546 nm (520-560).

REAGENT COMPOSITION –

R1 Copper II Sulphate 12 mmol/l

Potassium Sodium Tartrate 31.9 mmol/l

Potassium Iodide 30.1 mmol/l

Sodium Hydroxide 0.6 mol/l

R2- standard - See bottle label

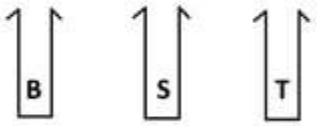
Procedure

Using the following steps, you can easily conduct this test.

- First, take 3 dry and clean test tubes.and mark blank, test , sample respectively.

PROCEDURE:

1. Take 3 cuvettes, label them as Blank (B), Standard (S), Test (T)



- Then fill the test tube mark with blank,test,sample with biuret reagent
- Then add standard reagent in standard mark test tube

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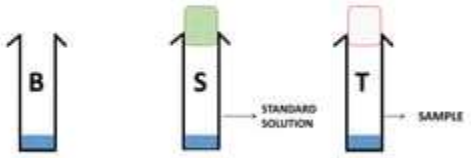
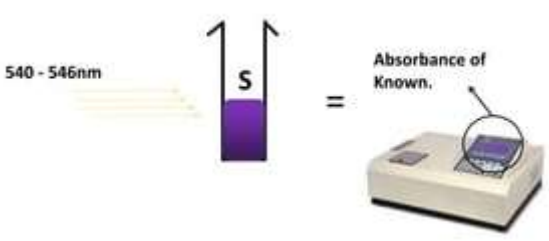
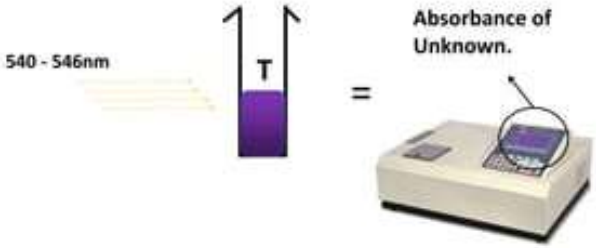


	Blank(ml)	Sample(ml)	Test(ml)
Reagent	1ml	1 ml	1 ml
Distilled water	.02 ml	-	-
Sample	-	.02 ml	-
standard	-		.02 ml

- Mix thoroughly and incubate at reaction temperature for 60 seconds. Read the initial

- absorbance and the change of absorbance at 340nm
- Then take absorbance in semi auto analyser





Calculation-

$$\text{Conc. of Sample} = \frac{\text{Absorbance of Unknown}}{\text{Absorbance of Known}} \times \text{Conc. of Standard Solution}$$

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RESULT: .....

Normal value: 6-8g/dL

Plasma Protiens: **Albumin, Globulin and fibrinogen**.

Interpretation - …….

Estimation of serum creatinine by jaffe's method –

INTRODUCTION –

Creatinine is the result of the degradation of the creatine, component of muscles, it can be transformed into ATP, that is a source of high energy for the cells. The creatinine production depends on the modification of the muscular mass, and it varies little and the levels usually are very stable. Is excreted by the kidneys. With progressive renal insufficiency there is retention in blood of urea, creatinine and uric acid. Elevate creatinine level may indicative renal insufficiency. Clinical diagnosis should not be made on a single test result; it should integrate clinical and other laboratory data.

PRINCIPLE OF THE METHOD –

The assay is based on the reaction of creatinine with sodium picrate as described by Jaffe. Creatinine reacts with alkaline picrate forming a red complex. The time interval chosen for measurements avoids interferences from other serum constituents. The intensity of the color formed is proportional to the creatinine concentration in the sample.

Reagents –

R 1 - Picric Reagent	Picric acid - 17.5 mmol/L
R 2 Alkaline Reagent	Sodium hydroxide- 0.29 mol/L
CREATININE STD	Creatinine aqueous primary standard - 2 mg/dL

PREPARATION –

Working reagent (WR): Mix equal volumes of R1 Picric Reagent and R2 Alkaline reagent.

The working reagent is stable for 15 days at (2- 8°C) or 7 days at room temperature (15-25 °C).

SAMPLES –

Serum or heparinized plasma Creatinine . stability: 24 hours at 2-8°C.

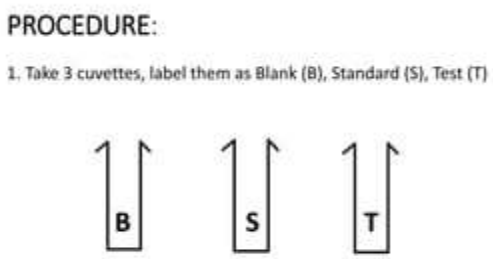
- Urine: Dilute sample 1/50 with distilled water. Mix. Multiply results by 50 (dilution factor);

Creatinine stability: 7 days at 2-8°C.

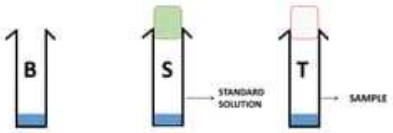
Procedure

Using the following steps, you can easily conduct this test.

- First, take 3 dry and clean test tubes.and mark blank, test , sample respectively.



- Then fill the test tube mark with blank,test,sample with WR reagent
- Then add standard reagent in standard mark test tube
- Then add serum sample in test marked test tube



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	Blank(ml)	Sample(ml)	Test(ml)
Reagent (wr)	1ml	1 ml	1 ml
Sample	-	.02 ml	-
standard	-		.02 ml

Calculation –

$$= \frac{T-B}{S-B} \times \frac{\text{Concentration of standard}}{\text{Volume of test sample}} \times 100$$

$$= \frac{T-B}{S-B} \times \frac{0.5}{0.5} \times 100$$

$$= \frac{T}{S} \times 100$$

$$= \ldots\ldots \text{ x mg/100 mL.}$$

Reference value –

	Male	Female
Serum/plasma	0.7 – 1.4 mg/dL = 61.8 – 123.7 µmol/L	0.6 – 1.1 mg/dL =53.0 -97.2 µmol/L
Urine	10 – 20mg	8 – 18 mg

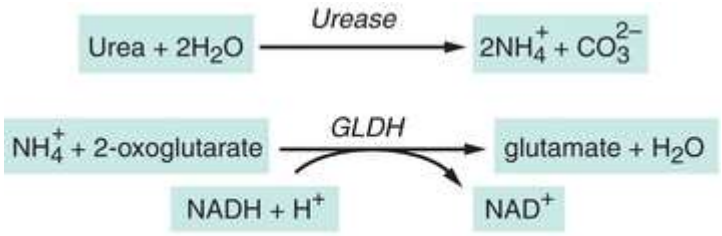
Interpretation - …….

Estimation of urea in blood sample

Introduction:

- Urea is the chief nitrogenous waste formed during protein metabolism in man.
- It is devised principally from the amino groups of amino acids.
- Liver is the main organ where urea is synthesized by a process called ornithine cycle.
- After urea is formed in the liver, it passes into the blood and then excreted in the urine.
- The concentration of urea in blood depends upon the relationship between urea production and urea excretion.

- PRINCIPLE:- This kit employs the urease method with a stabilized NADH cofactor analog for improved performance. Urea is hydrolyzed by urease to give ammonia which reacts with 2-oxoglutarate and the reduced cofactor in the presence of the enzyme glutamate dehydrogenase (GLDH) to yeild an oxidized cofactor. The resulting decrease in color due to the reduction of the cofactor is monitored spectrophotometrically at 340 nm and is directly proportional to the urea in the sample.



Normal value of blood urea

- The normal blood (serum) urea = 10-50 mg/100ml of blood.
- This value varies directly with the protein intake of the individual.
- The urea molecule contains two nitrogen atoms.
- So, the concentration of urea is expressed as blood urea nitrogen (BUN).
- The conversion of blood urea nitrogen (BUN) value to the blood urea is done by the following formula.
- Value of blood urea = BUN X 2.14
- Hence the normal BUN = 6-22mg/100ml of serum.

Method for estimation of blood urea

- Blood urea can be estimated by the following method.
- **Berthelot method:**
  - In this method to estimate blood urea, blood plasma or serum is used.
  - It is based on the principle that urea is hydrolysed into carbonic acid and ammonia by enzyme urease.

- The ammonia produced is measured photometrically after its reaction with phenol in the presence of hypochlorite (Berthelot reaction).
- This blue color reaction product is determined photometrically.
- This method is widely used because of absolute specificity of enzyme urease.

- Components
  Reagent 1 : Urea (Enzymes/Co-Enzymes)
  Reagent 2 : Urea (Buffer)
  Reagent 3 : Urea Standard (50 mg/dl)

Reagent	COMPONENT & concentration
Reagent1	Urease- >10000 U/L Glutamate Dehydrogenase (GLDH)- >1400 U/L NADH - 0.3 mmol/L
Reagent 2	Tris Buffer 200 mmol/L a-ketoglutarate 13 mmol/L
standard	Urea - 50 mg/dl

Reagent Preparation : Reagent1 & Reagent 2, Ready to use Dissolve one bottle of Reagent1 with Reagent2 (refer to Reagent1 label) as working solution..

Wavelength : 340 nm

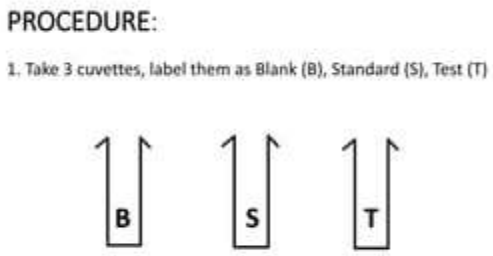
Temperature : 37°C

Cuvette : 1 cm

Procedure

Using the following steps, you can easily conduct this test.

- First, take 3 dry and clean test tubes.and mark blank, test , sample respectively.



- Then fill the test tube mark with blank,test,sample with WR reagent
- Then add standard reagent in standard mark test tube
- Then add serum sample in test marked test tube

	Blank(ml)	Sample(ml)	Test(ml)
Reagent (wr)	1ml	1 ml	1 ml
Sample	-	.02 ml	-
standard	-		.02 ml

Mix thoroughly and incubate at reaction temperature for 60 seconds. Read the initial absorbance and the change of absorbance at 340nm

Calculation -

$$\text{Blood urea (mg\%)} = \frac{\text{OD of test}}{\text{OD of std.}} \times \frac{\text{Conc of std}}{\text{Effective volume}} \times 100$$

$$= \frac{\text{OD of test}}{\text{OD of std}} \times \frac{0.01 \times 100}{0.01}$$

$$= \frac{\text{OD of test}}{\text{OD of std}} \times 100$$

Blood Urea Nitrogen (BUN) (mg/dl)
= 0.467 × blood urea mg/dl

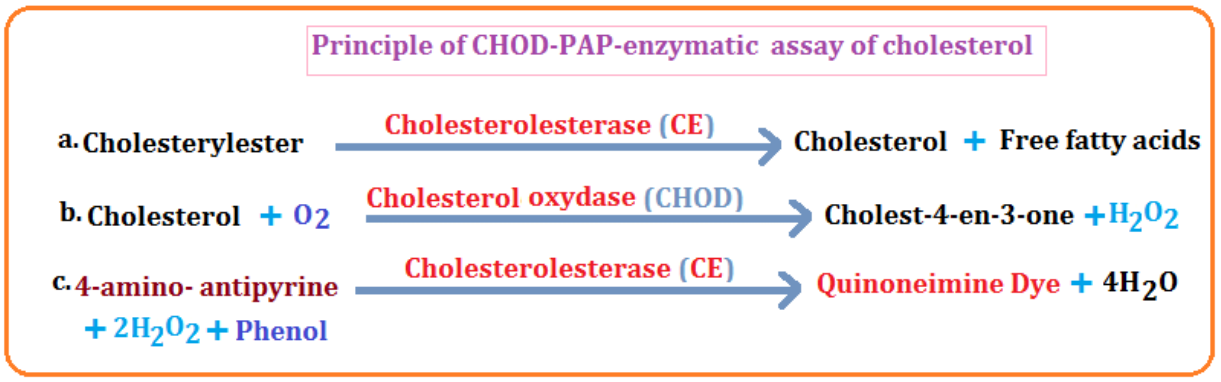
The normal blood (serum) urea = 10-50 mg/100ml of blood. This value varies directly with the protein intake of the individual. The urea molecule contains two nitrogen atoms. So, the concentration of urea is expressed as blood urea nitrogen (BUN).

Interpretation - …….

Estimation of cholesterol by CHOD/POD method

INTRODUCTION Cholesterol is one of the lipids found in the blood stream. High level of cholesterol in blood - hypercholesterolemia - is a major risk factor of coronary heart disease, which may lead to heart attacks. METHODOLOGY: CHOD/POD method.

PRINCIPLE OF THE METHOD The enzyme cholesterol esterase is used to hydrolyze the cholesterol esters present in serum into free cholesterol and free fatty acids. The enzyme cholesterol oxidase, in the presence of oxygen, oxidizes cholesterol to cholest-4-en-3one and hydrogen peroxide. Hydrogen peroxide oxidizes phenol and 4-aminoantipyrine to produce red color that can be measured spectrophotometrically.



The intensity of the color formed is proportional to the cholesterol concentration in serum.

REAGENTS COMPOSITION

WORKING REAGENT	PIPES PH 6.8 90mmol/L Phenol 26mmol/L 4-Aminophenazone(4-AA) 0.4mmol/L Cholesterol esterase(CHE) 1000U/L Cholesterol oxidase(CHOD) 300U/L Peroxidase(POD) 650U/L
STANDARD	Cholesterol aqueous primary standard - 200mg/dl

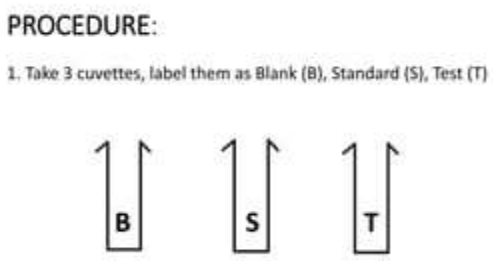
COLLECTING AND HANDLING OF SPECIMENS-

Use serum, or plasma preserved in EDTA. Determination of lipid constituent in plasma or serum are normally done on blood drawn from patients fasting for 12 to 16 hours. Stability of the sample is 7 days at 2-8°C. Freezing at -20ºC will keep samples stable for 3 months.

Protocol

Using the following steps, you can easily conduct this test.

- First, take 3 dry and clean test tubes.and mark blank, test , sample respectively.



- Then fill the test tube mark with blank,test,sample with WR reagent
- Then add standard reagent in standard mark test tube
- Then add serum sample in test marked test tube

	Blank(ml)	Sample(ml)	Test(ml)
Reagent (wr)	1ml	1 ml	1 ml
Sample	-	.02 ml	-
standard	-		.02 ml

- Mix well and incubate at 37C for 5min or at R.T. (25C) for 10min.
- Measure the absorbance of the standard and test sample against blank.
- After incubation the color is stable for at least 60 min.

CALCULATIONS

Cholesterol (mg/dl) = (A) Sample / (A) Standard * 200 mg/dl (STD Conc.)

REFERENCE VALUES Serum or plasma:

Classification	Total cholesterol (mg/dl)
Desirable	<200mg
Borderline to high risk	200-239
Risk	>240

Interpretation - …….

Estimation SGOT/AST IN BLOOD SAMPLE

SGOT

Introduction - Aspartate aminotransferase (AST) (formerly referred to as glutamate oxalacetate transaminase, GOT) belongs to the transaminases, which catalyze the interconversion of amino acids and a-ketoacids by transfer of amino groups. Aspartate aminotransferase is commonly found in human tissue. Although heart muscle is found to have the most activity of enzyme, significant activity has also been seen in the brain, liver, gastric mucosa, adipose tissue, skeletal muscle and kidney. AST is present in both the cytoplasm and mitochondria of cells. In cases involving mild tissue injury, the predominant form of AST is that from the cytoplasm, with a smaller amount coming from the mitochondria. Several tissue damage results in more of the mitochondrial enzyme released. Elevated levesl of the transaminases can signal myocardial infarction, hepatic disease, muscular dystrophy and organ damage. AST is a sensitive indicator of hepatic damage, Including hepatitis, cirrhosis and necrosis. It is often elevated before clinical signs and symptoms of liver disease appear. The determination of serum AST activity has also been used in the diagnosis and prognosis of myocardial infarction, muscular dystrophy and dermatomyositis. Elevated levels hve also been observed in cases where infectious mononucleosis, acute pancreatitis, traumatic muscle injury and hemolytic disease have been diagnosed.

Principle - In the reaction, AST catalyzed the reaction of a-ketoglutarate and L-aspartate to L-glutamate and oxalacetate. Malate dehydrogenase (MDH) catalyzes the oxidation of NADH to NAD. The rate of decrease in absorbance of the reaction mixture at 340 nm, due to the oxidation of NADH is directly proportional to the AST activity of the sample AST.

$$\text{L-Aspartate} + \alpha\text{ Ketoglutarate} \xrightarrow{\text{SGOT}} \text{Oxaloacetate} + \text{L-Glutamate}$$

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{Malate} + \text{NAD}^+$$

Content - Reagent 1 : SGOT (Enzyme/Co-Enzyme)

Reagent 2 : SGOT (Buffer)

Components

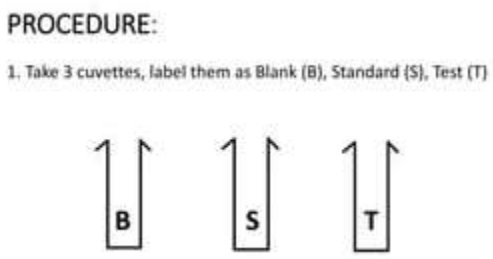
Reagents	Composition & concentration
Reagent 1	a-ketoglutaric acid 12 mmol/L NADH 0.18 mmol/L Malic dehydrogenase (MDH) >1000 U/L Lactic dehydrogenase (LDH) >5000 U/L
Reagent 2	Tris Buffer 80 mmol/L L-aspartic acid 240 mmol/L

PROCEDURE : Reagent Preparation : Reagent 1 & Reagent 2: Ready to use. Dissolve one bottle of Reagent 1 with Reagent 2 (refer to Reagent 1 Label) as working solution
Wavelength : 340 nm
Temperature : 37 C
Cuvette : 1 cm

Protocol

Using the following steps, you can easily conduct this test.

- First, take 3 dry and clean test tubes.and mark blank, test , sample respectively.



- Then fill the test tube mark with blank,test,sample with WR reagent
- Then add standard reagent in standard mark test tube
- Then add serum sample in test marked test tube

	Blank(ml)	Sample(ml)	Test(ml)
Reagent (wr)	1ml	1 ml	1 ml
Sample	-	.02 ml	-
standard	-		.02 ml

Mix thoroughly and incubate at reaction temperature for 60 seconds. Read the initial absorbance and the change of absorbance at 340nm

CALCULATION-

SGOT Conc. = (DAsample/min - DAblank/min) x K

K = (Vtotal /Vsample) x Extinction coefficient X 1000

Millimolar extinction coefficient of NADH at 340nm is 6.3.

- Expected range in Healthy adult < 40 U/L E

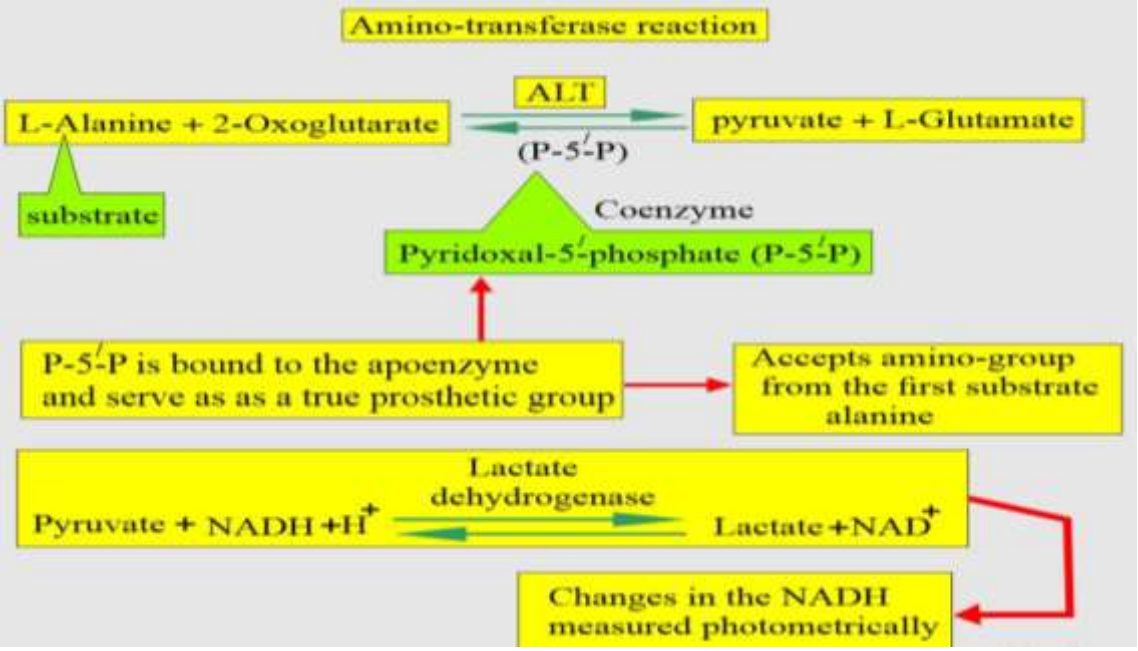
Interpretation - ……..

SGPT ESTIMATION TEST

INTRODUCTION & CLINICAL SIGNIFICANCE: SGPT is found in a variety of tissues but is mainly found in the liver. Increased levels are found in hepatitis, cirrhosis, obstructive jaundice and other hepatic diseases. Slight elevation of the enzymes is also seen in myocardial infraction.

PRINCIPLE: SGPT (ALT) catalyzes the transfer of amino group betwee L-Alanine and a-ketoglutarate to form pyruvate and glutamate. The pyruvate formed reacts with NADH in the presence of Lactate Dehydrogenase to form NAD. The rate of oxidation of NADH to NAD is measured as a decrease in absorbance which is proportional to the SGPT (ALT) activity in the sample.

Reaction -



CONTENTS: Reagent 1 : SGPT Enzyme Reagent

Reagent 2 : SGPT Substrate Reagent

SAMPLES: Serum free of hemolysis. SGPT (ALT) is reported to be stable in serum for 3 days at 2 - 8°C.

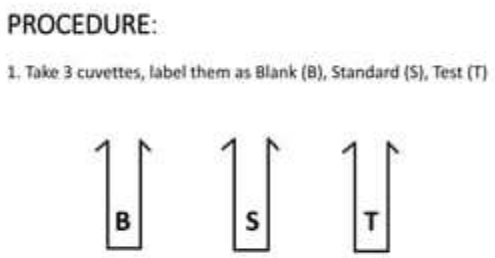
Reagents	Composition & concentration
Reagent 1	Buffer Tris, pH = 7.5 125 mmol/L L-alanine 600 mmol/L LDH ≥ 1.7 KU/L Detergent, preservative
Reagent 2	NADH - 0.18 mmol/L α-ketoglutarate - 15 mmol/L

Working Reagent: (4+1) Mix 4 volumes of bottle R1 with 1 volume of bottle R2. Avoid direct exposure to light. Stability: 3 days at 2 - 8 °C.

Protocol

Using the following steps, you can easily conduct this test.

- First, take 3 dry and clean test tubes.and mark blank, test , sample respectively.



- Then fill the test tube mark with blank,test,sample with WR reagent
- Then add standard reagent in standard mark test tube
- Then add serum sample in test marked test tube

	Blank(ml)	Sample(ml)	Test(ml)
Reagent (wr)	1ml	1 ml	1 ml
Sample	-	.02 ml	-
standard	-		.02 ml

Mix thoroughly and incubate at reaction temperature for 60 seconds. Read the initial absorbance and the change of absorbance at 340nm

CALCULATIONS –
SGPT(ALT) Activity in IU/L 37°C - Abs.. / min. x Factor (1746).

NORMAL REFERENCE VALUES :
Serum (Males) - Upto 40 IU/L at 37°C
Serum (Females) - Upto 31 IU/Lat 37°C

Interpretation - …….

ESTIMATION OF BILIRUBIN (TOTAL AND DIRECT)

Introduction - Bilirubin is mainly formed from the heme portion of aged or damaged RBC'S. It then combines with albumin to form a complex, which is not water-soluble. This is referred to as indirect or unconjugated Bilirubin. In the liver this Bilirubin complex is combined with glucuronic acid into a water-soluble conjugate. This is referred to as conjugated or direct Bilirubin. Elevated levels of bilirubin are found in liver diseases (Hepatitis, cirrhosis), excessive haemolysis / destruction of RBC (hemolytic jaundice) obstruction of the biliary tract (obstructive jaundice) and in drug induced reactions. The differentiation between the direct bilirubin hyperbilirubinemia.

PRINCIPLE: - Bilirubin reacts with diazotized sulphanilic acid to produce azobilirubin (violet colour). DMSO catalyzes the formation of azobilirubin from free bilirubin. The violet color is proportional to bilirubin concentration measured at 546 nm (530-550nm).

REACTION:

Total Bilirubin- Bilirubin+Sulphanilic acid+Sodium ~~Nitrite~~ → Azobilirubin

Direct Bilirubin - Bilirubin + Sulphanilic acid + Sodium ~~Nitrite~~ → Azobilirubi

CONTENTS:-

Reagent 1 : Total Bilirubin Reagent

Reagent 2 : Direct Bilirubin Reagent

Reagent 3 : Total Bilirubin Activator

Reagent 4 : Direct Bilirubin Activator

Reagent 5: Bilirubin Artificial Standard (10.0 mg/dl)

MATERIALS REQUIRED:- Clean & Dry Glassware.

Laboratory Glass Pipettes or Micropipettes & Tips. Bio-Chemistry Analyzer.

- **PROCEDURE:** Pipette into clean dry test tubes labeled as Blank (B) and Test(T) :
  Total bilirubin –

Addition sequence	Blank	Test
Total Bilirubin Reagent	1 ml	1 ml
Total Bilirubin activator	-	.02ml
Serum / Plasma	.05ml	.05ml

Direct Bilirubin

Addition sequence	Blank	Test
Total Bilirubin Reagent	1 ml	1 ml
Total Bilirubin activator	-	.02ml
Serum / Plasma	.05ml	.05ml

Mix well and incubate at 37°C for exactly 5 minutes. Measure the absorbance of the Test Samples (Abs. T) immediately against their respective Blanks at 530nm-550nm.

CALCULATION : Bilirubin mg/dl (Total or Direct)=Abs. of Test -Abs. Blank x Factor

NORMAL VALUE :
Serum : Total Bilirubin : upto 1.0 mg/dl
Direct Bilirubin : upto 0.3 mg/dl

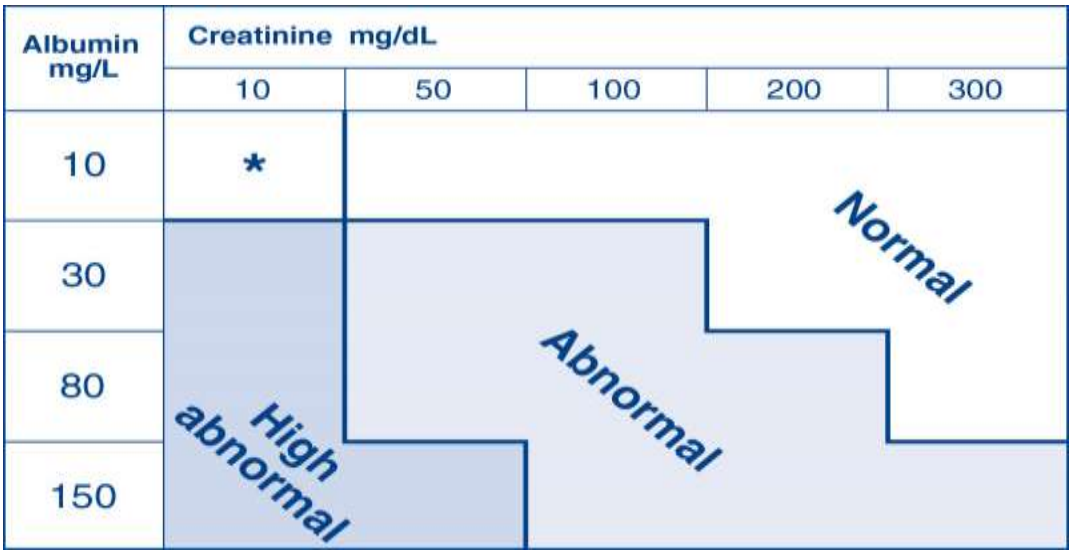
Interpretation - ……

Estimation of common parameters in urine through Use of strips

Albumin

Principle

The test is based on the principle of the "protein error" of indicators, i.e. at a constantly buffered pH, albumin reacts with a tetrabromophenol sulphonephthalein derivative resulting in a color change from yellow-green to green-blue.



Ascorbic acid (Vitamin C)

Modern Medi-Test urine test strips have the best available protection against influences of ascorbic acid (vitamin C) in the sample. For historic reasons, many test strips still feature a test pad for ascorbic acid.

Principle

The detection is based on the de-coloration of Tillman's reagent. The blue colored 2,6-dichlorophenol indophenol sodium salt is reduced to the colorless leuco form by ascorbic acid. In the presence of ascorbic acid a color change takes place from blue to red.

Evaluation

The color fields correspond to the following values:
0 (negative), 10 (+), and 20 (++) mg/dL or
0 (negative), 0.6 (+), and 1.1 (++) mmol/L.

Diagnosis

The wide spread intake of ascorbic acid (e.g. in vitamin C therapy, as a therapeutical ingredient and stabilizer of numerous medicaments, oxidation inhibitors and preservatives in food industry) causes a rapid saturation of the organism, and a renal excretion of the excess. Interfering ascorbic acid concentrations may be reached after the

ingestion of fruit juice or plenty of fruit. Therefore, the ascorbic acid test zone minimizes falsely negative results.

Bilirubin

Principle

A red azo compound is obtained in the presence of acid by the coupling of bilirubin with a diazonium salt.

Evaluation

The minimum sensitivity of the test strip is 0.5 to 0.75 mg bilirubin/dL urine. The color fields correspond to the following values:
0 (negative), 1 (+), 2 (++), 4 (+++) mg/dL or
0 (negative), 17 (+), 35 (++), 70 (+++) µmol/L.
Some urine components can produce a yellow coloration of the test strip. Ascorbic acid and nitrite in higher concentrations inhibit the test. Longer exposure of the urine to light leads to lowered, or falsely negative results.

Diagnosis

Only conjugated (water soluble) bilirubin is excreted by the kidneys. Normally bilirubin is undetectable in urine. Bilirubinuria generally indicates liver parenchyma damage (e.g. acute virus hepatitis and other forms of hepatitis, liver cirrhosis, toxic liver cell damage) or biliary obstructions (e.g. cholangitis, obstructive jaundice).
Unconjugated bilirubin, which is detectable in serum, indicating hemolytic jaundice is not excreted by the kidneys and is absent from urine.

- Detection of liver diseases
- In combination with urobilinogendifferentiation betwaeen different forms of jaundice

Blood

Principle

The detection is based on the pseudoperoxidative activity of hemoglobin and myoglobin, which catalyze the oxidation of an indicator by an organic hydroperoxide, producing a green color.

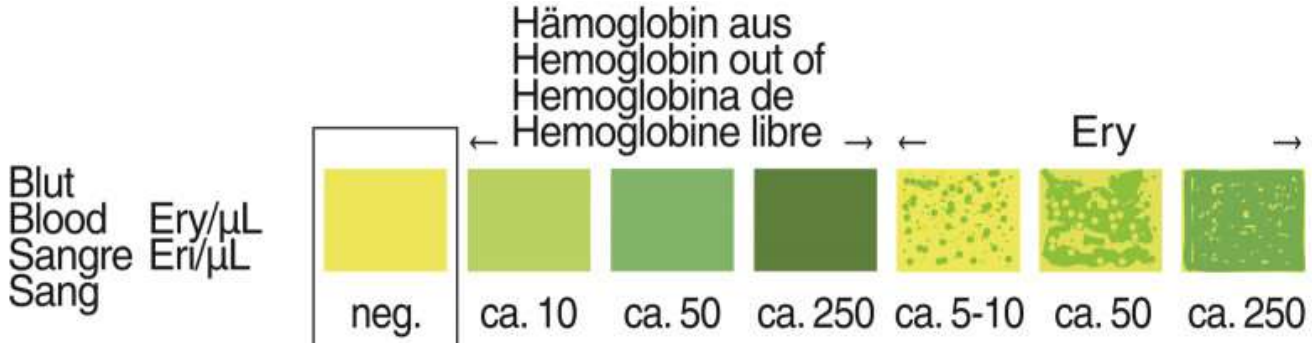
Evaluation

The minimum sensitivity of the test strip is 5-10 erythrocytes/µL urine corresponding to approx. 0.015 mg hemoglobin or myoglobin/dL urine. Intact erythrocytes are indicated by flecked discolourations of the test field. The color fields correspond to the following values: 0 (negative), ca. 5–10, ca. 50, ca. 250 Ery/µL, or
a hemoglobin concentration out of ca. 10, ca. 50, ca. 250 Ery/µL.
The blood test on Medi-Test urine test strips is optimally protected against interferences by ascorbic acid. Normal concentrations of vitamin C (< 40 mg/dL) do not influence the test result. However gentisic acid still shows an inhibitory effect. Falsely positive reactions can be produced by a residue of peroxide-containing cleansing agents.

Diagnosis

Every positive reaction should be taken as a pathological finding requiring further diagnostic examinations. Hematuria (hemolysis of intact erythrocytes occurs on the test field), hemoglobinuria or myoglobinuria are frequently caused by:
Serious infections of the kidneys and urinary tract, kidney and bladder calculi, serious poisonings (e.g. benzene and aniline derivatives, chlorate, bacteria toxins, poisonous mushrooms and snake poison), heart attack, hemolysis after transfusion incident, cold hemoglobinuria or march hemoglobinuria (after strong physical exertion), different paroxysmal hemoglobinurias and serious hemolytic anemias.

- Indicator for several serious diseases



Glucose

Principle

The detection is based on the glucose oxidase-peroxidase-chromogen reaction. The oxidation of glucose by atmospheric oxygen is catalyzed by glucose oxidase to form gluconic acid lactone and hydrogen peroxide. Peroxidase catalyzes the reaction of hydrogen peroxide with the chromogen. Apart from glucose, no other compound in urine is known to give a positive reaction.

Evaluation

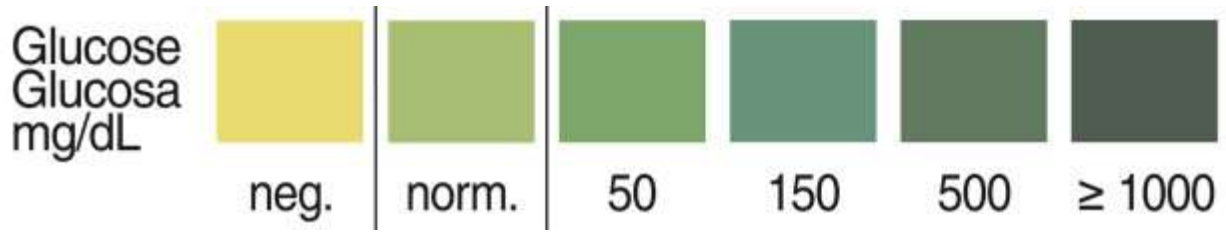
Pathological glucose concentrations are indicated by a color change from green to bluish green. Yellow or greenish test fields should be considered negative or normal. All test fields

which have an intensity greater than the greenish negative color field must be considered positive. The color fields correspond to the following ranges of glucose concentrations:
neg. (yellow), neg. or normal (greenish), 50, 150, 500, and ≥ 1000 mg/dL or
neg. (yellow), neg. or normal (greenish), 2.8, 8.3, 27.8, and ≥ 55.5 mmol/L.
An inhibitory effect is produced by gentisic acid. Falsely positive reactions can also be produced by a residue of peroxide-containing cleansing agents. The test is not influenced by vitamin C.

Diagnosis

Because of the clear distinction between physiological and pathological glucosuria, the test is especially suitable for the detection of diabetes mellitus and for supervising (and self-supervising) of diabetes. Apart from diabetes mellitus, renal glucosuria with increased glucose concentrations may be noted during pregnancy, and after a meal with excessive carbohydrates. Every positive test reaction requires further diagnosis.

- Early detection of diabetes mellitus
- Supervision of type-II-diabetic



Ketones

Principle

The test is based on the principle of Legal's test. Acetoacetic acid and acetone form a violet colored complex with sodium nitroprusside in alkaline medium.

Evaluation

Acetoacetic acid reacts more sensitively than acetone. Values of 5 mg/dL of acetoacetic acid or 50 mg/dL acetone are indicated. The color fields correspond to the following acetoacetic acid values:
0 (negative), 25 (+), 100 (++) and 300 (+++) mg/dL or
0 (negative), 2.5 (+), 10 (++) and 30 (+++) mmol/L
Phenylketones in higher concentrations interfere with the test, and will produce deviating colors. ß-hydroxybutyric acid (not a ketone) is not detected. Phthalein compounds interfere by producing a red coloration.

Diagnosis

Ketone bodies including acetoacetic acid, acetone, and ß-hydroxybutyric acid are only produced in the liver. Ketones in the urine are caused by an abnormal carbohydrate metabolism. Frequently, ketonuria is a sign of diabetic ketosis, which in connection with other metabolic abnormalities may cause diabetic coma. Ketonuria may also be noted in case

of insulin overdoses, starvation (e.g. slimming diet, calorie free diet), dangerous metabolic abnormalities during pregnancy (hyperemesis gravidarum), acetonemic vomiting of infants and fever caused especially by infections.

- Early detection of ketosis / acidosis
- Control-parameter for diabetes mellitus

Leukocytes

Principle

The test is based on the esterase activity of granulocytes. This enzyme splits a carboxylic acid ester. The alcohol component formed during this step reacts with a diazonium salt to form a violet dye.

Evaluation

The test detects values from about 10 leukocytes/µL urine. Discolorations, which can no longer be correlated to the negative test field, and weakly violet discolorations after 120 seconds are to be considered positive. The color fields correspond to the following leukocyte concentrations:
negative (normal), 25, 75, 500 leukocytes/µL
A diminished reaction can result for protein excretion above 500 mg/dL, and a glucose concentration above 2 g/dL as well as during therapy with preparations containing cephalexin or gentamycin. Bacteria, trichomonades and erythrocytes do not give a positive reaction with this test. Formaldehyde (a preservative) can cause falsely positive reactions. Boric acid used as preservative decreases the sensitivity of the reaction.
Excretion of bilirubin, nitrofurantoin, or other strongly colored compounds can cover the reaction color. For samples from female patients vaginal secretion can simulate a falsely positive reaction. In order to avoid falsely positive results, the urine should only be sampled afterthorough cleaning of the genitals.

Diagnosis

An increased excretion of leukocytes in urine (leukocyturia) is an important symptom for infectious diseases of the kidneys and/or urinary tract (incl. the prostate).
Leukocyturia is especially important for diagnosis of chronic pyelonephritis. Often it is the only symptom between acute attacks. Other causes for leukocyturia may be: analgetic nephropathia, glomerulopathia and intoxications, cystitis, urethritis, kidney or urogenital tuberculosis, fungus and trichomonade infections, gonorrhoea, urolithiasis, tumors with obstructions.

- Symptomatic of renal and urinary tract diseases

Nitrite

Principle

Microorganisms, which are able to reduce nitrate to nitrite, are indicated indirectly with this test, which is based on the principle of Griess reagent. The test paper contains an amine and a coupling component. Diazotization and subsequent coupling result in a red colored azo compound. Only nitrite can produce a diazonium salt for coupling reaction, therefore falsely positive results are virtually impossible in this case.

Evaluation

The test detects concentrations from 0.05-0.10 mg nitrite/dL urine. Any pink color indicates a bacterial infection of the urinary tract. The color intensity only shows the nitrite concentration, and does therefore not provide information about the extent of the infection. A negative result does not preclude an infection of the urinary tract, if bacteria, which cannot produce nitrite are present. Falsely negative results can be produced by high doses of ascorbic acid, by antibiotics therapy, and by very low nitrate concentrations in urine as the result of low nitrate diet or strong dilution (diuresis). Falsely positive results can be caused by the presence of diagnostic or therapeutic dyes in the urine.

Diagnosis

Bacteria, which cause infections, and can produce nitrite in the urine are e.g. *E. coli* (bacteria which causes most frequently infections), Aerobic Bacteria, Citrobacteria, Klebsiella, Proteus, Salmonellae and in part Enterococci, Pseudomonas and Staphylococci. If the test is positive a microscopic examination and determination of susceptibility of pathogenic bacteria to chemotherapeutic agents should follow.

- Bacterial infection of the kidneys or urinary tract

pH value

Principle

The test paper contains indicators, which clearly change color between pH 5 and pH 9 (from orange to green to turquoise).

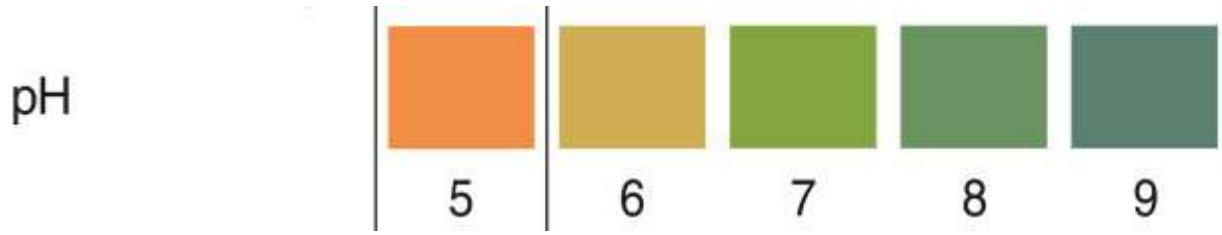
Evaluation

The pH value of fresh urine from healthy people varies between pH 5 and pH 6. The color scale gives a clear distinction of pH value between pH 5 and pH 9. The pH should always be measured in fresh urine, since bacterial decomposition may increase the pH of the urine to values > 9.

Diagnosis

The pH value is only of significance in relation to other parameters. More acid urine (lower pH values) is found in case of an increased protein metabolism, high fever, serious diarrhoea and metabolic acidosis (serious form of diabetes mellitus). Alkalinity (increased pH value) may be noted in urinary tract infections, respiratory or metabolic alkalosis.

- Diseases of the urogenital tract
- Supplement for other parameters
- Backup for microscopic results



Protein

Principle

The test is based on the "protein error" principle of indicators. The test zone is buffered to a constant pH value and changes color from yellow to greenish blue in the presence of albumin. Other proteins are indicated with less sensitivity.

Evaluation

The test strip detects values above 10 mg protein/dL urine. The color fields correspond to the following ranges of albumin concentrations:
negative, 30, 100 and 500 mg/dL or negative, 0.3, 1.0, and 5.0 g/L.
Falsely positive results are possible in strongly alkaline urine samples (pH > 9), after infusions with polyvinylpyrrolidone (blood substitute), after intake of medicaments containing quinine, and also by disinfectant residues in the urine sampling vessel. The protein coloration may be masked by the presence of medical dyes (e.g. methylene blue) or beetroot pigments.

Diagnosis

The limit of a physiological proteinuria lies between 10 and 30 mg/dL. It differentiates between:

1. Benign proteinuria is observed after physical strain, orthostatic proteinuria, with fever and during pregnancy. In such cases the protein excretion rate is usually normal in the first morning urine, however in the course of the day values can vary greatly.
2. Extrarenal proteinuria frequently appears with acute diseases like heart insufficiency, colics, liver cirrhosis, plasmocytoma, and carcinomas.
3. Renal proteinuria is caused by increased permeability of the glomerular filter and may indicate pyelonephritis, glomerulonephritis, tuberculosis of the kidneys, kidneys participation at infections and poisonings, cystic kidneys, gouty kidney. Every positive test reaction requires further diagnostic examinations.

- Symptomatic of renal and urinary tract diseases

Urobilinogen

Principle

The test paper contains a stable diazonium salt producing a reddish azo compound with urobilinogen.

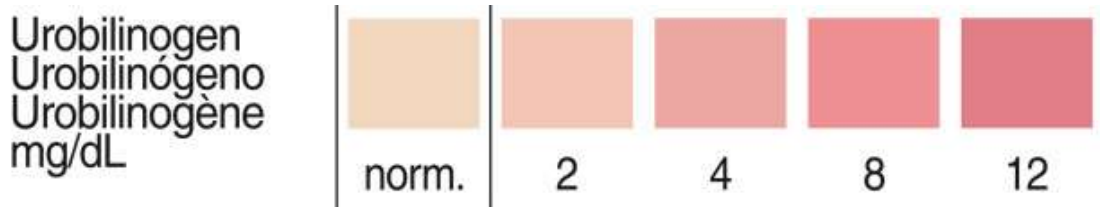
Evaluation

Depending on the urine color 0.5 to 1 mg urobilinogen/dL urine are indicated. 1 mg/dL is considered to be the normal excretion rate. Higher values are pathological. A complete absence of urobilinogen in the urine, which is likewise pathological, cannot be detected with the strips. The color fields correspond to the following urobilinogen concentrations:
normal (0–1), 2, 4, 8, 12 mg/dL or
normal (0–17), 34, 70, 140, 200 µmol/L.
The test is inhibited by higher concentrations of formaldehyde. Longer exposure of the urine to light leads to lowered or falsely negative results. Higher, or falsely positive results, can be caused by the presence of diagnostic or therapeutic dyes in the urine. Larger amounts of bilirubin produce a yellow coloration.

Diagnosis

An increased urobilinogen concentration in urine is a sensitive index of liver dysfunction or hemolytic diseases. Urobilinogen uria is caused by e.g. virus hepatitis, chronic hepatitis, liver cirrhosis, infections, poisonings, congestion or carcinoma of liver, hemolytic, and pernicious anemina, polycythemia and pathological state of the intestinal tract with an increased resorbence.

- Detection of liver diseases
- In combination with bilirubin differentiation betwee



Sodium & Potassium

(For the analyser/Colorimetric estimation of Na^+/K^+) In VITRO USE Only.

PRINCIPLE FOR SODIUM :

The sodium and the proteins are Precipitated Simultaneously by means of a reagent containing magnesium uranyl acetate containing alcohol. The precipitate is seperated by centrifugation. The content of sodium is calculated from the loss in the concentration of magnesium uranyl acetate in the reagent solution in comparison to a standard sodium solution treated similarly. The residusal amount of magnesium uranylacetate is estimated by forming brown (dark) ferrous uranyl acetate. Which is read in a colorimeter.

PRINCIPLE FOR POTASSIUM :

Potassium can be determined by a number of different methods. It can be directly estimated by flame photometry, colorimetry. It can also be measured by the use of ion selective electrode. The method is based on the measurement of turbidity of the reaction mixture containing Sodium Tetraphenyl Boron, Alkaline EDTA, Formaldehyde and sample containing potassium or standard potassium salt. The method accurate within the concentration of 2.0 to 7.0 mmol/L. There is a good agreement with flame photometry.

REAGENTS:

1. Sodium Precipitating Reagent 33 ml.
2. Standard Sodium /Potassium 3 ml.
3. Sodium Color Reagent 10 ml.
4. Potassium Reagent 45 ml.

The reagents are ready to use and usable to the expiration date when stored at 2-8°C, if contamination is avoided.

SAMPLE:

Serum (Haemolysed sera should not be used)

1. Serum should be seperated from the cloted blood without delay to prevent any leakage of potassium from the RBC,which contains 23 times higher concentration of potassium than the serum.
2. Lipemic samples should be avoided. Turbid or icteric samples produced falsely elevated potassium results.
3. Serum urea level higher than 150 mg% will produce elevated potassium results.

EXPECTED RANGE :

Potassium : 3.5 to 5.5 mmol/L
Sodium : 135 to 155 mmol/L

LINEARITY:

Potassium : This method is linear between 2 to 7 mmol/L Sodium : This method is linear between 100 to 200 mmol/LSTRICT ADHERENCE TO THE INSTRUCTIONS AND TO THE PROCEDURES ALONE GIVE THE PROPER RESULTS.

INSTRUCTIONS:

Contamination of glassware usually from detergents, results in falsely elevated concentrations. Therefore glassware should be washed with 1N Nitric Acid rinsed with high purity deionized water before use. Slowly transfer standard/serum in reagent (4) of the respective test tubes by dipping the micropipette/glass pipette tips in the solution for potassium test.
Sodium assay is an inverse reaction, hence blank is higher than the standard and test.

SODIUM ASSAY :

Step I - Precipitation of sodium and proteins.

Pipette into two clean dry test tubes labelled standard (S) and test (T)

	S	T
Sodium PPT Reagent(l) Standard	1.0 ml	1.0 ml
Sodium/Potassium(2)Serum	0.02 ml	.....
	.....	0.02 ml

Mix well on vortex for one minute and wait for five minutes at room temperature. Centrifuge for one minute at 3000 rpm.

Step II - Color Development.

Pipette into three clean dry test tubes labelled blank (B), standard (S) and test (T)

	B	S	T
Distilled Water Supernatant	3ml	3ml	3ml
from step I Sodium PPT	...	0.05ml	0.05ml
Reagent (1) Sodium Color	0.05ml	....	....
Reagent (3)	0.2ml	0.2ml	0.2ml

Mix well and allow it to stand at room temperature for five minutes. Then measure absorbance of B,S,T against distilled water on a photocolorimeter at 540 nm within 10 minutes.

Calculation :

Sodium in mmol/L =
(Absorbance of B-T/
Absorbance of B-S) X 150
(Standard concentration)

POTASSIUM ASSAY:

Pipette into two clean dry test tubes labelled standard (S) and test (T)

	S	T
Potassium Reagent (4) Standard	1.0 ml	1.0 ml
Sodium/Potassium(2)Serum	0.05 ml	....
	...	0.05 ml

Mix gently wait for five minutes at room temperature and read the absorbance of standard and test against distilled water on a photocolorimeter at 620 nm within 10 minutes.

CALCULATION :

Potassium in mmol/L =
(Absorbance of T/
Absorbance of S) x 5
(standard concentration)

ALKALINE PHOSPHATASE ESTIMATION

KINETIC METHOD

PRINCIPLE :

Phenyl phosphate —(Alkaline phosphatase, pH 10.0)→ phenol + phosphate

The liberated phenol is measured colorimetricallyin the presence of 4-aminophenazone and potassium ferricyanide .

SAMPLE :

Serum, Hemolysis will interfere .

REAGENTS :

1.	**Standard phenol**	1.59	mmol / L
2.	**Buffer – Substrate :**		
	Buffer pH 10.0	50	mmol / L
	Phenyl phosphate	5	mmol / L
3.	**Enzyme Inhibitor :**		
	EDTA	100	mmol / L
	4-Aminophenazone	50	mmol / L
4.	**Color Reagent :**		
	Potassium ferricyanide	200	mmol / L

STABILITY :

The reagents are stable up to the expiry datespecified when stored at +4 to +8 °C .

PROCEDURE :

Mix , let stand for 5 min. at room temp. in the dark. Read the absorbances of sample (A_{Sample}) and standard ($A_{standard}$) against reagent blank at 510 nm. The color is stable for one hour . The reaction is linear up to 250 IU / L .

CALCULATION

$$\text{Enzyme activity (IU / L)} = \frac{A_{Sample}}{A_{Standard}} \times 75$$

NORMAL VALUES :

Children : 70 – 140 IU / L .

REFERENCE :

Belfield A. and Goldberg D.M. (1971), Enzyme . 12,561.

QUALITY CONTROL :

For accuracy and reproducibility control:- Assayed Multi – Sera, Normal and Elevated.

	Standard (mL)	Sample (mL)	Blank (mL)
Reagent 1	0.025	-	-
Serum	-	0.025	-
Reagent 2	0.50	0.50	0.50
Incubate for 20 min. exactly at 37°C, then add:			
Reagent 3	0.25	0.25	0.25
Mix well, then add :			
Reagent 4	0.25	0.25	0.25

SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDSPAGE)

AIM:

SDS-PAGE was performed to separate and observe the protein pattern of the sample by the method of Lammeli (1970).

PRINCIPLE:

SDS-PAGE was performed to accomplish the following:

a) To observe the protein pattern of the enzyme mixture.

b) To determine the homogeneity of the purified enzyme mixture.

c) To determine the molecular weight of the purified enzyme.

REAGENTS REQUIRED:

1. Preparation of stock solution and buffers:

30% acrylamide

a) Acrylamide: 29.2g

b) N, N-methelyne–bis–acrylamide: 0.8g Added water, dissolved andmade upto 100mL and filtered with Whatman no.1 filter paper.

2. Separating gel buffer:

a) Tris-HCl: 1.5M, pH 8.8

18.171g of Tris was dissolved in 60mL of water and adjusted the pH to 8.8 with HCl and finally made upto 100mL with water.

3. Stacking gel buffer:

a) Tris-HCl: 1M, pH 6.8

6.057g of Tris was dissolved in 60mL water and adjusted the pH to 6.8with HCl and upto 100mL with water.

4. 10% SDS solution: 1g of SDS in 10mL of distilled water.

5. N,N,N’N’-Tetra methylene diammine(TEMED)

6. 10% Ammonium per sulphate (APS): 1g of APS in 10mL of distilled water.

7. Electrophoresis Buffer:

a) Tris: 25mM, pH 8.3

b) glycine: 250mM,pH 8.3

c) SDS: 0.1%: Dissolved in minimum amount of water (500mL)and then added SDS.

Allowed to settle and dissolved. This was finally made upto 2.5liters.

8) Sample buffer 4x: 5.0mL

a) Tris (1M, pH 6.8): 2.1mL

b) 2% SDS: 100mg

c) Glycerol (100%): 1.0mL

d) b-mercaptoethanol: 0.5mL

e) Bromophenol blue: 2.5mg

f) Distilled water: 0.4mL

9) Staining solution (100mL):

a) Alcohol: 40%

b) Acetic acid: 10%

c) Commassie Brilliant Blue (CBB): 259mg

d) Distilled water: 50%

10) Destaining solution (100mL)

a) Alcohol: 50%

b) Acetic acid: 10%

c) Distilled water: 40%

PROCEDURE

Preparation of gel:

The glass plates were washed in warm detergent solution, rinsed subsequently in tap water, deionised water and ethanol and dried. The unnotched outer plates were laid on the table and Vaseline (or grease) was

coated. Spacer strips were arranged approximately at the sides and bottom of the plates. The notched inner plates were laid in position, resting on the spacer strips and the arrangement was mounted vertically. Sealing was done properly to avoid leakage. The volume of the gel solution required for making separating gel was calculated as follows (the reagents in the following table yield 20mL of solution after the addition of APS and TEMED)

Reagents	8%	10%	15%
H2O (ml)	9.3	7.9	4.6
30% acrylamide mix (ml)	5.3	6.7	10.0
1.5M Tris (pH 8.8) (ml)	5.0	5.0	5.0
10% SDS (ml)	0.2	0.2	0.2
10% APS (ml)	0.2	0.2	0.2
TEMED (ml)	0.012	0.008	0.008

APS and TEMED were added just prior to the pouring of gel. The solution was mixed well and poured into the space between the two plates leaving an inch of the upper space unfilled. Water was carefully laid over the surface of the poured gel mixture to avoid air contact, which reduces the polymerization reaction. The gel mixture was allowed to polymerize, undisturbed at room temperature for 60 minutes. In the mean time gel mixture for stacking gel was prepared. (The reagents in the following table yield 10mL of solution after the addition of APS & TEMED)

After the separating gel was polymerized the over laid water was removed carefully with filter paper and an appropriate comb was inserted between the plates. 0.1mL of 10% APS and 10 l of TEMED were added to the stacking gel mixture. It was mixed well and poured immediately (to the brim) over the separating gel. The stacking gel was allowed to polymerize. Additional gel mixture was added when gel retracted significantly.

Preparation of protein samples:

The required volume of sample buffer was added to protein samples and they were loaded (the final concentration of sample buffer in the prepared sample should come to 1x. If the protein was dried suspend it in 1x buffer). Thesamples were incubated

Reagents	**Volume (ml)**
H2O	6.8
30% acrylamide mix	1.7
1M Tris-Cl, pH 6.8	1.25
10% SDS	0.1
10% APS	0.1
TEMED	0.01

for 2min in a boiling water bath prior to loading. When thepolymerization was completed the comb was removed and the lower spacer strip was carefully removed. The Vaseline (or grease) from the bottom was removed with a piece of tissue paper. The gel was attached to the electrophoresis tank using appropriate clips/clamps. The lower reservoir was filled with 1x electrophoresis buffer, using a bent Pasteur pipette or syringe needle to remove any air bubble trapped beneath the bottom of the gel. The protein samples were loaded using a micropipette and the wells were completely and carefully filled with 1x electrophoresis buffer. The upper reservoir was also carefully filled with 1x electrophoresis buffer. The electrodes were connected to a power pack.

The gel was run at constant current (20 milli ampere 100 volts) for 4-6 hrs at room temperature. Electrophoretic mobility of the samples was determined by bromophenol blue front. At the end of the run the power pack was switched off. The gel and plates were laid flat on the table and a corner of the upper glass plate was lifted up and the gel was carefully removed.

Staining of the gel:

After the completion of the electrophoresis, the gel was fixed with 10% trichloroacetic acid for 5minutes and stained with CBB. The CBB staining

solution was prepared using methanol, acetic acid and double distilled water in the ratio of 4:1:5 and 0.25gm of CBB was added and the gel was stained over night.

Destaining of the gel:

The destaining of CBB stained gel was done by using methanol, aceticacid and double distilled water in the ratio of 5:1:4 till the appearance of clearbands on the gel.

RESULT:

The sample proteins are separated by Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis. The proteins appeared as discrete bands in the gel. The relative molecular weights of the protein with respect to their bands were observed in Kilo Daltons.