BACHELOR OF MEDICAL LABORATORY TECHNOLOGY LAB MANUAL

4th Semester

Prepared By

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BMLT

MIDNAPORE CITY COLLEGE

Clinical Pathology: Paper – X, Unit – 20

EXPERIMENT 1: Physical and Microscopic examination of Urine

AIM: To examine the physical characteristics of normal and abnormal urine, wet preparation of urine and its microscopic examination and interpretation.

INTRODUCTION: Examination of urine is important for diagnosis and assistance in the diagnosis of various diseases. Routine (complete) examination of urine must start from physical and microscopic examination.

PRINCIPLE: The specimen should be properly collected in a clean container which should be properly labelled with name of the patient, age, sex, identity number with date and time of collection. It should not show signs of contamination. Physical examination of urine consists of volume, colour, odour, reaction/pH and specific gravity. After that observe by eye and light microscope.

SPECIMEN COLLECTION:

For routine examination a clean glass tube or capped jar is used; for bacteriologic examination a sterilized container is required.

For routine urinalysis: first morning sample is best since it is most concentrated.

For bacteriologic examination: mid-stream sample is preferable, i.e. first part of urine is discarded and mid-stream sample is collected.

For 24-hour sample: Collection of urine is started in the morning at 8 AM (first voided sample is discarded) and all subsequent samples are collected till next day 8 AM.

RESULTS & INTERPRETATION:

A. VOLUME:

Normal range: 700-2500 ml (average 1200 ml) of urine in 24 hours

Interpretation:

i) Nocturia	Excess of 500 ml during night with specific gravity of less than 1.018.	Sign of early renal failure.
ii) Polyuria	Excess of urine is passed in 24 hr (> 2000 ml) with low specific gravity.	Physiological: excess water intake, may be seasonal (e.g. in winter). Pathological: Diabetes insipidus, diabetes mellitus.
iii) Oliguria	Less than 500 ml of urine is passed in 24 hr.	Less intake of water, dehydration, renal ischaemia.
iv) Anuria	Almost complete suppression of urine (< 150 ml in 24 hours).	Renal stones, tumours, renal ischaemia

B. COLOUR:

Interpretation:

Normal: urine is clear, pale or straw-coloured due to pigment urochrome.

Colour	Inference
i) Colourless	diabetes mellitus, diabetes insipidus, excess intake of water.
ii) Deep amber	good muscular exercise, high grade fever.
iii) Orange	increased urobilinogen, concentrated urine
iv) Smoky urine	small amount of blood, administration of vitamin B12, aniline dye
v) Red	haematuria, haemoglobinuria
vi) Yellow-brown	bile and its derivatives
vii)Milky	pus, fat
viii) Green	putrefied sample, phenol poisoning

C. ODOUR:

Interpretation:

Normal: Faint aromatic odour.

Odour	Inference
i) Pungent	ammonia produced by bacterial contamination
ii) Putrid	UTI.
iii) Fruity	ketoacidosis
iv) Mousy	phenylketonuria

D. REACTION/pH:

Measured by pH indicator paper or by electronic pH meter.

Interpretation:

Normal urine: Slightly acidic and its pH ranges from 4.6-7.0 (average 6.0).

pH	Inference
Acidic urine	i. High protein intake, e.g. meats. ii. Ingestion of acidic fruits. iii. Respiratory and metabolic acidosis. iv. UTI by *E. coli.*
Alkaline urine	i. Citrus fruits. ii. Vegetables. iii. Respiratory and metabolic alkalosis. iv. UTI by *Proteus, Pseudomonas.*

E. SPECIFIC GRAVITY:

This is the ratio of weight of 1 ml volume of urine to that of weight of 1 ml of distilled water. It depends upon the concentration of various particles/solutes in the urine. Specific gravity is used to measure the concentrating and diluting power of the kidneys. Measured by urinometer, refractometer or reagent strips.

Assay by Urinometer:

Procedure:

1. Fill urinometer container 3/4th with urine.

2. Insert urinometer into it so that it floats in urine without touching the wall and bottom of container.
3. Read the graduation on the arm of urinometer at lower urinary meniscus.
4. Add or substract 0.001 from the final reading for each 3°C above or below the calibration temperature respectively marked on the urinometer.

Interpretation:

Normal specific gravity of urine: 1.003 to 1.030.

Specific Gravity	**Inference**
Low specific gravity	i. Excess water intake; ii. Diabetes insipidus
High specific gravity	i. Dehydration; ii. Albuminuria; iii. Glycosuria
Fixed specific gravity (1.010)	i. ADH deficiency; ii. Chronic nephritis

INFERENCE:

Record your observations in the following tabular format and write the inference from supplied sample.

Property	Results	Inference
Volume		
Colour		
Odour		
pH		
Specific gravity		

MICROSCOPIC ANALYSIS OF URINE:

Microscopic analysis of urine sample has great clinical importance. Important structures like different casts, erythrocytes, leukocytes, and bacteria need to be observed.

PROCEDURE:

i. Agitate the urine sample to avoid any sediment that may settled to bottom.
ii. Fill the centrifuge tube with urine and centrifuge for 3 min at low rate of speed.
iii. Pour all the urine and there is sufficient urine on the slide to drain to the bottom and suspend the sediment.
iv. Pour a drop of sediment on a glass slide, and cover with a cover glass that has been wiped clean of oil and lint.
v. Examine under microscope with the lower power objective.
vi. Finding should be reported as few, many or abundant.
vii. If necessary, stain with new methylene blue.
viii. Organized sediments, epithelial cells.

OBSERVATION & INFERENCE:

Observe the followings and draw on your observation note book:

1. Cells - *RBCs, WBCs, epithelial cells.*
2. Casts -*Hyaline Cast, Red Cell Cast, Leucocyte Cast, Granular Casts, Waxy Casts, Fatty Cast, Epithelial Cast, Pigment Cast.*
3. Crystals - *Calcium oxalate, Uric acid, Amorphous urate, Tyrosine, Cystine, Cholesterol crystals, Sulphonamide, Amorphous phosphate, Triple phosphate, Calcium carbonate, Ammonium biurate*
4. Miscellaneous structures- *Spermatozoa, Parasites, Fungus, Tumour cells.*

EXPERIMENT 2: Bio-chemical estimation of glucose in urine.

AIM: To study glucose present in supplied urine sample.

INTRODUCTION: Glucose is the most important of the sugars which may appear in urine. Normally, approximately 130 mg of glucose in urine is passed per 24 hours which is undetectable by qualitative tests. Above that level is called glucosuria that may be detected or estimated by qualitative or quantitative analysis.

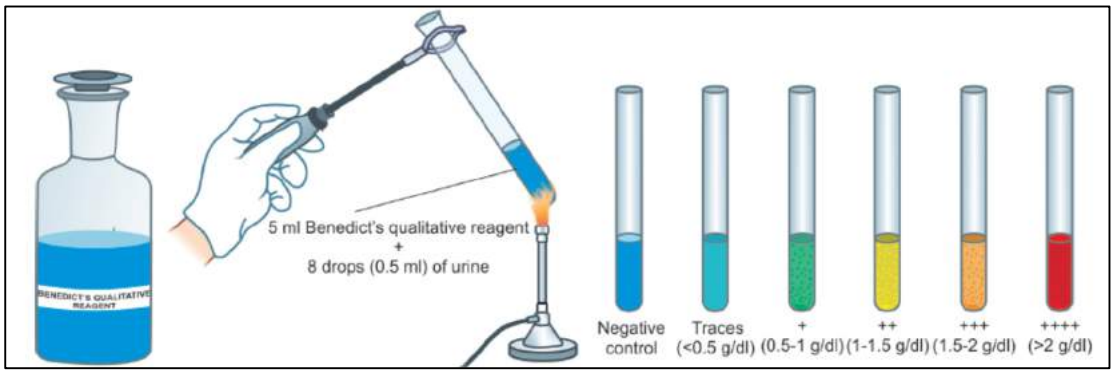
PRINCIPLE:

Cupric ion is reduced by glucose to cuprous oxide and a coloured precipitate is formed.

<u>QUALITATIVE TESTS</u>*: BENEDICT'S TEST:*

PROCEDURE: Benedict's test:

1. Take 5 ml of Benedict's qualitative reagent in a test tube.
2. Add 8 drops (or 0.5 ml) of urine.
3. Heat to boiling for 2 minutes (Fig. 42.5).
4. Cool in water bath or in running tap water and look for colour change and precipitation.



RESULTS & INTERPRETATION: Benedict's test:

No change of blue colour	= Negative
Greenish colour	= Traces (< 0.5 g/dl)
Green/cloudy green ppt	= + (0.5-1 g/dl)
Yellow ppt	= ++ (1-1.5 g/dl)
Orange ppt	= +++ (1.5-2 g/dl)
Brick red ppt	= ++++ (> 2 g/dl)

NOTE: Benedict's test is for reducing substances excreted in the urine, the test is positive for all reducing sugars (glucose, fructose, maltose, lactose but not for sucrose which is a nonreducing sugar) and other reducing substances (e.g. ascorbic acid, salicylates, antibiotics, L-dopa).

QUANTITATIVE TEST:

PROCEDURE:

1. Take 25 ml of quantitative Benedict's reagent in a conical flask.
2. Add to it 15 gm of sodium carbonate (crystalline) and some pieces of porcelain.
3. Heat it to boil.
4. Add urine to it from a burette slowly till there is disappearance of blue colour of Benedict's reagent. This is same as common titration procedure.
5. Record the volume of urine used from burette.
6. Note: 0.05 gm of glucose reduces 25 ml of Benedict's reagent

CALCULATION: The amount of glucose present in urine as under: (0.05 × 100)/Amount of urine.

RESULTS & INFERENCE:
Record your results, calculate and interpret it.

Considering the other physical factor, causes of glucosuria may be due to diabetes mellitus, renal glucosuria, severe burns, administration of corticosteroids, severe sepsis, pregnancy.

EXPERIMENT 3: Bio-chemical estimation of protein and ketone bodies in urine, bile salt, bile pigment, urobilinogen and blood in urine.

AIM: To learn the principle, perform the procedure and interpret the results of various routine tests for chemical
constituents of a urinary sample.

INTRODUCTION: Chemical constituents frequently tested in urine are: proteins, ketones, bile derivatives and blood. If urine is not clear, it should be filtered or centrifuged before testing.

TESTS FOR PROTEINURIA:

Urine may be tested for proteinuria by qualitative tests and quantitative methods.

Qualitative Tests:

1. Heat and Acetic Acid Test

PRINCIPLE: Heat causes coagulation of proteins.

PROCEDURE:

i. Take a 5 ml test tube.
ii. Fill 2/3rd with urine.
iii. Acidify by adding a few drops of 3% acetic acid if urine is alkaline.
iv. Boil upper portion for 2 minutes (lower part acts as control).
v. If precipitation or turbidity appears, add a few drops of 3% acetic acid.

INTERPRETATION:

Turbidity or precipitation disappears on addition of acetic acid, it is due to phosphates; if it persists after addition of acetic acid, then it is due to proteins.

The test is semi-quantitative and can be graded from traces 0 to ++++ depending upon amount of protein as under:

No cloudiness	= Negative
Faint cloudiness	= Traces (less than 0.1 g/dl).
Cloudiness without granularity	= +(0.1 g/dl).
Granular cloudiness	= ++(0.1-0.2 g/dl)
Precipitation and flocculation	= +++(0.2-0.4 g/dl).
Thick solid precipitation	= ++++ (> 0.5 g/dl).

2. Sulphosalicylic Acid Test

PROCEDURE:

i. Make urine acidic by adding 3% acetic acid.
ii. With 2 ml of urine add a few drops (4-5) of 20% sulphosalicylic acid.

INTERPRETATION:

Appearance of turbidity which persists after heating indicates presence of proteins.

3. Heller's Test

PROCEDURE:

1. Take 2 ml of concentrated nitric acid in a test tube.
2. Add urine drop by drop by the side of test tube.

INTERPRETATION:

Appearance of white ring at the junction indicates presence of protein.

Quantitative Estimation:

1. Esbach's albumin meter method:

i. Fill the albuminometer with urine up to mark U.
ii. Add Esbach's reagent (picric acid + citric acid) up to mark R.
iii. Stopper the tube, mix it and let it stand for 24 hours.

iv. Take the reading from the level of precipitation in the albuminometer tube and divide it by 10 to get the percentage of proteins.

2. Turbidimetric method:

i. Take 1 ml of urine and 1 ml standard in two separate tubes.
ii. Add 4 ml of trichloroacetic acid to each tube.
iii. After 5 minutes take the reading with red filter (680 nm).

RESULTS & INFERENCE:

Write your results based on the different procedure and interpret as stated:

i. Normal: A very scanty amount of protein in urine (< 150 mg/day).
ii. *Heavy proteinuria* (> 3 gm/day) occurs due to nephrotic syndrome, renal vein thrombosis, diabetes mellitus and SLE.
iii. *Moderate proteinuria* (1-3 gm/day) due to Chronic glomerulonephritis, nephrosclerosis, multiple myeloma, pyelonephritis.
iv. *Mild proteinuria* (< 1.0 gm/day) occurs due to hypertension, polycystic kidney, chronic pyelonephritis, UTI, fever.
v. *Microalbuminuria* is excretion of albumin 30-300 mg/day or random urine albumin/ creatinine ratio of 30-300 mg/gm creatinine and is indicative of early and possibly reversible glomerular damage from hypertension and risk factor for cardiovascular disease. *Microalbuminuria* is estimated by radioimmunoassay.

TESTS FOR KETONURIA:

AIM: To detect the ketone bodies present in urine.

INTRODUCTION:

Ketone present in urine because of incomplete fat metabolism. The three ketone bodies excreted in urine are: acetoacetic acid (20%), acetone (2%), and β-hydroxybutyric acid (78%).

TEST PROCEDURE:

1. Rothera's Test:

Principle: Ketone bodies (acetone and acetoacetic acid) combine with alkaline solution of sodium nitroprusside forming purple complex.

Procedure:

i. Take 5 ml of urine in a test tube.
ii. Saturate it with solid ammonium sulphate salt; it will start settling to the bottom of the tube when saturated.
iii. Add a few crystals of sodium nitroprusside and shake.
iv. Add liquor ammonia from the side of test tube.

Interpretation:
Appearance of purple or permanganate coloured ring at the junction indicates presence of ketone bodies.

2. Gerhardt's Test:
Procedure:

i. Take 5 ml of urine in a test tube.
ii. Add 10% ferric chloride solution drop by drop.
iii. Filter it and add more ferric chloride.

Interpretation: Brownish-red colour indicates presence of ketone bodies.

RESULTS & INFERENCE:
Write your results/observation and interpret as stated below:
Ketone bodies found in urine sample may be due to:

i. Diabetic ketoacidosis
ii. Dehydration
iii. Hyperemesis gravidarum
iv. Fever
v. Cachexia
vi. After general anaesthesia.

TEST FOR BILE DERIVATIVES IN URINE:

AIM: To detect the bile derivatives (salts, pigments and urobilinogen) present in urine.

INTRODUCTION: Three bile derivatives excreted in urine are: urobilinogen, bile salts and bile pigments. While urobilinogen is normally excreted in urine in small amounts, bile salts and bile pigments appear in urine in liver diseases only.

A. TESTS FOR BILE SALTS:
Bile salts excreted in urine are cholic acid and chenodeoxycholic acid.

METHODS:

Hay's Test:

Principle. Bile salts if present in urine lower the surface tension of the urine.
Procedure:

i. Take a 50 ml beaker or 15 ml test tube.
ii. Fill the beaker or test tube upto 2/3rd with urine.
iii. Sprinkle finely powdered sulphur powder over it.

Interpretation: If bile salts are present in the urine then Sulphur powder sinks, otherwise it floats.

Cause for Bile Salts in Urine: Obstructive jaundice.

B. TESTS FOR UROBILINOGEN:

A small amount of urobilinogen is excreted in urine (4 mg/24 hr) normally. The sample should always be collected in a dark coloured bottle as urobilinogen gets oxidized on exposure to light. Urobilinogen in urine are detected by Ehrlich's test.

Ehrlich's Test:
Principle: Urobilinogen in urine combines with Ehrlich's aldehyde reagent to give a red purple coloured compound.

Procedure:

i. Take 10 ml of urine in a test tube.
ii. Add 1 ml of Ehrlich's aldehyde reagent.
iii. Wait for 3-5 minutes.
iv. If the test is positive, the test is repeated by preparing multiple dilutions, e.g. 1:10, 1:20, 1:40 and so on.

Interpretation:

i. Development of red purple colour indicates presence of urobilinogen.
ii. A positive test is subsequently done in dilutions; normally it is positive in up to 1:20 dilution.
iii. Increased urobilinogen in urine indicated haemolytic jaundice and haemolytic anaemia.
iv. Causes for absent urobilinogen in urine: Obstructive jaundice.

C. TESTS FOR BILIRUBIN (BILE PIGMENT):

Bilirubin is breakdown product of haemoglobin. Normally, no bilirubin is passed in urine.

1. Fouchet's Test:
Principle: Ferric chloride oxidizes bilirubin to green biliverdin.

Procedure:

i. Take 10 ml of urine in a test tube.
ii. Add 3-5 ml of 10% barium chloride.
iii. Filter through filter paper.
iv. To the precipitate on filter paper, add a few drops of Fouchet's reagent (ferric chloride + trichloroacetic acid).

Interpretation: Development of green colour indicates bilirubin.

2. Foam Test

Procedure:

i. Take 5/10 ml of urine in a test tube.
ii. Shake it vigorously.

Interpretation: Presence of yellow foam at the top indicates presence of bilirubin.
Causes of bilirubinuria: Obstructive jaundice, Hepatocellular jaundice.

TESTS FOR BLOOD IN URINE:

Presence of blood can be detected in urine by two important chemical test: Benzidine test and Orthotoluidine test

1. Benzidine Test

Procedure:

i. Take 2 ml of urine in a test tube.
ii. Add 2 ml of saturated solution of benzidine with glacial acetic acid.
iii. Add 1 ml of H2O2 to it.

Interpretation: Appearance of blue colour indicates presence of blood.

2. Orthotoluidine Test

Procedure:

i. Take 2 ml of urine in a test tube.
ii. Add a solution of 1 ml of orthotoluidine in glacial acetic acid.
iii. Add a few drops of H2O2.

Interpretation: Blue or green colour indicates presence of blood in urine.
Note: Causes of blood in urine:
i. Renal stones
ii. Renal tumours
iii. Polycystic kidney
iv. Bleeding disorders
v. Trauma.

EXPERIMENT 4: Laboratory testing of Serus fluid, Gastric juice, and Synovial fluid.

AIM: To study the different pathological conditions of serus fluid.

INTRODUCTION:

The commonly examined body fluids in the laboratory are (1) Serous fluids such as (a) Pleural (around the lungs) (b) Pericardial (around the heart) (c) Peritoneal fluids (around the abdominal

and pelvic cavities) and (2) Synovial fluids (around the joints). These fluids are grouped as extravascular fluids as they exist outside the body vessels. These specimens are collected by the attending physician or the registered nurse. The fluids collected in the intercellular spaces (or body cavities) come from fluid of the blood (vascular spaces). There is normally an exchange of fluid between the vascular space and the extravascular space. The amount of fluid entering the tissue spaces and cavities is generally equal to the amount of fluid which leaves these spaces, so that there is no accumulation of fluid in these spaces. Most of the fluid which moves back and forth through the capillary membrane is water containing salts and low molecular weight organic substances such as glucose, urea, etc. The larger molecules largely stay inside the vascular lumen.

PLEURAL FLUID EXAMINATION:

SAMPLE COLLECTION:

Perform the thoracocentesis i.e. the process to collect the pleural fluid. It is collected in 3 aseptic test tube:

Tube 1: 15 mg fluoride oxalate (sodium fluoride: potassium oxalate = 1:3). Collect 5 ml sample for glucose and protein estimation.

Tube 2: 15 mg EDTA, collect 5 ml for microscopy

Tube 3: Without anti-coagulant; Collect 5 ml for Clot and bacteriological test.

EXPERIMENTS:

1. Perform the physical observation for color, turbidity, appearance- cloudy/bloody.
2. Perform the microscopic examination for WBC - RBC count, differential WBC count.
3. Perform different chemical Examination for protein, glucose.
4. Perform cytological examination for tumor cells.

RESULTS & INTERPRETATION:

Note your observation and interpret the results

PERICARDIAL FLUID EXAMINATION:

Under normal circumstances the pericardial sac contains 20-50 ml of clear, straw colored fluid. Collection of pericardial fluid known as pericardio-centesis, also called a pericardial tap.

EXPERIMENTS:

1 Check the appearance and color

2 Study the ability to clot

3 Determine the specific gravity

4 Perform the microscopic examination: WBC count, RBC count, Differential WBC count.

5 Perform the chemical examination: Protein determination, Glucose determination

6 Prepare smear for cytological examination and observe.

RESULTS & INTERPRETATION:

Note your observation and interpret the results.

SYNOVIAL FLUID EXAMINATION:

Synovial fluid is found around the joints such as knee, ankle, hip, elbow, wrist and shoulder. The laboratory examination of this fluid helps to assist to diagnosis of joint arthritis, gout or Infection of the Joint (septic arthritis).

SPECIMEN COLLECTION:

The specimen is collected in the following sterile tubes.

1 EDTA tube: For cell counts and microscopic examination.

2 Plain tube (without anticoagulant): Gross examination, mucin clot test, evaluation of viscosity.

and for microbiological and serological tests.

3 Fluoride-oxalate tube for glucose determination.

PROCEDURE:

Physical Examination:

A. Color and appearance: The various observations are as follows:

a) Normal synovial fluid is clear, straw colored and viscous. It does not clot.

b) Turbid appearance: Inflammatory and infected conditions. It may be due to presence of crystals, amyloid and cartilage fragments. Highly purulent fluid with increased leukocyte count indicates acute septic arthritis.

c) A gross red or dark brown supernatant (or the presence of black streaks).

B. Viscosity test

Synovial fluid is viscous and the viscosity is due to the presence of hyaluronic acid. The viscosity decreases due to the breakdown of hyaluronic acid by the enzyme hyaluronidase in inflammatory disorders.

Procedure: Drop the fluid from a syringe and note the length of the tenacious string formed. Use a scale to measure the length.

C. Mucin clot test

Hyaluronlc acid forms compact clot in the presence of acetic acid. Low concentrations of hyaluronic acid does not allow the formation of firm clot.

Procedure:

i. Take 20 ml of 5% acetic acid (v/v) in a beaker
ii. Add 1.0 ml synovial fluid
iii. Observe the formation of clot: firm clot, soft clot, friable clot, no clot formation.
iv. After agitation observe the following: clot does not break, clot break into small pieces.

Microscopic examination:

i. WBC count, RBC count, Differential WBC count.
ii. Crystals: Urate crystals (Gouty arthritis), Rhomboid calcium and pyrophosphate crystals. (Pseudo gout), Cholesterol crystals (Rheumatoid arthritis)
iii. Gram's staining, acid fast stain to identify bacteria.

Chemical examination:

Protein determination and Glucose determination

RESULTS & INTERPRETATION:

Note your observation and interpret the results on the following tables:

Synovial fluid examination observations

Test	Normal	Non Inflammatory	Inflammatory mild	Inflammatory severe	Infectious
Appearance	Clear yellow	Clear yellow	Hazy	Turbid	Turbid to purulent
Viscosity	Normal/ high	Normal	Decreased	Decreased	Decreased
Mucin clot	Good	Good	Fair	Fair to poor	Poor
WBC count	0-200	0-5000	0-10 ,000	500-50,000	500-200,000
Neutrophil (%)	0-25	0-25	0-50	0-90	40-100
Protein (g/dl)	1 -3	1- 3	>3	>4	>4 to 5
Glucose (mg/dl)	60-100	60-100	Average 60	Average 40	0-70

Synovial fluid analysis in diseases

Test	Rheumatic fever	Rheumatoid arthritis	Gouty arthritis	Pseudo gout	Tuberculous arthritis
Appearance	Slightly Turbid	Turbid, Yellow, milky	Turbid, Yellow, milky	Clear or slightly Turbid yellow	Turbid
Viscosity	Variable	Decreased	Decreased	Decreased	Decreased
Mucin clot	Good/Fair	Fair/poor	Fair/poor	Fair/poor	Poor
WBC count	50-50,000	200-80,000	100-100,000	50-75,000	2000-100,000
Neutrophils (%)	0-60	0-90	0-90	0-90	20-95
Glucose (mg/dl)	50-70	10-70	0-80	50- 70	0-70

GASTRIC JUICE ANALYSIS:

INTRODUCTION:

The main constituents of gastric secretions are HCl from parietal cells; pepsinogen from chief cells; Renin; Hemopoietic factor and mucus.

Gastric acid secretions have three pahse: 1. Cephalic phase: Caused by sight, smell, taste, or thought of food; 2. Gastric Phase: Caused by entry of food into stomach and increased pH caused by food; 3. Interstital phase: Hormones produced by small intestine. Gastric juice analysis involves quantification of gastric acid produced by the stomach. It is usually collected by inserting a nasogastric tube/Ryle's tube into the stomach and aspirating the contents for analysis. Chemical examination of gastric contents has limited but specific value in diagnosis & assessment of disorders of upper GIT, diagnosis of gastric ulcers, to exclude the diagnosis of pernicious anaemia & peptic ulcer, for presumptive diagnosis of Zollinger Ellison Syndrome, and to determine the completeness of Surgical Vagotomy. Normal fasting gastric juice per day is about 1.0 L. Stomach of a person taking a normal diet secretes 2.0 L-3.0 L of gastric juice per day. Collected gastric juice are analyzed for the presence of Free acid, Combined acid, Bile, blood, Lactic acid etc.

PRINCIPLE:

1. The strength of the acid is found out by titrating against 0.1 N NaOH. Titration carried out by two stage:
   i) Determination of strength of free acid by 0.1 N NaOH using Topfer's reagent as indicator.
   ii) Determination of combined acids using phenolphthalein as indicator.
2. Occult blood test by Guaiac reagent.
3. Determination of starch by addition of Iodine solution.
4. Lactic acid test by addition of few drops of 10% ferric chloride.

PROCEDURE:

1. DETERMINATION OF FREE ACID:
   i. Add 1.0 ml gastric sample in a glass test tube.
   ii. Add 5.0 ml of distilled water.
   iii. Add 2 drop of Topfer's reagent.
   iv. Perform the titration against 0.1 N NaOH till color changes from red to yellow.
   v. Note the titration reading in ml.
2. DETERMINATION OF COMBINED ACID:
   i. Same as free acid (i – iv steps).
   ii. Continue the titration using 2 drop of phenolphthalein till color changes from yellow to pink.
   iii. Note the reading.

Calculation:
Free acid (meq/L) = Titration reading in ml X 100
Total acid (meq/L) = Titration reading in ml X 100
Combined acid (meq/L) = Total acid – Free acid.

3. OCCULT BLOOD TEST BY GUAIAC REAGENT:
   i. In a test tube take a pinch of benzidine powder.
   ii. Add 3-4 drop of glacial acetic acid and 0.5 ml of H_2O_2 sequentially.
   iii. Transfer few drops of gastric juice specimen and few drops of freshly prepared reagent.
   iv. Observe the color change green to blue.

5. DETERMINATION OF STARCH:
   Take few drops of gastric juice specimen and few drop of Iodine solution (if starch present, blue color develops).
6. DETERMINATION OF LACTIC ACID:
   With 1.0 ml of gastric juice specimen add few drops of 10% ferric chloride. (If lactic acid present yellow color is produced).

RESULTS & INTERPRETATION:

Write your results and infer it.

EXPERIMENT 5: Collection and processing of CSF and its laboratory investigation.

AIM:

To learn the method of specimen collection of CSF and to know the various parameters of normal physical and chemical composition and microscopic findings.

INTRODUCTION:

Most of the CSF (70%) is produced by choroid plexus in the lateral, third and fourth ventricle while the remainder is produced by the surface of brain and spinal cord. CSF examination is an important part of neurologic evaluation in non-neoplastic and neoplastic diseases of CNS. It includes its physical characters, chemical composition, and microscopic findings, and in some instances microbiologic and immunologic tests.

Normal Composition of CSF:

Appearance	: Clear and colourless
Rate of production	: 500 ml/day
Total volume	: 120-150 ml in adults, 10-60 ml in neonates
Specific gravity	: 1.006-1.008
Normal pressure	: 60-150 mm of water in adults, 10-100 mm water in infants
Sugar	: 50-80 mg/dl (i.e. 60% of plasma value)
Proteins	: 15-45 mg/dl
Chloride	: 720-760 mg% (i.e. same as in plasma)
Cells	: 0-4 leucocytes/ml in Adult; 0-30 nos/ml in neonates
Bacteria	: Nil

SPECIMEN COLLECTION:

CSF is obtained by the following techniques:

i. Lumbar puncture: Insert the specialized lumber puncture needle (10 cm long, a bore of 1 – 1.5 mm) into the L3 – L4 or L4-L5 space.
ii. Cisternal puncture
iii. Ventricular cannulas or shunts
iv. Lateral cervical puncture

Collect 2 ml of CSF by any one of the technique. CSF tap is done by lumbar puncture for which indications can be divided into following 4 categories:

a. Meningeal infection.
b. Subarachnoid haemorrhage.
c. CNS malignant tumours.
d. Demyelinating diseases.

The specimen should be transported to the laboratory immediately and processed within one hour, otherwise cellular degradation occurs giving incorrect results. In case delay in examination of CSF is anticipated, the sample may be refrigerated, except for microbial culture.

MICROSCOPIC EXAMINATION:

Total Leucocyte Count (TLC): TLC in CSF done by in Neubauer's chamber.

Differential Leucocyte Count (DLC): Centrifuge a small amount of CSF then prepare smears from the sediment. Stain one of the smears with any of the Romanowsky stain and examine under high power and oil immersion of microscope for the presence of various cells. The various cells which may be seen in CSF are as under: neutrophils, lymphocytes, plasma cells, monocytes, and malignant cells.

RESULTS & INTERPRETATION:

Write your results and infer it.

Note:

Conditions causing increased neutrophils in CSF: Bacterial meningitis, Brain abscess, Brain infarct, Repeated lumbar puncture.

Conditions causing increased lymphocytes in CSF: Viral meningitis, Tuberculous meningitis, Parasitic meningitis, Fungal infections.

Conditions causing plasma cells in CSF: Tuberculous meningitis, Syphilitic meningoencephalitis, Multiple myeloma, Malignant brain tumours.

Conditions causing lymphocytes and monocytes in CSF: Viral meningitis, Degenerative brain disorders, Tuberculous meningitis, Fungal meningitis, Sarcoidosis of meninges.

Conditions causing malignant cells in CSF: Metastatic cancers, Leukaemias, Lymphomas, Medulloblastoma, Ependymoma

CHEMICAL EXAMINATION:

Sugar, proteins, chloride and enzymes, ammonia and amines, electrolytes and acid-base balance and tumour markers can be estimated in CSF.

RESULTS & INTERPRETATION:

Write your results and identify the meningitis as summarized in the table:

Feature	Normal	Acute pyogenic (bacterial meningitis)	Acute lymphocytic (viral meningitis)	Chronic (tuberculosis meningitis)
1. Naked eye appearance	Clear and colourless	Cloudy or frankly purulent	Clear or slightly turbid	Clear of slightly turbid, forms fibrin coagulum on standing
2. CSF pressure	60-150 mm water	Above 180 mm water	Above 250 mm water	Above 300 mm water
3. Cells	0-4 lympho-cytes /ml	1,000-100,000 neutrophils /ml	10-100 mono-nuclears /ml	100-1000 mono-nuclears /ml
4. Proteins	15-45 mg/dl	Raised	Raised	Raised
5. Glucose	50-80 mg/dl	Reduced (less than 40 mg/dl)	Normal	Reduced (less than 45 mg/dl)
6. Bacteriology	Sterile	Causative organisms present	Sterile	Tubercle bacilli present

MICROBIOLOGICAL EXAMINATION:

Perform the Gram's stain for bacteria and Ziehl-Neelsen's stain for AFB and India ink for the capsule of *Cryptococcus* from the smears from CSF.

RESULTS & INTERPRETATION: Write your results and identify the microorganism.

IMMUNOLOGICAL EXAMINATION:

Perform the following immunotechniques to identify different pathological condition:

i. Viral inclusions by immunostains
ii. PCR for viral DNA and tuberculosis
iii. ELISA for tuberculosis
iv. VDRL for syphilis.

RESULTS & INTERPRETATION:
Document your results and make inference on it.

EXPERIMENT 6: Routine test and microscopical test for stool and Occult blood test.

6A. Routine test and microscopical test for stool

AIM:
To analyze the stool (feces) by a series of tests done on it to help diagnose certain conditions affecting the digestive tract. These conditions can include infection (such as from parasites, viruses, or bacteria), poor nutrient absorption, or cancer and presence of hidden blood.

INTRODUCTION:
Feces analysis can be done by Physical Examination for color, volume, consistency, odour, mucus, pus, helminths; chemical examination for occult blood, fat, carbohydrate, protein etc.; and microscopic examination for cells, bacteria, protozoa, helminths etc.

SAMPLE:
Collect the stool in a dry, clean, leak proof container.
Make sure no urine, water, soil, toilet paper, soap gets in the container.
Morning Specimen is preferable.

PHYSICAL EXAMINATION:

PROCEDURE:
1. Note the color of the specimen.
2. Note the consistency of the specimen. Mushy or liquid stools suggest the possible presence of trophozoites or intestinal protozoa. Protozoan cystsare found most frequently in formed stools. Helminth eggs and larvae maybe found in either liquid or formed stools.
3. Examine the surface of the specimen for parasites (e.g., tape worm proglottids or, less commonly, adult pinworms).
4. Examine the stool for blood and/or mucous.
    a. Fresh blood (bright red) indicates acute lower intestinal tract bleeding.

b. Bloody mucus suggests ulceration, and some of this material should be examined microscopically for trophozoites.

c. Black, tarry stools are indicative of occult (hidden) blood from higher up in the intestinal tract.

5. Break up the stool with applicator sticks to check for the presence of adult helminths (e.g., *Ascaris*).

6. Feces should be sieved after drug treatment for tapeworm infections to assure recovery of the scolex.

MACROSCOPIC EXAMINATION: Protozoa and Helminths:

PROCEDURE:

1. Direct saline wet mount

i. Place a drop of saline on the slide.

ii. Pick up a small amount of fecal material on the end of an applicator stick. NOTE: Take small amounts of material from several different areas, especially from bloody and/or mucoid areas.

iii. Emulsify in the saline and cover with a coverslip. Examine on low and high power. NOTE: A smear should be thin enough so that a printed page can be read through it.

iv. The entire preparation must be examined for the presence of eggs, larvae and protozoa. Low power is used to scan for large helminth eggs or larvae. High power is used to detect and identify smaller parasites and larger helminth eggs and larvae.

v. Any parasites detected are reported out by their scientific name and quantity observed. If no parasites are observed report out as “No parasites seen.”

Density	Protozoa	Helminths
Rare	2 to 5 organisms per 22 mm square coverslip	2 to 5 organisms per 22mm square coverslip.
Few	1 organism per 5 to 10 high-power fields (40x)	1 egg/larva per 5 to 10 low-power fields (10x)
Moderate	1 to 2 organisms per high-power field, to as few as 1organism per 2 to 3 in high-power fields	1 to 2 eggs/larvae in low-power field
Many	Several organisms in every high-power field	Several eggs/larvae inevery low-power field

2. Iodine Wet mount

i. Place a drop of Lugol's iodine solution on a slide.

ii. Pick up a small amount of fecal material on an applicator stick using the same criteria in the saline procedure for selection of the proper areas.

iii. Emulsify in the iodine solution and cover with a coverslip.

iv. Examine on low and high power as described in the previous procedure.

RESULTS & INTERPRETATION:

Note your observation and interpret the results.

6B. Detection of Occult Blood:

AIM: to detect the Occult blood (hidden) in supplied sample.

INTRODUCTION:

As blood passes through the intestinal tract it changes color and consistency due to the digestive processes. Stools with large amounts of occult blood are black and have a tarry consistency. Smaller amounts of blood may not be visibly detectable. Occult blood may indicate lesions in the intestinal tract due to parasitic infections, cancerous tumors, bleeding ulcers or other disease processes.

PRINCIPLE:

The occult blood test is primarily done to screen for colorectal cancer. It is an easy inexpensive procedure and the specimen is easily obtained by the patient. The chemical reaction involved is as follows:

Hemoglobin + Developer

$Hb + 2\ H_2O_2 = 2H_2O + O_2$

Oxidation of Guaiac

O_2 + Guaiac = Oxidized Guaiac

(Colorless) (Blue)

The presence of a blue color upon completion of the test is a positive test. A positive and negative control must be run concurrently with the patient sample. Most manufacturers now include this as part of the patient test card.

DIET recommendations/restrictions:

It is recommended that the patient be placed on a high residue diet starting 2 days before and continuing through the test period.

DIET MAY INCLUDE:

1. Meats: Only small amounts of chicken, turkey and tuna.
2. Vegetables: Generous amounts of both raw and cooked vegetables including lettuce, corn, spinach, carrots and celery. Avoid those with high peroxidase activity.
3. Fruits: Plenty of fruits, especially prunes and apples.
4. Cereals: Bran and bran-containing cereals.
5. Moderate amounts of peanuts and popcorn daily.

DIET TO BE AVOIDED:

1. Meat: Diet should not include any red or rare meat.
2. Fruits and vegetables containing high peroxidase activity:

Turnip Cauliflower, Broccoli Cantaloupe, Horseradish Parsnip

Alternately, the special diet may be omitted initially with dietary restrictions imposed upon the re-testing of all positive results.

Other factors which affect the test:

1. Medications: Do not ingest aspirin, tonics or vitamin preparations which contain Vitamin C (ascorbic acid) in excess of 250 mg per day.
2. Bleeding hemorrhoids
3. Collection of specimen during menstrual cycle.
4. Improper specimen collection
5. Other diseases of the gastrointestinal tract such as colitis, gastritis, diverticulitis and bleeding ulcers.

PROCEDURE:

1. Take a small test tube.
2. Pour a pinch of benzidine powder on it.
3. Add 2 -3 drops of glacial acetic acid and mix well.
4. Add 1.0 ml of H_2O_2 and mix well.
5. Take a small quantity of stool specimen on a clean glass slide.
6. One or two drop of benzidine-glacial acetic acid – H_2O_2 mixture on the stool specimen on the glass slide.
7. Observe the color change.

RESULT & INTERPRETATION:

Observe the color change and draw your inference as below:

i) Occult blood absent: No color change.

ii) Occult blood present: Color changes to green to blue.

Clinical Biochemistry: Paper – XI, Unit – 22

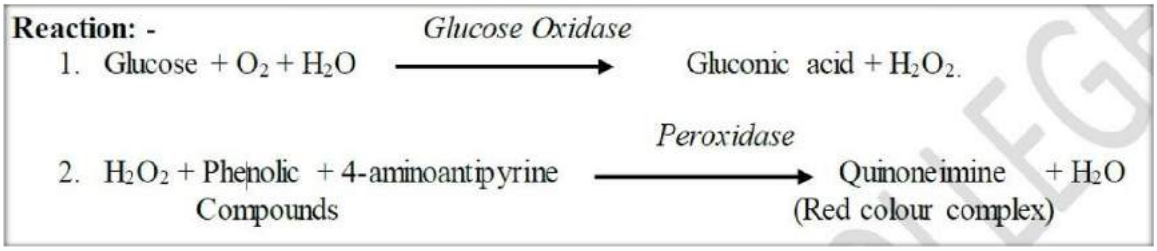
ESTIMATION OF PLASMA GLUCOSE BY GOD -POD METHOD

Principal:

Glucose oxidase (GOD) oxidizes the specific substrate β-D- glucose to gluconic acid and hydrogen peroxide (H2O2) is liberated. Peroxidase (POD) enzyme acts on hydrogen peroxide to liberate nascent oxygen (O2), then nascent oxygen couples with 4- amino antipyrine and phenol to form red quinoneimine dye.

The intensity of the colour is directly proportional to the concentration of glucose present in plasma. The intensity of colour is measured by colorimeter at 530 nm or green filter and compared with that of a standard treated similarly. Final colour is stable for at least 2 hours if not exposed to direct sunlight.

Reaction:



Reagents:

1. Glucose colour reagent; it contains GOD, POD, 4- amino antipyrine, phenol & phosphate buffer (pH 7.5)

2. Glucose standard solution, Concentration = 100 mg/dl. 100 mg of anhydrous glucose is dissolved in 100 ml of distilled water

Procedure:

Pipette into clean, dry test tube labelled as Blank (B), Standard (S) and Test (T).

Then add the solution in each of test tubes separately as shown in table below

	BLANK	STANDARD	TEST
Glucose colour Reagent	**1000 µl**	**1000 µl**	**1000 µl**
Distilled Water	**10 µl**	-----	-----
Standard	-----	**10 µl**	-----
Plasma	-----		**10 µl**
Mix thoroughly and keep the tubes at 37˚C for 15 minutes.			
OD at 530 nm	0.02	0.45	0.58

Data

Plasma Glucose standard concentration is 100 gm/dl

CALCULATION

Concentration of Glucose =

$$\frac{O.D.of\ Test - O.D.of\ Blank}{O.D.\ of\ Standard - O.D.of\ Blank} \times Concentration\ of\ Standard$$

Result

Plasma glucose concentration in given unknown blood sample = mg/dl.

Normal Range:

	Fasting	After eating	2-3 hrs after eating
Normal	80 - 100	170 - 200	120 - 140
Pre Diabetic	101 - 125	190 - 230	140 - 160
Diabetic	126+	220 - 300	200+

Interpretation:

- Hyperglycemia:
- It is found in following conditions
- **I. Physiological:**
- 1. Alimentary : After high carbohydrate diet
- 2. Emotional: Stress, anger, anxiety etc.
- **II. Pathological:**
- 1. Diabetes mellitus
- 2. Hyperadrenalism
- 3. Hyperpituitarism
- Hypoglycemia:
- It is found in following conditions:
- **I. Physiological:**
  - During starvation
  - After Severe Exercise
- **II. Pathological:**
  - Prolonged fasting
  - Due to excess of insulin e.g.
- Excessive dose of insulin
- No food intake after insulin administration
- Tumours of pancreas (insulinoma)
  - Glycogen storage disease
  - Hypoactivity of adrenal and pituitary gland

Glucose tolerance test

GTT short for Glucose Tolerance Test is a test designed to assess the body response to glucose. In GTT, the patient is given a glucose solution and blood samples are drawn afterword at intervals to measure how well the body cells are able to absorb glucose. There are several variations to the glucose tolerance test used in different conditions but, the most common one of them is the Oral glucose tolerance test or OGTT.

The OGTT is mainly used in the diagnosis of gestational diabetes. For OGTT the patient is required to fast for 8 hours and then a fasting plasma glucose is tested, after that oral glucose solution is given. After that blood samples can be drawn up to 4 times at different intervals to measure the blood glucose. A OGTT is usually performed in the morning as glucose levels usually fall by afternoon.

OGTT used to be the gold standard in the diagnosis of diabetes type 2 but, is now being replaced with other GTT methodology. The GTT is primarily used for the diagnosis of diabetes, insulin resistance, impaired beta cell function, carbohydrate metabolism disorder and also reactive hypoglycaemia and acromegaly.

The GTT is usually given to pregnant women during 24th and 28th week of pregnancy. This test is also given to pregnant who has diabetes symptoms or have the risk of developing diabetes prior to the pregnancy.

Besides that the GTT is also given to other patients who are experiencing symptoms of varied diseases that can cause high glucose levels in the blood stream or restrict the proper absorption of glucose by the body cells.

Principle:

A glucose tolerance test is the administration of glucose in a controlled and defined environment to determine how quickly it is cleared from the blood. The test is usually used to test for diabetes, insulin resistance, and sometimes reactive hypoglycemia. The glucose is most often given orally.

Preparation for GTT

TT is an elaborate blood test, that requires frequent testing and as the special requirements need for GTT are as follows;

- Have a normal diet like any other day.
- Inform the doctor about the varied prescription drugs you are taking, as certain drugs like corticosteroids, diuretics and anti- depressants can cause false results.
- Fasting is required for 8 to 10 hours prior to the test and only water is allowed during this period.
- You might want to avoid using the washroom prior to testing as urine samples might be needed
- On the morning of the test do not smoke or have coffee or caffeine based product.
- The GTT is not to be done on a sick person

GTT procedure

The GTT procedure is as follows;

- At first a zero -time or baseline blood sample is drawn.
- Then the patient is given a specific dose of glucose solution to drink
- After that the blood samples are drawn at regular intervals to measure the blood sugar levels and also insulin levels in certain cases. The blood sampling can be done as requested by the doctor and could involve up to 6 hours of testing.

GTT normal values:

The GTT normal value is lower than 140 mg/dL and if the blood glucose level is between 140 and 199 mg/dL then it is a strong indication of prediabetes.

The OGTT normal range for fasting results is between 100 – 125 mg/dL for prediabetes, 126 mg/dL or greater for diabetes and greater than 92 mg/dL for gestational diabetes.

The OGTT normal range for after 2 hour test results is between 140 – 199 mg/dL for pre diabetes, 200 mg/dL or greater for diabetes and greater than 153 mg/dL for gestational diabetes.

GTT result interpretation

For Gestational diabetes no further test is required and proper medication and treatment can start. In case of diabetes, further testing is advised to confirm the diagnosis. In case of pre diabetes doctors start the treatment with medication and dietary changes along with lifestyle changes.

Plasma and Serum Preparation

How to separate serum and plasma from blood?

Serum is the liquid fraction of whole blood that is collected after the blood is allowed to clot. The clot is removed by centrifugation and the resulting supernatant, designated serum, is carefully removed using a Pasteur pipette. Plasma is produced when whole blood is collected in tubes that are treated with an anticoagulant. The blood does not clot in the plasma tube. The cells are removed by centrifugation. The supernatant, designated plasma is carefully removed from the cell pellet using a Pasteur pipette.

Serum preparation

Collect whole blood in a covered test tube. If commercially available tubes are to be used, the researcher should use the red topped tubes. These are available from Becton Dickinson (BD). BD's trade name for the blood handling tubes is Vacutainer. After collection of the whole blood, allow the blood to clot by leaving it undisturbed at room temperature. This usually takes 15–30 minutes. Remove the clot by centrifuging at 1,000–2,000 x g for 10 minutes in a refrigerated centrifuge.
The resulting supernatant is designated serum. Following centrifugation, it is important to immediately transfer the liquid component (serum) into a clean polypropylene tube using a Pasteur pipette. The samples should be maintained at 2–8°C while handling. If the serum is not analyzed immediately, the serum should be apportioned into 0.5 ml aliquots, stored, and transported at –20°C or lower. It is important to avoid freeze-thaw cycles because this is detrimental to many serum components. Samples which are hemolyzed, icteric or lipemic can invalidate certain tests.

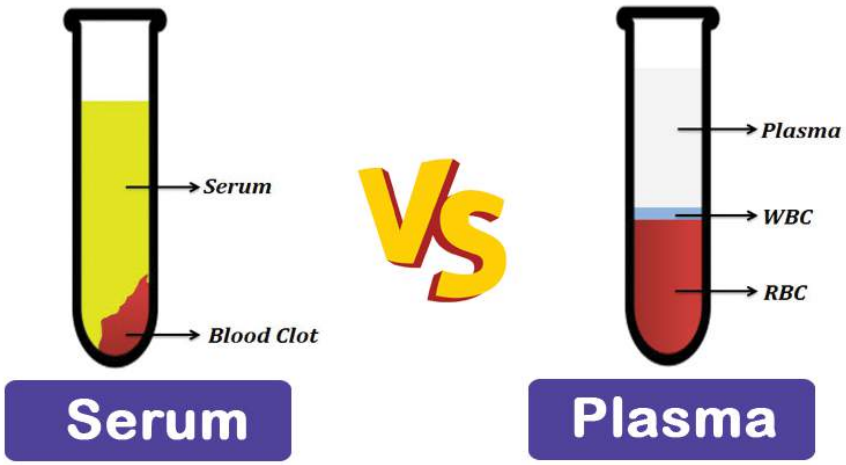
Plasma preparation

Collect whole blood into commercially available anticoagulant-treated tubes e.g., EDTA-treated (lavender tops) or citrate-treated (light blue tops). Heparinized tubes (green tops) are indicated for some applications; however, heparin can often be contaminated with endotoxin, which can stimulate white blood cells to release cytokines. Cells are removed from plasma by centrifugation for 10 minutes at 1,000–2,000 x g using a refrigerated centrifuge. Centrifugation for 15 minutes at 2,000 x g depletes platelets in the plasma sample.

The resulting supernatant is designated plasma. Following centrifugation, it is important to immediately transfer the liquid component (plasma) into a clean polypropylene tube using a Pasteur pipette. The samples should be maintained at 2–8°C while handling. If the plasma is not analyzed immediately, the plasma should be apportioned into 0.5 ml aliquots, stored, and transported at –20°C or lower. It is important to avoid freeze-thaw cycles. Samples which are hemolyzed, icteric, or lipemic can invalidate certain tests. There are other commercially available tubes for blood sample collection. Invitrogen has not evaluated some of these tubes for compatibility with our ELISA kits.

Serum and plasma tubes

The commercially available serum tubes are as follows:	
Red	No anticoagulant.
Red with black	Treated with gel to help to separate the clot (not evaluated).
The commercially available plasma tubes are as follows:	
Lavender	Treated with EDTA.
Blue	Treated with citrate.
Green	Treated with heparin.
Grey	Treated with potassium oxalate/sodium fluoride (not evaluated).
Yellow	Treated with potassium oxalate/sodium fluoride (not evaluated).



Preparation of protein free filtrates of Blood

Importance:

Blood (Plasma) or serum specimens containing high concentration of lipids, bilirubin or hemoglobin are not suitable for quantitative mono-step photometric assays. Proteins are precipitated by the action of tungstic acid. After filtration, clear filtrate can be used for the quantitative determinations such as glucose, urea, creatinine etc.

Principle:

The reagents , 2/3N sulfuric acid and 10g/dl sodium tungstate are used for the deproteinization of blood. Tungstic acid and sodium sulphate formed in the chemical reaction , are responsible for the precipitation of proteins in the specimen. Clear filtrate can be obtained by filtration of the reaction mixture. The chemical reactions involved in the deproteinization of blood are as follows.

$H_2SO_4 + Na_2WO_4 \rightarrow H_2WO_4 + Na_2SO_4$

Proteins + Tungstic acid → Precipitate + Filtrate

Whatman No. 44 Filter paper

Clear filtrate

Requirements

1. 1.0 ml volumetric pipette
2. 25 ml conical flask
3. 2.0 serological pipettes
4. Test Tubes (15x125mm)
5. Whatman Filter paper 44
6. Funnel
7. 2/3N Sulfuric acid
8. 10g/dl Sodium tungstate

9. Distilled water
10. Specimen Blood

Procedure:

In a 25 ml conical flask pipette out the following:

1. Distilled water: 7ml
2. Blood: 1ml
3. 2/3N sulfuric acid: 1ml
4. 10 g/dl Sodium Tungstate: 1.0 ml

Mix thoroughly. Keep at room temperature for 5 mins. Filter, using a whatman No. 44 filter paper, observe the filtrate. It should be clear.

Diagnosis of Hepatitis A, B, C, E

Blood tests are used to look for signs that a viral infection is present and to evaluate liver function. Your doctor draws a small amount of blood from a vein in your arm and sends it to a laboratory for testing.

The results of a blood test can confirm the type of viral hepatitis, the severity of the infection, whether an infection is active or dormant, and whether a person is currently contagious. A blood test can also confirm whether a virus is acute, meaning short term, or chronic, meaning long term.

Doctors may recommend different blood tests throughout the diagnostic process. For example, many blood tests look for antibodies, which are substances that the body produces in response to viruses or bacteria. If the results of a blood test indicate the presence of hepatitis antibodies in the bloodstream, the same blood sample is tested to evaluate whether the virus is still present—and if so how much is present—in the bloodstream. Your doctor may also recommend a blood test to determine how significantly the virus has affected the liver.

The results of blood tests are available in one or two days, and your doctor can discuss the results with you to explain how they affect your health and whether they indicate the need for further testing or treatment.

Imaging Tests

If the results of blood tests confirm a diagnosis of hepatitis A, B, or C, your doctor may recommend one or more imaging tests of the liver to assess liver damage.

Ultrasound

A doctor may recommend an ultrasound to see whether the liver is inflamed. An ultrasound image may also reveal large areas of scar tissue within the liver, which may suggest cirrhosis.

Ultrasound uses high-frequency sound waves to create images of structures inside the body. A specialist places a handheld probe called a transducer against your abdomen, and the transducer sends an image of the liver to a computer monitor, where it can be analyzed. This is a painless test often performed in a doctor’s office.

Liver Biopsy

Rarely, the results of imaging studies are not detailed enough to show the extent of liver damage, and a doctor may recommend a liver biopsy. A biopsy can determine the extent of scarring, or fibrosis, in a liver affected by viral hepatitis. Information provided by a biopsy can be used to guide treatment.

The doctor injects a local anesthetic into the skin to numb it, and then inserts a needle through the skin and into the liver, removing a tiny sample of liver tissue. Often, doctors use imaging, such as ultrasound or CT scanning, to guide the position of the needle. This tissue sample is sent to a laboratory for testing, and results are usually available in one week.

The viruses are spread in different ways and cause different symptoms:

Hepatitis A is most often spread by contact with contaminated feces (stool) or by eating tainted food. Though uncommon, it can also be spread through sexual contact with an infected person. Most people recover from hepatitis A without any lasting liver damage.

Hepatitis B is spread through contact with infected blood, semen, or other bodily fluids. Some people recover quickly from a hepatitis B infection. For others, the virus can cause long-term, chronic liver disease.

Hepatitis C is most often spread by contact with infected blood, usually through sharing of hypodermic needles. Though uncommon, it can also be spread through sexual contact with an infected person. Many people with hepatitis C develop chronic liver disease and cirrhosis.

Surface antigen (A,B,C,E) of Hepetitis is detected by ELISA for blood test.

Hepatitis E virus spreads through poop. You can catch it if you drink or eat something that has been in contact with the stool of someone who has the virus. Hepatitis E is more common in parts of the world with poor handwashing habits and lack of clean water. It happens less often in the U.S., where water and sewage plants kill the virus before it gets into the drinking supply.

You also can get hepatitis E if you eat undercooked meat from infected animals, such as pigs or deer. Less often, you can get the virus from raw shellfish that comes from tainted water.

Hepatitis A

Principle

IgM Rapid Test is a lateral flow immunoassay for the qualitative detection of IgM antibodies to hepatitis A virus (HAV) in human serum, plasma or whole blood. It is intended as a screening test by professionals and as an aid in the diagnosis of infection with HAV.

Hepatitis B

Principle

HBsAb Rapid Test is a qualitative, lateral flow immunoassay for the detection of HBsAb in serum or plasma. The membrane is pre-coated with HBsAg on the test line region of the strip. During testing, the serum or plasma specimen reacts with the particle coated with HBsAg.

Hepatitis C

Principle

HCV Rapid Test is a qualitative, membrane based immunoassay for the detection of antibodies to HCV in samples. The membrane is pre-coated with recombinant HCV antigen on the test line region of the cassette. During testing, the specimen reacts with recombinant HCV antigen conjugate colloid gold.

Hepatitis E

Principle

HEV IgM Rapid Test is a lateral flow chromatographic immunoassay for the qualitative detection of anti-hepatitis E virus (HEV) IgM in human serum or plasma. It is intended to be used as a screening test by professionals and provides a preliminary test result to aid in the diagnosis of infection with HEV.

Preparation for the Test

There are no special preparations necessary for this test. You should inform your doctor if you are taking any blood thinning medications. Your doctor may advise you to stop taking certain medications.

Normal Results

If your results are normal, you don't have hepatitis and have never been infected with hepatitis or been vaccinated for it.

Abnormal Results

If your blood sample tested positive for antibodies, it may mean a few things:

You have a hepatitis infection. It may be a recent infection or you may have had it for a long time. You have had a hepatitis infection in the past, but you do not have it now. You are not contagious. You have been vaccinated for hepatitis.

Hepatitis A (HAV) Test Results

IgM HAV antibodies mean that you have recently been infected with HAV. IgM and IgG HAV antibodies mean that you have had HAV in the past or been vaccinated for HAV. If both tests are positive, you have an active infection.

Hepatitis B (HBV) Test Results

HBV surface antigen means you are currently infected with HBV. This may be a new or chronic infection. Antibody to HBV core antigen means you have been infected with HBV. This is the first antibody to appear after infection. Antibody to HBV surface antigen (HBsAg) means you have been vaccinated for or infected with hepatitis B. HBV type e antigen means you have HBV and are currently contagious.

Hepatitis C (HCV) Test Results

Anti-HCV test means you have been infected with HCV or are currently infected. HCV viral load means there is detectable HCV in your blood and you are contagious.

Hepatitis E (HEV) Test Result

Positive results confirm the presence of acute or recent (in the preceding 6 months) hepatitis E infection.

Negative results indicate absence of acute or recent hepatitis E infection.

Indeterminate results may be seen in: 1) acute hepatitis E infection with rising level of anti-hepatitis E virus (HEV) IgM; 2) recent hepatitis E infection with declining level of anti-HEV IgM; 3) acute hepatitis E infection due to HEV genotype 2 strains; or 4) cross-reactivity with nonspecific antibodies (ie, false-positive results). Repeat testing of serum for anti-HEV IgM and anti-HEV IgG in 4 to 6 weeks is recommended to determine the definitive HEV infection status.

CHOLESTEROL KIT

(CHOD / PAP method)

For the determination of Cholesterol in serum or plasma.
(For Invitro Diagnostic Use Only)

Summary

Cholesterol is the main lipid found in blood, bile and brain tissues. It is the main lipid associated with arteriosclerotic vascular diseases. It is required for the formation of steroids and cellular membranes. The liver metabolizes the cholesterol and it is transported in the blood stream by lipoproteins. Increased levels are found in hypercholesterolaemia, hyperlipidaemia, hypothyroidism, uncontrolled diabetes, nephrotic syndrome, and cirrhosis. Decreased levels are found in malabsorption, malnutrition, hyperthyroidism, anemias and liver diseases.

Principle

Cholesterol esterase hydrolyses esterified cholesterols to free cholesterol. The free cholesterol is oxidised to form hydrogen peroxide which further reacts with phenol and 4-aminoantipyrine by the catalytic action of peroxidase to fom a red coloured quinoneimine dye complex. Intensity of the colour formed is directly proportional to the amount of cholesterol present in the sample.

$$\text{Cholesterol esters} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{Fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholestenone} + H_2O_2$$

$$H_2O_2 + \text{4Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Red Quinoneimine dye} + H_2O$$

Nomal reference values

Serum/Plasma	(Suspicious)	220 mg/dl and above
	(Elevated)	260 mg/dl and above

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	75ml	2 x 75ml	2 x 150 ml	2 x 250 ml
L1: Enzyme Reagent 1	60 ml	2X60 ml	2x120 ml	2x200 ml
L2: Enzyme Reagent2	15ml	2x15ml	2x30ml	2x50ml
S :Cholesterol Standard (200mg/d)	5ml	Sml	Sml	5ml

Storage / stability

Contents are stable at 2-8'C till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use.

Working reagent : Pour the contents of 1 bottle of L2 (Enzyme Reagent 2) into 1 bottle of L1 (Enzyme Reagent 1). This working reagent is stable for at least 8 weeks when stored at 2-8° C. Upon storage the working reagent may develop a slight pink colour however this does not affect the performance of the reagent.

Alternatively for flexibility as much of working reagent may be made as and when desired by mixing together 4 parts of L1 (Enzyme Reagent 1) and 1 part of L2(Enzyme Reagent 2). Alternatively 0.8 ml of L1 and 0.2 ml of L2 may also be used instead of 1 ml of the working reagent directly during the assay.

Sample material

Serum, EDTAplasma. Cholesterol is reported tobestablein the sample for 7 days when stored at 2-8'C. The sample should preferablybeof12 to14 hoursfasting.

Procedure

Wavelength/filter	505 nm(Hg 546 nm)/Green
Temperature	37'C/R.T.
Lightpath	1cm

Pipette into clean dry test tubes labelled as Blank (B), Standard (S), and Test(T):

Addition Sequence	B (ml)	S (ml)	T (ml)
Workingreagent	1.0	1.0	1.0
Distilled water	0.01		
Cholesterol Standard(S)		0.01	
Sample			0.01

Mix well and incubate at37°Cfor5min. or at R.T. (25'C) for 15 min. Measure the absorbance of the Standard (Abs.S), and Test Sample(Abs.T) against the Blank, within 60 Min.

Calculations

$$\text{Cholesterol in mg/dl} = \frac{\text{Abs.T}}{\text{Abs.S}} \times 200$$

Linearity

This procedure is linear upto 750 mg/dl. If the value exceeds this limit, dilute the serum with normal saline (NaCl 0.9%) and repeat the assay. Calculate the value using the proper dilution factor.

Note

Anticoagulants such as flourides and oxalates result in false low values. The test is not influenced by Hb values upto 20 mg/dl and bilirubin upto 10 mg/dl.

References

Trinder, P., (1969)Ann. Clin. Biochem. 6:24
Allain, C.C., etal,(1974) Clin. Chem.20 :470
Flegg, H.M., (1972)Ann. Clin.Biochem. 10: 79

CREATININE KIT

(Alkaline Picrate method)

For the determination of Creatinine in serum and urine.
(For Invitro Diagnostic Use Only)

Summary

Creatinine is the catabolic product of creatinine phosphate which is used by the skeletal muscle. The daily production depends on muscular mass and it is excreted out of the body entirely by the kidneys. Elevated levels are found in renal dysfunction, reduced renal blood flow (shock, dehydration, congestive heart failure) diabetes acromegaly. Decreased levels are found in muscular dystrophy.

Principle

Picric acid in an alkaline medium reacts with creatinine to form a orange coloured complex with the alkaline picrate. Intensity of the colour formed is directly proportional to the amount of creatinine present in the sample.

Creatinine + Alkaline Picrate - Orange Coloured Complex

Reference values

	Serum	Urine, 24hrs. collection
Males	0.6-1 .2mg%	1.1- 3 .0gm
Females	0.5-1 .1mg%	1.0- 1 .8gm

It is recommended that each laboratory establish its own normal range representing its patient population

Contents	15 Tests	35 Tests	70 Tests
L1 : Picric Acid Reagent	60 ml	140 ml	2 x 140ml
L2 : Buffer Reagent	5ml	12 ml	25 ml
S : Creatinine Standard (2 mg/di)	5ml	5ml	10ml

Storage / stability

All reagents are stable at R.T. till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use. Do not pipette with mouth.

Sample material

Serum or Urine.
Creatinine is stable in serum for 1 day at 2-8°C
Urine of 24 hours collection is preferred. Dilute the specimen 1:50 with distilled/ deionised water before the assay.

Procedure

Wavelength/ filter	520nm (Hg546 nm)/ Green
Temperature	R.T.
Light path	1cm

Deproteinization of specimen:

Pipette into a clean dry test tube

Picric acid reagent (L1)	2.0ml
Sample	0.2ml

Mix well and centrifuge at 2500 - 3000 rpm for 10 min. to obtain a clear supernatant

Colour development :

Pipette into clean dry test tubes labelled as Blank (B), Standard (S), and Test (T):

Addition Sequence	B (ml)	S (ml)	T (ml)
Supernatant			1.1
Picric Acid Reagent (L1)	1.0	1.0	
Distilled water	0.1		
Creatinine Standard (S)		0.1	
Buffer Reagent (L2)	0.1	0.1	0.1

Mix well and keep the test tubes at R.T. for exactly 20 minutes. Measure the absorbance of the Standard (Abs.S), and Test Sample (Abs.T) against the Blank.

Calculations

$$\text{Creatinine in mg\%} = \frac{\text{Abs.T}}{\text{Abs.S}} \times 2.0$$

$$\text{Urine Creatinine in gm/Lit.} = \frac{\text{Abs.T}}{\text{Abs.S}} \times 1.0$$

Urine Creatinine gm/24Hrs. = Urine Creatinine in gm/L x *Vol.* of urine in 24 Hrs.

Linearity

The procedure is linear upto 8 mg/di of creatinine.
If values exceed this limit, dilute the sample with distilled water and repeat the assay. Calculate the value using the proper dilution factor.

Note

Maintain the reaction time of 20 min. as closely as possible since a longer incubation causes an increase in the values due to the reaction of pseudochromogens.
The determination is not specific and may be affected by the presence of large quantities of reducing substances in the sample.
The reaction is temperature sensitive and all the tubes should be maintained at a uniform temperatur.e

Ketones

- Chemically, ketones are the organic compounds containing carbon, oxygen various carbon-containing substituents.
- Ketones consist of a carbonyl (C=O) functional group.
- In our body, ketones are the metabolic end products of fatty acid metabolism.

Ketone Bodies

- Ketone bodies are the water-soluble particles formed in the liver when the acetyl-CoA formed in the liver during the oxidation of fatty acids undergoes ketogenesis instead of entering the citric acid cycle when carbohydrate is not available or poorly utilized.
- Ketone bodies are produced in the liver and are used as an energy source when glucose is not readily available.
- The two main ketone bodies are acetoacetate (AcAc) and 3-beta-hydroxybutyrate (3HB), while acetone is the third, and least abundant, ketone body.
- AcAc accumulates during fatty acid metabolism under low carbohydrate conditions.
- 3-β-hydroxybutyrate is formed from the reduction of AcAc in the mitochondria.
- Acetone is generated by spontaneous decarboxylation of AcAc and is responsible for the sweet odor on the breath of individuals with ketoacidosis.
- During periods of glucose deficiency, ketone bodies play a crucial role in sparing glucose utilization and reducing proteolysis.

Ketonuria

- The medical condition associated with the excretion of more than usual quantities of the ketone bodies in the urine is called ketonuria.
- This usually occurs as a result of the utilization of ketone bodies as a source of energy.
- Under the reasonable condition, the body utilizes carbohydrates as a source of energy, and the ketones formed in the liver are almost entirely metabolized. Thus, the only negligible amount of ketones are observed in the urine.
- However, when the carbohydrates are unavailable, fat functions as the predominant fuel and results in the formation of a large number of ketones as a by-product.
- It is seen during starvation or more commonly in type 1 diabetes mellitus.
- Overproduction of ketone bodies in uncontrolled diabetes or severely reduced calorie intake can lead to acidosis or ketosis (severe condition).

Methods for detection of ketone bodies in urine are Rothera's test, Acetest tablet method, and reagent strip test.

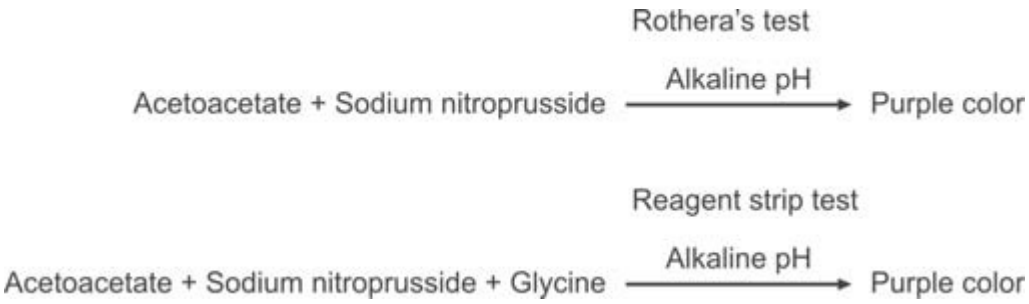
Principle:

- The ketone in the urine test detects acetoacetic acid and acetone in the urine.
- The principle of the ketone in the urine test is that the keto-group of acetone and acetoacetic acid reacts with alkaline nitroprusside to form a purple-colored complex.

- Based on the intensity of the color, the quantitative or qualitative analysis of ketone in urine can be done.

ROTHERA'S' TEST (Classic Nitroprusside Reaction)

Acetoacetic acid or acetone reacts with nitroprusside in an alkaline solution to form a purple-colored complex (Figure 822.1). Rothera's test is sensitive to 1-5 mg/dl of acetoacetate and 10-25 mg/dl of acetone.



Method

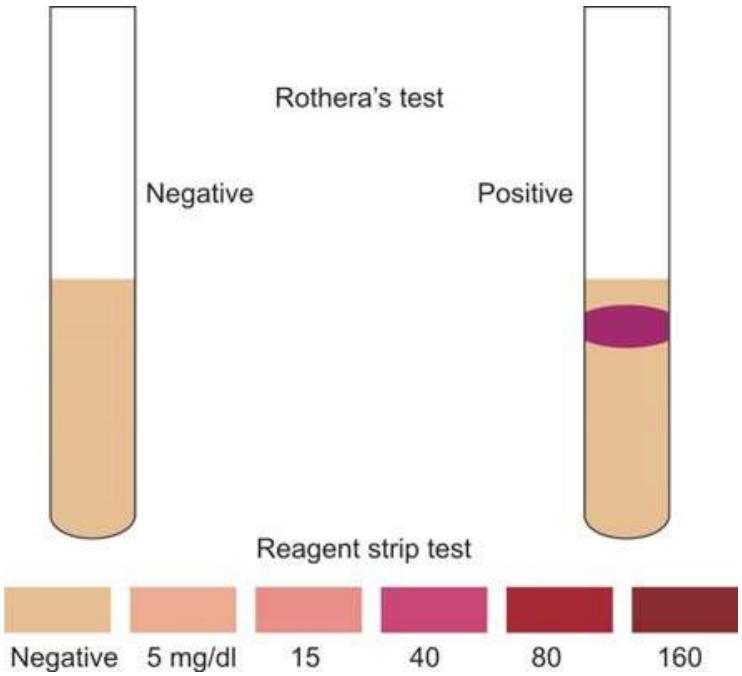
1. Take 5 ml of urine in a test tube and saturate it with ammonium sulphate.
2. Add a small crystal of sodium nitroprusside. Mix well.
3. Slowly run along the side of the test tube liquor ammonia to form a layer.
4. Immediate formation of a purple permanganate-colored ring at the junction of the two fluids indicates a positive test .

A false-positive test can occur in the presence of L-dopa in urine and phenylketonuria.

2. **ACETEST TABLET TEST**

This is Rothera's test in the form of a tablet. The Acetest tablet consists of sodium nitroprusside, glycine, and an alkaline buffer. Purple lavender discoloration of the tablet indicates the presence of acetoacetate or acetone (≥ 5 mg/dl). A rough estimate of the amount of ketone bodies can be obtained by comparison with the color chart provided by the manufacturer.

The test is more sensitive than the reagent strip test for ketones.



Rothera's tube test and reagent strip test for ketone bodies in urine

REAGENT STRIP TEST

Reagent strips tests are modifications of the nitroprusside test. Their sensitivity is 5-10 mg/dl of acetoacetate. If exposed to moisture, reagent strips often give a false-negative result. The ketone pad on the strip test is especially vulnerable to improper storage and easily gets damaged.

Procedure:

1. Before the sample collection, hands should be washed.
2. Then the genital area is cleaned with a cleansing pad. In the case of men, the tip of the penis is wiped. In the case of women, labia should be cleaned from front to back.
3. At least an ounce or two of urine is then collected into the container, which should have markings to indicate the amount.
4. One of the urine dipsticks is taken and is dipped in the container.
5. The strip is taken out and dried by shaking lightly.
6. The color of the strip is observed once the strip is dry.

Result:

The color observed in the strip is then tallied against the chart that comes with the test kit.

- Based on the intensity of the purple color, the quantitative analysis of the ketone bodies can be done.
- In the case of qualitative analysis the following, the concentration can be detected as follows:

Small: 20 mg/dl

Moderate: 30 to 40 mg/dl

Large: >80 mg/dl

Uses:

- This test is usually used to monitor people at a higher risk of developing ketones in urine like people with type 1 or type 2 diabetes.
- The detection of ketone bodies in urine is essential as the presence of ketones in urine can be a symptom of a more severe condition like toxic ketoacidosis.

Limitations

- Because of the imbalance between the concentration of acetone and β-hydroxybutyrate, the number of ketones measured in the urine cannot be considered as the total plasma ketone
- The ketone in the urine test only detects acetoacetic acid and not β-hydroxybutyrate.
- The ketone strips usually have a short shelf life.
- Interference by other substances like vitamin C might give inaccurate results.

Phospholipid Assay

Phospholipids are important structural lipids that are the major component of cell membranes and lipid bilayers. They contain a hydrophilic head and a hydrophobic tail which give the molecules their unique amphiphilic characteristics. Most phospholipids contain one diglyceride, a phosphate group, and one choline group. Sphingomyelin, lysophosphatidylcholine (lysolecithin), and phosphatidylcholine (lecithin) are predominantly choline-based phospholipids which account for 95% of phospholipids in human serum and plasma. Phosphatidylcholine is the foremost membrane phospholipid in eukaryotic cell membranes and is present in every cell in the body.

Determining circulatory levels of phospholipids and lipoproteins is critical to the diagnosis of lipid transport disorders and may be used as markers for heart, liver, or lung diseases. Phospholipid biosynthesis and metabolism is important to membrane structure maintenance and function. Many exertconsiderable influence on lipid homeostasis. They serve as a pool for many lipid messengers and are a source for bioactive lipids such as phosphatidic acid, diacylglycerol, lysophosphatidylcholine, and others. Phospholipids supply choline in the body, which itself and its derivative compounds form cell signaling molecules such as acetylcholine, platelet activating factor and sphingophosphorylcholine.

Energy production and storage, prostaglandin production, blood clotting, cholesterol solubility, fat and bile emulsification, and antioxidant activity are pathways involving phospholipid activity.

Principle:

Phospholipid Assay Kit measures phospholipids through a series of enzyme driven reactions. First, a hydrolyzing enzyme reacts with phospholipids, such as lecithins and sphingomyelins, breaking the phosphodiester bond within their structure. Next, an oxidoreductase reacts to generate a peroxide, which is subsequently detected with a highly specific fluorescence probe. Samples and standards are incubated for 30 minutes and then read with a standard 96-well fluorometric plate reader (Ex. 530-570 nm/Em. 590-600 nm). Sample phospholipid levels are determined by comparison with known phospholipid standards.

Kit Components

Box 1 (shipped at room temperature)

1. 96-well Microtiter Plate : One 96-well clear bottom black plate.
2. Assay Buffer (10X) : One 25 mL bottle.
3. Fluorescence Probe (100X) : One 50 µL tube in DMSO.
4. HRP (Part No. 234402): One 100 µL tube of 100 U/mL HRP solution in glycerol.

Box 2 (shipped on blue ice packs)

1. Phospholipid Standard : One 50 µL amber tube of a 2 mM solution.
2. Hydrolyzing Enzyme (400X) : One 15 µL amber tube.
3. Oxidoreductase (200X) : One 25 µL tube.

Materials Not Supplied

1. Distilled or deionized water
2. 10 µL to 1000 µL adjustable single channel micropipettes with disposable tips
3. 50 µL to 300 µL adjustable multichannel micropipette with disposable tips
4. Multichannel micropipette reservoir
5. Fluorescence microplate reader capable of reading excitation in the 530-570 nm range andemission in the 590-600 nm range.
6. (optional) Chloroform
7. (optional) Superoxide dismutase

Storage

Upon receipt, store the Hydrolyzing Enzyme at -80ºC and the Phospholipid Standard, FluorescenceProbe, HRP, and Oxidoreductase at -20ºC. The Fluorescence Probe is light sensitive and must be stored accordingly. Avoid multiple freeze/thaw cycles. Store the remaining kit components at 4ºC.

Preparation of Reagents

- 1X Assay Buffer: Warm the Assay Buffer (10X) to room temperature prior to using. Dilute the Assay Buffer (10X) with deionized water by diluting the 25 mL Buffer with 225 mL deionized water for 250 mL total. Mix to homogeneity. Store the 1X Assay Buffer at 4ºC up to six months.
- Detection Reagent: Prepare a Detection Reagent by diluting the Hydrolyzing Enzyme 1:400, Oxidoreductase 1:200, HRP 1:500, and Fluorescence Probe 1:100 in 1X Assay Buffer. (e.g. for 50 assays, combine 6.3 µL Hydrolyzing Enzyme, 12.5 µL of Oxidoreductase, 5 µL of HRP, and 25 µLFluorescence Probe, and with 1X Assay Buffer to 2.5 mL total solution). See Table 1 below for examples of Detection Reagent preparation based on the number of assays employed. Mix thoroughly and protect the solution from light. For best results, place the Detection Reagent on iceand use within 30 minutes of preparation. Do not store the Detection Reagent solution.

Hydrolyzing Enzyme (µL)	Oxidoreductase (µL)	HRP (µL)	Fluorescence Probe (µL)	1X Assay Buffer (µL)	**Number of Assays(50 µL/well)**
12	24	9.6	48	4,755	**96**
6.3	12.5	5	25	2452	**50**
2.5	5	2	10	981	**20**

Table 1. Preparation of Detection Reagent

Preparation of Samples

Samples should be assayed immediately or stored at -80ºC prior to performing the assay. Optimal experimental conditions for samples must be determined by the investigator. The following recommendations are only guidelines and may be altered to optimize or complement the user's experimental design. A set of serial dilutions is recommended for samples to achieve optimal assay results and minimize possible interfering compounds. Run proper controls as

necessary. Always run astandard curve with samples.

- Serum: Collect blood without using an anticoagulant. Allow blood to clot for 30 minutes at room temperature. Centrifuge at 2000 x g and 4ºC for 10 minutes. Remove the serum layer and store on ice. Take care to avoid disturbing the white buffy layer. Aliquot samples for testing and store remaining solution at -80ºC. Perform serum dilutions in 1X Assay Buffer. Serum samples must be diluted at least 1:50 to 1:400 with Assay Buffer. This will provide values within the range of the standard curve.
- Plasma: Collect blood with heparin or citrate and centrifuge at 1000 x g and 4ºC for 10 minutes. Remove the plasma layer and store on ice. Take care to avoid disturbing the white buffy layer. Aliquot samples for testing and store remaining solution at -80ºC. Perform plasma dilutions in 1X Assay Buffer. Plasma samples must be diluted at least 1:50 to 1:400 with Assay Buffer. This will provide values within the range of the standard curve.

  Notes:

  1. *Samples with NADH concentrations above 10 µM and glutathione concentrations above 50 µM will oxidize the probe and could result in erroneous readings. To minimize this interference, it is recommended that superoxide dismutase (SOD) be added to the reaction at a final concentration of 40 U/mL.*
  2. *Avoid samples containing DTT or β-mercaptoethanol since the fluorescence probe is not stable in the presence of thiols (above 10 µM).*
  3. *Choline can generate high background if present in samples. If choline may be present, run a background control without the Hydrolyzing Enzyme. Subtract this value from sample reading values.*

Preparation of Phospholipid Standard Curve

1. Prepare fresh phospholipid standards by first diluting a portion of the 2 mM Phospholipid Standard stock solution 1:80 in 1X Assay Buffer. (e.g. Add 5 µL of Phospholipid Standard in 395 µL 1X Assay Buffer). Vortex thoroughly. This provides a 25 µM concentration. Use this solution to prepare a series of the remaining phospholipid standards according to Table 2 below.

Tubes	2mM Phospholipid Standard (µL)	1X Assay Buffer (µL)	Resulting Phospholipid Concentration (µM)
1	5	395	25
2	200 of Tube #1	200	12.5
3	200 of Tube #2	200	6.25
4	200 of Tube #3	200	3.13
5	200 of Tube #4	200	1.56
6	200 of Tube #5	200	0.78
7	200 of Tube #6	200	0.39
8	200 of Tube #7	200	0.20
9	0	500	0

Table 2. Preparation of Phospholipid Standards.

Note: Do not store diluted phospholipid standard solution

Assay Protocol

Each phospholipid standard and sample should be assayed in duplicate or triplicate. A freshly preparedstandard curve should be used each time the assay is performed.

1. Add 50 µL of the diluted phospholipid standards or samples to the 96-well microtiter plate.
2. Add 50 µL of the prepared Detection Reagent to each well and mix the well contents thoroughly.
3. Cover the plate wells to protect the reaction from light. Incubate the plate for 30 minutes at 37ºC.
4. Read the plate with a fluorescence microplate reader equipped for excitation in the 530-570 nmrange and for emission in the 590-600 nm range.

Calculate the concentration of phospholipid within samples by comparing the sample RFU to thephospholipid standard curve.

Example of Results

The following figures demonstrate typical Phospholipid Assay results. One should use the data belowfor reference only. This data should not be used to interpret or calculate actual sample results.

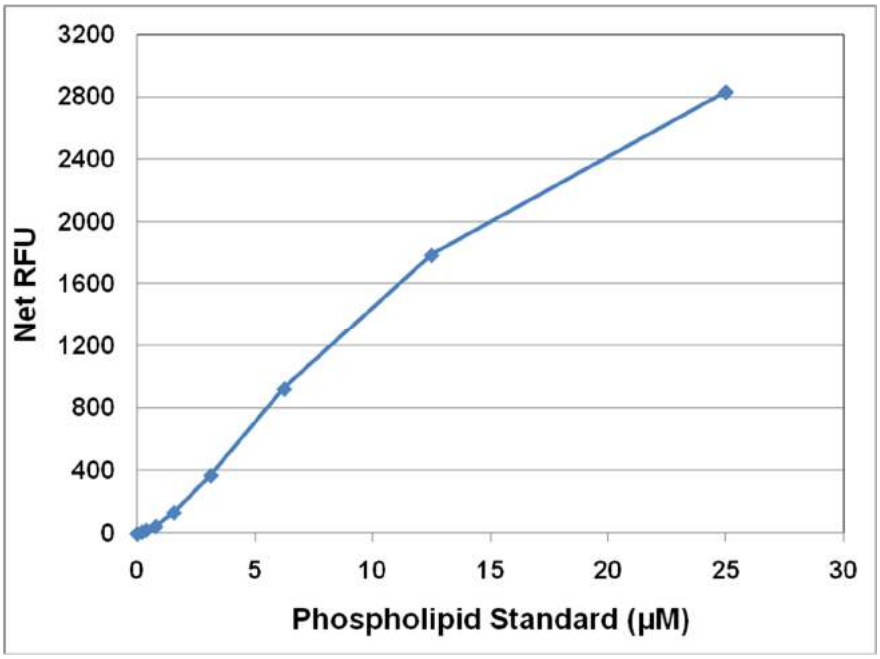


Figure 2: Phospholipid Standard Curve.

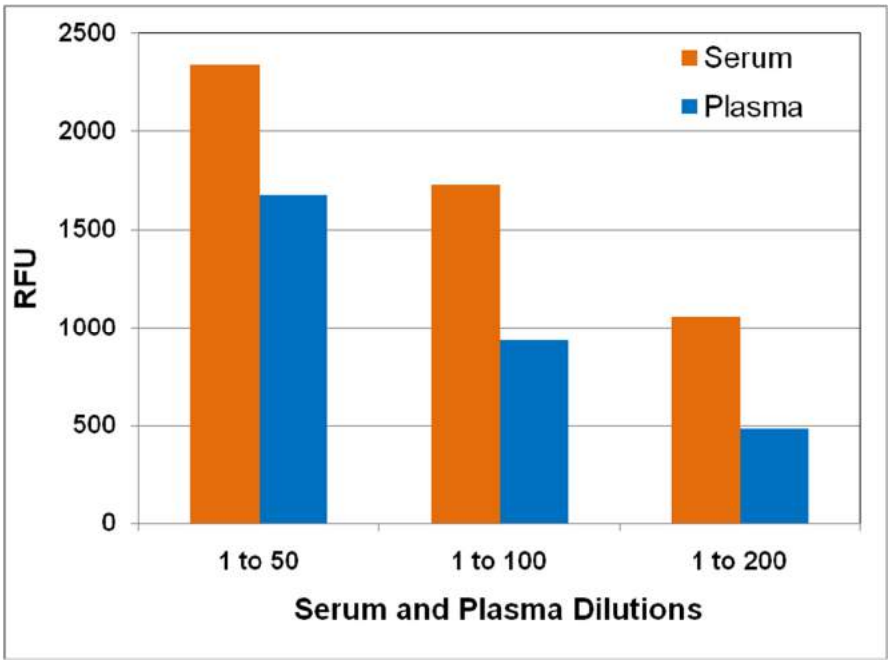


Figure 3: Human Serum and Plasma. Human plasma and serum were both diluted in 1X AssayBuffer and tested according to the assay protocol.

Calculation of Results

1. Calculate the average fluorescence values for every standard, control, and sample. Subtract the average zero standard value from itself and all standard and sample values. This is the correctedfluorescence.
2. Plot the corrected fluorescence for the standards against the final concentration of the phospholipidstandards from Table 2 to determine the best curve. See Figure 2 for an example standard curve.
3. Determine the phospholipid concentration of the samples with the equation obtained from the linear regression analysis of the standard curve. Substitute the corrected fluorescence values for each sample. Remember to account for dilution factors.

$$\text{Phospholipid } (\mu M) = \left[\frac{\text{Sample corrected fluorescence}}{} \right] \times \text{Sample dilution}$$

Protein Estimation

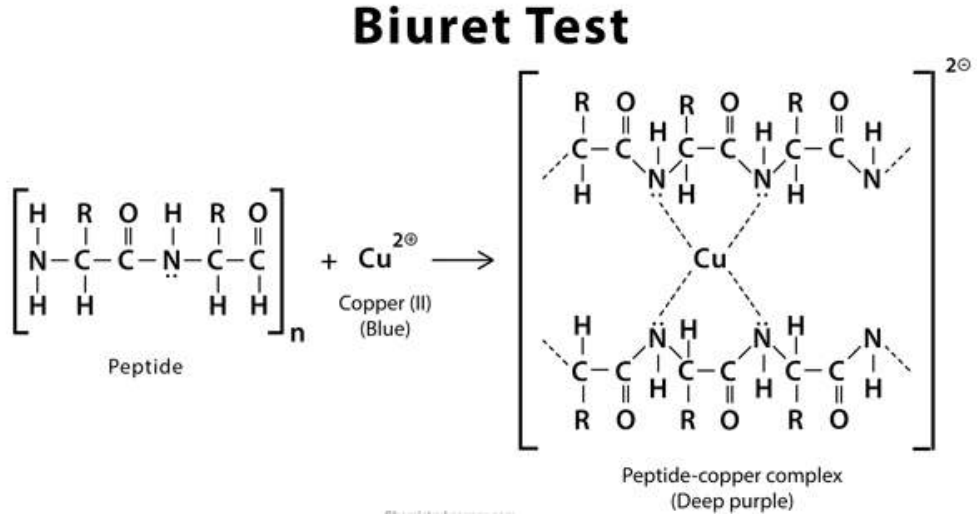
CLINICAL SIGNIFICANCE:

Proteins are constituents of muscle, enzymes, harmones and several other key functional and structural entities in the body. They are involved in the maintenance of the normal distribution of water between blood and the tissues. Consisting mainly of albumin and globulin in the fractions vary independently and widely in diseases. Increased levels are found mainly in dehydration. Decreased levels are found mainly in malnutrition, impaired synthesis, protein losses as in hemorrhage or excessive protein catabolism.

PRINCIPLE:

Proteins, in an alkaline medium, bind with the cupric ions present in the biuret reagent to form a blue-violet coloured complex. The intensity of the colour formed is directly proportional to the amount of proteins present in the sample.

REACTION:



CONTENTS:

Reagent 1 : Biuret Reagent Reagent 2 : Protein Standard 6 g/dl

MATERIALS REQUIRED BUT NOT PROVIDED:-

Clean & Dry Glassware.

- Laboratory Glass Pipettes or Micropipettes & Tips.

- Colorimeter or Bio-Chemistry Analyzer.

SAMPLES:

Serum, Heparinized/EDTA Plasma. Proteins are reported to be stable in the sample for 6 days at 2-8°C

PREPARATION OF REAGENT & STABILITY :

All reagents are stable till the expiry date mentioned on the label at room temperature. Standard vial once opened should be stored at 2 -8°C, it is stable till the expiry date mentioned on the vial. All reagents are in ready to use form.

GENERAL SYSTEM PARAMETERS:

Reaction type : End point

Wave length : 546 nm (530 - 570 nm)

Temperature : Room temperature

Incubation : 5 minutes

Reagent volume : 1.0 ml

Sample volume : 10 µl

Standard concentration : 6 gm/dl.

Zero setting : Reagent blank

Light path : 1 cm

PROCEDURE:

Pipette into clean dry test tube labeled as Blank (B), Standard (S) and Test (T).

Addition sequence	**B**	**S**	**T**
Biuret Reagent	1ml	1ml	1ml
Standard		10µl	
Sample			10µl

Mix well, Incubate for 5 minutes at Room temperature. Measure the absorbance of the standard Abs. S and sample Abs. T against the reagent blank , within 60 minutes.

CALCULATION :

Total Protein Conc. (gm/dl) = Abs. T/ Abs. S x 6

NORMAL VALUE :

Serum : 6.0 - 8.0 gm/dl It is recommended that each laboratory establish its own normal range.

LINEARITY : This procedure is linear upto 10 gm/dl. Samples above this concentration should be diluted with normal saline and results should be multiplied by dilution factor.

QUALITY CONTROL :

For accuracy it is necessary to run known controls with every assay.

LIMITATION & PRECAUTIONS :

1. Storage condition mentioned on the kit must be adhered.

2. Do not in any case freeze or expose reagent to high temperature as it may effect the performance of the kit.

3. Before the assay bring all the reagents to room temperature.

4. Avoid contamination of the reagents during the assay process.

5. Use clean glassware free from dust or debris

TRIGLYCERIDES KIT

(GPO / PAP method)

For the determination of Triglycerides in serum or plasma.

(For Invitro Diagnostic Use Only)

Summary

Triglycerides are a form of fatty acid esters. They are produced in the liver by binding glycerol and other fatty acids. They are transported by VLDL and LDL and act as a storage source for energy. Increased levels are found in hyperlipidemias, diabetes, nephrotic syndrome, hypothyroidism. Increased levels are risk factor for arteriosclerotic coronaiy disease and peripheral vascular disease. Decreased levels are found in malnutrition and hyperthyroidism.

Principle

Lipoprotein lipase hydrolyses triglycerides to glycerol and free faky acids' The glycerol formed with ATP in the presence of glycerol kinase forms glycerol 3 phosphate which is oxidised by the enzyme glycerol phosphate oxidase to form hydrogen peroxide. The hydrogen peroxidefurther reacts with phenolic compound and 4-aminoantipyrine by the catalytic action of peroxidase to form a red coloured quinoneimine dye complex. Intensity of the colour formed is directlyproportional totheamountof triglycerides presentinthesample.

LipoproleinLipase
Triglycerides ——— Glycerol +Free fatty acids

GlycefiolKinase
Glycerol +ATP ——— Glycerol 3 Phophate + ADP

G/ycero/3PO
Glycerol 3 Phophate +O, ——— Dihydroxyacetonephos.+ H,O,

Peroxidase
H,O, +4Aminoantipyrine ——— Red Quinoneimine dye +H,O +Phenol

Normal reference values

Serum/Plasma (Suspicious) : 150 mg/dl and above
(Elevated) : 200 mg/dl and above

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	is=i	7smi	2x75ml 2x150ml
L1 : Enzyme Reagent 1	20 ml	60 ml	2 x 60 i lx 120 ml
L2 : Enzyme Reagent 2	5 ml	15 ml	2 x 15 l2ik 30 ml
5 : Triglycerides Standard (200 mg/dl)	5 ml	5 ml	5 6fril

Storage / stability

Contents are stable at 2-8'C till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use.

Working reagent : Pour the contents of 1 bottle of L2 (Enzyme Reagent 2) into 1 bottle of L1 (Enzyme Reagent 1).This working reagent is stable for at least 8 weeks when stored at 2-8' C. Upon storage the working reagent may develop a slight pink colour however this does not affect the performance of the reagent.

Alternatively for flexibility as much of working reagent may be made as and when desired by mixing together 4 parts of L1 (Enzyme Reagent 1) & 1 part of L2 (Enzyme Reagent 2). Alternatively0.8 ml ofL1 and 0.2 ml ofL2 may also be used instead of 1 ml of the working reagent directly during the assay.

Sample material

Serum, plasma. Triglycerides is reported to be stable in the sample for Sdayswhenstoredat 2-8°C.

Procedure

Wavelength/filter	505nm(Hg 546 nm)/Green
Temperature	37 C/R.T.
Light path	1 cm

Pipette into clean dry test tubes labelled as Blank (B), Standard (S), and Test(T):

Additiion Sequence	B (ml)	S (ml)	(ml)
Working reagent	1.0	1.0	1.0
Distilled water	0.01		
Triglycerides Standard(S)		0.01	
Sample			001

Mix well and incubate at 37 C for 5 min. or at R.T. (25'C) for 15 min. Measure the absorbance of the Standard (Abs.S), and Test Sample (Abs.T) against the Blank, within 60 Min.

Calculations

$$\text{Triglycerides in mg/dl} = \frac{\text{Abs.T}}{\text{Abs.S}} \times 200$$

Linearity

This procedure is linear upto 1000 mg/dl. If values exceed this limit, dilute the serum with normal saline (NaCl 0.9%) and repeat the assay.

Note

Fasting samples of 12 to 14 hrs. are preferred. Fatty meals and alcohol may cause elevated results. Patient should not drink alcohol for 24 hrs. before the test

UREA KIT

(GLDH Kinetic method)

For the determination of Urea in serum or plasma.
(For Invitro Diagnostic Use Only)

Summary

Urea is the end product of protein metabolism. It is synthesised in the liver from the ammonia produoed by the catabolism of amino acids. It is transported by the blood to the kidneys from where it is excreted. Increased levels are found in renal diseases, urinary obstructions, shock, congestive heart failure and burns. Decreased levels are found in liverfailure and pregnancy.

Principle

Urease hydrolyzes urea to ammonia and CO,. The ammonia formed further combines with a Ketoglutarate and NADH to form Glutamate and NAD.The rate of oxidation of NADH to NAD is measured as a decrease in absorbance in a fixed time which is proportional to the urea concentration in the sample.

$$\text{Urea} + H_2O + 2H^+ \xrightarrow{\text{tfrease}} 2NH_4^+ + CO_2$$

$$2NH_4^+ + 2\text{-Ketoglutarate} + 2NADH \xrightarrow{GLDH} 2\text{L-glutamate} + 2NAD^+ + 2H_2O$$

Normal reference values

Serum/Plasma	14 - 40 mg/dl
Urine	Upto 20 g/l

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	75 ml	2 x 75 ml	2 x 150 ml
L1 : Enzyme Reagent	60 ml	2 x 60 ml	2 x 120ml
L2 : Starter Reagent	15 ml	2 x 15 ml	2 x 30ml
S : Urea Standard (40 mg/dl)	5 ml	5 ml	5 ml

Storage / stability

Contents are stable at2-8'C till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use.
Workingreagent: For sample startassays a singlereagent is required. Pour the contents of 1 bottle of L2 (Starter Reagent) into 1 bottleofL1 (Enzyme Reagent). Thisworking reagent isstableforatleast 10dayswhen stored at2-8'C. Alternatively for flexibility as much of working reagent may be made as and when desired by mixing together 4 parts of L1 (Enzyme Reagent) and 1 part of L2 (Starter Reagent). Alternatively0.8mlofL1 and 0.2 ml ofL2 may also be used instead of 1 ml of the working reagent directly during the assay.

Allow the Working Reagent to stand for 30 min. before use.

Sample material

Serum, plasma, Urine. Dilute urine 1+ 49 with distilled water before the assay(results x 50). Urea is reported to be stable in the serum for 5 days when stored at2-8'C.

Procedure

Wavelength/filter	340 nm
Temperature	37°C/30'CI25'C
Light path	1 cm

Substrate StartAssay:

Pipette into a clean dry test tube labelled Standard (S) or Test(T):

AdditionSequence	\Sjl\fl 37'C/30'CI25'C
Enzyme Reagent (L1)	0.8 ml
Urea Standard/Serum/Diluted urine	0.01 ml
Incubate at the assay temperaturefor 5 minute	

Mix well and read the initial absorbanceA,forthe Standard and Test after exactly 30 seconds. Read another absorbance A, of the Standard and Testexactly 60 seconds later. Calculate the change in absorbance A A for both the Standard and Test.

Sample StartAssay :
Pipette into a clean dry test tube labelled Standard (S) or Test(T):

Addition Sequence	(S)/(T) 37°C/30'CI25°C
Working reagent	1.0 ml
Bringtoassaytemperatureand add Urea Standard/Serum/ Dilutedurine	0.01ml

Mix well and read the initial absorbanceA,for the Standard and Test after exactly 30 seconds. Read another absorbanceA, of the Standard and Testexactly 60 seoonds later. Calculate the change in absorbance AA for both the Standard and Test.

ForStadad	dAS	@s	A,s
ForTest	bAT	QT	A,T

Calculations

$$\text{Urea in mg/dl} = \frac{AAT}{AAS} \times 40$$

Linearity

This procedure is linear upto 250 mg/dl. If values exceed this limit, dilute the serum with normal saline (NaCl 0.9%) and repeat the assay. Calculate the value using the proper dilutionfactor.

Note

Plasma should not be collected with Flouride or Heparin salts as contamination by ammoniaorammonium salts lead toerroneousresults.

Reaction		Fixed Time Kinetic
Wavelength		340 nm
Zero Setting		
Incub. Temp.		Distilled Water
Incub. Time		
Delay Time		30 sec.
Read Time No. of read.	:	60 sec.
Interval		
Sample Vol.		2
Reagent Vol.		
Standard		60 sec.
Factor React.		
Slope		Decreasing
Linearity		250 mg/dl
		mg/dl

UBE(UV):02(P)

Coral

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URIC ACID KIT

(Uricase / PAP method)

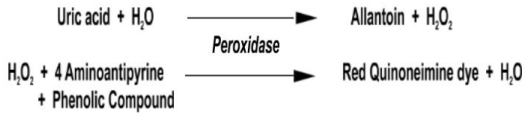
For the determination of Uric Acid in serum or plasma.
(For Invitro Diagnostic Use Only)

Summary

Uric acid is the end product of purine metabolism. Uric acid is excreted to a large degree by the kidneys and to a smaller degree in the intestinal tract by microbial degradation. Increased levels are found in Gout, arthritis, impaired renal functions,and starvation. Decreased levels are found in Wilson'sdisease, Fanconis syndrome and yellow atrophy of the liver.

Principle

Uricase converts uric acid to allantoin and hydrogen peroxide. The hydrogen peroxide formed further reacts with a phenolic compound and 4 aminoantipyrine by the catalytic action of peroxidase to form a red coloured quinoneimine dye complex. Intensity of the colour formed is directly proportional to the amount of uric acid present in the sample.



Normal reference values

Serum/Plasma	(Males)	3.4 - 7.0 mg/dl
	(Females)	2.5 - 6.0 mg/dl

It is rec0mmended that each laboratory establish its own normal range representing its patient population.

Contents	25 ml	75 ml	2 x 75 ml	2 x 150 ml
L1 : Buffer Reagent	20 ml	60 ml	2 x 60 ml	2 x 120 ml
L2 : Enzyme Reagent	5 ml	15 ml	2 x 15 mg	x 30 ml
S : Uric Acid Standard (8 mg/dl)	5 ml	5 ml	5 ml	5 ml

Storage / stability

Contents are stable at 2-8'C till the expiry mentioned on the labels.

Reagent Preparation

Reagents are ready to use.
Working reagent : Pour the contents of 1 bottle of L2 (Enzyme Reagent) into 1 bottle of L1 (Buger Reagent). This working reagent is stable for at least 4 weeks when stored at 2-8°C. Upon storage the working reagent may develop a slight pink colour however this does not a8ect the performance of the reagent.
Alternatively for flexibility as much of working reagent may be made as and when desired by mixing together 4 parts of L1 (Buffer Reagent) and 1 part of L2 (Enzyme Reagent). Altematively 0.8 ml of L1 and 0.2 ml of L2 may also be used instead of 1 ml of the working reagent directly during the assay.

Sample material

Serum, plasma. Uric Acid is reported to be stable in the sample for 3-5 days when stored at 2-8'C.

Procedure

Wavelength / filter	520 nm (Hg 546 nm)/YellowGreen
Temperature	37'C/R.T.
Light path	1 cm

Pipette into clean dry test tubes labelled as Blank (B), Standard (S), and Test(T):

Addition **Sequence**	B (ml)	5 (ml)	T (ml)
Working reagent	1.0	1.0	1.0
Distilled water	0.02		
Uric Acid Standard (S)		0.02	
Sample			0.02

Mix well and incubate at 37'C for 5 min. or at R.T. (25'C) for 15 min. Measure the absorbance of the Standard (Abs.S), and Test Sample (Abs.T) against the Blank, within 30 Min.

Calculations

$$\text{Uric Acid in mg/dl} = \frac{\text{Abs.T}}{\text{Abs.S}} \times 8$$

Linearity

This procedure is linear upto 20 mg/dl. If values exceed this limit, dilute the serum with normal saline (NaCl 0.9%) and repeat the assay. Calculate the value using the proper dilution factor.

Ctyotechnology & Histotechnology: Paper – XII, Unit – 24

H & E Stain

Intended use H & E Staining method is used for the routine staining of the cationic and anionic tissue components in tissue sections. This is the standard reference stain used in the study of histochemical tissue pathology.

Summary

Hematoxylin and eosin stain (abbreviated as H & E stain) is one of the principal tissue stains used in routine histology staining methods. It is the most widely used stain in medical diagnosis and is often the gold standard; wherein, when a pathologist looks at a biopsy of a suspected cancer, the histological section is likely to be stained with H & E.

H & E Stain is the combination of two histological stains: Hematoxylin and Eosin. The hematoxylin is a selective nuclear stain which stains the cell nuclei blue, and eosin stains the extracellular matrix and cytoplasm pink, with other structures taking on different shades, hues, and combinations of these colors. The stain shows the general layout and distribution of cells and provides a general overview of a tissue sample's structure. Hence, there is a clear differentiation between the nuclear and cytoplasmic parts of a cell.

Principle

Hematoxylin and eosin are the principle stains used for the demonstration of nucleus and the cytoplasmic inclusions. Alum acts as a mordant and hematoxylin containing alum stains nucleus light blue which turns red in the presence of acid. The cell differentiation is achieved by treating the tissue with acid solution. Counter staining is performed by using eosin solution which imparts pink color to the cytoplasm.

Hematoxylin, a common nuclear stain, is isolated from an extract of logwood (Haematoxylon campechianum). Before hematoxylin can be used as a nuclear stain, it must be oxidized to hematein and combined with a metallic ion (mordant). Most successful mordants have been salts of aluminum or iron. Generally, hematoxylins are classified as progressive or regressive based on dye concentration. Progressive stains (e.g., Mayer’s hematoxylin) have a lower concentration of dye and selectively stain nuclear chromatin. The desired intensity is a function of time. Regressive stains (e.g., Harris hematoxylin) color all nuclear and cytoplasmic structures intensely. To arrive at correct chromatic response, excess dye must be removed by treatment with dilute acid (differentiation). Eosin is tetra bromofluorescein (a substituted xanthene), a red acidic dye and fluorochrome. The dye is very soluble in ethyl alcohol and also used for the staining of cytoplasm. Eosin Y is the most commonly used counterstain for hematoxylin.

Reagents / Contents

1. Hematoxylin Harris

Hematoxylin 5.0 g

Ammonium/ Potassium Alum 100 g

Mercuric Oxide 2.5 g

Alcohol 95% 50 mL

Distilled Water 1000 mL

Appearance: Maroon purplish solution.

2. **Eosin (AQU.) 2%**

   Eosin-Y 2.0 g

   Distilled water 100 mL

   Appearance: Dark reddish solution.

Storage and Stability

Store at 15°C-25°C away from bright light. Use before expiry date on label.

Type of Specimen

Histochemical tissues sections obtained from biopsy specimens

Procedure

1. Sections are deparaffinized (removal of wax) by placing in xylene for 10 - 15 minutes.

2. Rehydrate section by passing in a series of descending grades of alcohol, finally to water.

3. Place in Hematoxylin Harris solution for 8-10 minutes.

4. Rinse in water.

5. Differentiate the slide in a solution 1% acid alcohol for 10 seconds.

6. Rinse in tap water.

7. Blueing (brining the required blue color to section) is done by putting the section in a solution containing Sodium bicarbonate, $MgSO_4$ and saturated solution of Lithium carbonate.

8. Counter stain with aqueous Eosin (Aqu.) 2% for 1-3 minutes.

9. Rinse in tap water.

10. Section are dehydrated which is done by a series of ascending grades of alcohol and finally clearing in Xylene.

11. Dry the section by pressing on the filter paper.

12. Mount in DPX and observe under microscope, 40X and 100X under oil immersion lens.

Interpretation of Results

The nuclei of cells are stained blue or dark-purple along with a few other tissues, such as kerato hyalin granules and calcified material with Hematoxylin. The cytoplasm and some other structures including extracellular matrix such as collagen stains in up to five shades of pink with Eosin. Most of the cytoplasm is eosinophilic and is rendered pink. Red blood cells are stained intensely red. The background of the tissue remains colourless.

Periodic Acid Schiff's (PAS) Stain

Purpose:

Glycogen is present in skin, liver, parathyroid glands and skeletal and cardiac muscle. The PAS stain is used for demonstration of basement membranes, fungus secreting adenocarcinoma from undifferentiated squamous cell carcinoma, and mucosubstances secreted from the epithelia of various organs. A routine stain for liver and kidney biopsies.

Principle:

The PAS stain is a histochemical reaction in that the periodic acid oxidizes the carbon to carbon bond forming aldehydes which react to the fuchsin-sulfurous acid which form the magenta color.

Solutions and Reagents:

0.5% Periodic Acid Solution

Periodic acid ---------- 0.5 g
Distilled water ---------- 100 ml

Schiff's reagent

Schiff's reagent reagents: Test for Schiff's reagent: Pour 10 ml of 10% formalin to a beaker, add a few drops of the Schiff's reagent to be tested. Good Schiff's reagent will rapidly turn a red purple colour. A deteriorating Schiff's reagent will give a delayed reaction and the colour produced will be a deep blue-purple.

Mayer's hematoxylin

Procedure:

1. Deparaffinize and hydrate to water.
2. Oxidize in 0.5% Periodic Acid solution for 5 minutes.
3. Rinse in 3 changes of distilled water.
4. Place in Schiff's reagent for 15 minutes (Sections become light pink colour during this step).
5. Wash in tap water for 5 minutes (Sections immediately turn dark pink).
6. Counter stain in Mayer's haematoxylin for 1 minute.
7. Wash in tap water for 5 minutes then rinse in distilled water.
8. Dehydrate, cover slip and mount using Xylene based mounting mediaayer's modified haematoxylin

Results:

Glycogen, mucin and some basement membranes -------- Red/ purple

Fungi ---------- Red/ purple
Background. ---------- Blue

Trichrome Stain (TRI)

Purpose:

Used to differentiate between collagen and smooth muscle in tumors, and the increase of collagen in diseases such as cirrhosis. Routine stain for liver and kidney biopsies.

Principle:

As the name implies, three dyes are employed selectively staining muscle, collagen fibers, fibrin, and erythrocytes. The general rule in trichrome staining is that the less porous tissues are colored by the smallest dye molecule; whenever a dye of large molecular size is able to penetrate, it will always do so at the expense of the smaller molecule. Others suggest that the tissue is stained first with the acid dye, Biebrich Scarlet, which binds with the acidophilic tissue components. Then when treated with the phospho acids, the less permeable components retain the red, while the red is pulled out of the collagen. At the same time causing a link with the collagen to bind with the aniline blue.

Solutions:

Bouin's Solution

Bouin's solution

Modified Weigert's Iron Hematoxylin

Solution A

Hematoxylin crystals---------- 4.0 g

Alcohol, 80% ----------200 ml

Solution B

Ferric chloride (EMS 15510) ---------- 8.0 g

Distilled water ----------190 g

HCl concentrated 36.5-38% ----------2.0 ml

Working Modified Weigert's Iron Hematoxylin

Equal parts of Solution A and Solution B mixed well, keep in dark

Biebrich Scarlet-Acid Fuchsin Solution

Bibrich scarlet ----------2.25 gm

Acid fuchsin, ----------0.25gm

Acetic acid, glacial ----------2.5 gm

Distilled water ----------250 ml

1% Phosphomolybidic Acid Solution

Phosphomolybdic acid ---------- 2.5 gm

Distilled water ---------- 250 ml

1.8 % Aniline Blue Solution

Aniline blue ---------- 4.5 gm

Acetic acid, glacial ----------4.5 ml

Distilled water ----------250 ml

1% Acetic Acid Solution

Acetic acid, glacial ----------2.5 ml

Distilled water ----------250 ml

Acid-Alcohol Differentiation Solution

0.5% hydrochloric acid in 70% alcohol

Procedure

1. Deparaffinize sections if necessary and hydrate to distilled water.
2. Preheat Bouin's Fluid in a water bath to 56-64°C in a fume hood or very well ventilated area.
3. Place slide in preheated Bouin's Fluid for 60 minutes followed by a 10 minute cooling period.
4. Rinse slide in tap water until section is completely clear.
5. Rinse once in distilled water.
6. Mix equal parts of Weigert's (A) and Weigert's (B) and stain slide with working Weigert's Iron Hematoxylin for 5 minutes.
7. Rinse slide in running tap water for 2 minutes.
8. Apply Biebrich Scarlet / Acid Fuchsin Solution to slide for 15 minutes.
9. Rinse slide in distilled water.
10. Differentiate in Phosphomolybdic/Phosphotungstic Acid Solution for 10-15 minutes or until collagen is not red.
11. Without rinsing, apply Aniline Blue Solution to slide for 5-10 minutes.
12. Rinse slide in distilled water.
13. Apply Acetic Acid Solution (1%) to slide for 3-5 minutes.
14. Dehydrate very quickly in 2 changes of 95% Alcohol, followed by 2 changes of Absolute Alcohol.
15. Clear in Xylene or Xylene Substitute and mount with DPX.

Results:

Nuclei ----------black

Cytoplasm, muscle, erythrocytes----------red

Collagen----------blue

Iron-Hematoxylin Staining

Principle:

Hematoxylin is a natural dye that is extracted from *Haematoxylon campechianum*, a leguminous plant, which must undergo oxidation (Ripening) to form hematein. Oxidation is a slow process involving dissolving crystals of Hematoxylin in water and its then exposed to oxygen (Atmospheric) and the addition of hydrogen peroxide with close monitoring to avoid overoxidation. Over-oxidation leads to the formation of oxy-hematein which is not a dye.

A hematoxylin solution is made up of a combination of hematoxylin, hematein, oxihematein. Hematein acts as the mordant to produce a lake i.e it reacts with ferric ammonium sulfate to produce the ferric lake (iron-hematoxylin), a basic dye. The iron-hematoxylin compound is stable and stains sharply the structures used to identify intestinal protozoan.

It is mostly used to stain regressively, i.e the slides are first over stained and then differentiated. The use of Alcoholic iodine ensures complete fixation of the smear.

Preparation of solutions:

1. **Schaudinn's solution:** Into 80ml of distilled water, add a saturated solution of Mercuric Chloride, Ethyl alcohol 20ml (95%), Acetic acid 3 mL
2. **Iodine solution:** add 98ml of Ethyl alcohol (95%) and 2ml of Tincture of iodine (2%)
3. **The mordant-differentiating solution:** Iron alum (ferric ammonium sulfate) 2 g, 1ml of Hydrochloric acid, distilled water 98 mL
4. **Hematoxylin solution:** Hematoxylin 0.30 g, distilled water 100 mL

Staining Procedure

1. On clean microscopic glass slides, slides, prepare thin smears of the stool samples using an applicator stick and while still wet, dip the slides into Schaudinn's solution, for at least 30 minutes. This is called the Schaudinn's fixation
2. Place the Schaudinn's fixed smear in 70% alcohol to remove excess fixative.
3. Rinse the slide in smoothly flowing tap water at least three times
4. Place slide in iron haematoxylin working solution for 4 to 5 min
5. Wash slides with smooth running tap water (constant stream of water into the container) for 10 min.
6. Immerse the slides in 95% ethyl alcohol for 5 minutes.
7. Place the slides in 100% ethanol for 5 minutes.
8. Immerse the slides in two changes in xylene for 5 minutes for each change
9. Add a permount (Mounting Media is formulated for mounting and storing long-term slides) to the stain and cover with a cover slip.
10. Examine the smear under a microscope at 100x and also under oil immersion before reporting.

Papanicolaou Stain

Intended use

Papanicolaou stains are used for staining the vaginal smear to detect vaginal, cervical and uterine cancer. Papanicolaou EA – 35 / EA – 65 is use as counterstain with Hematoxylin Stains in Papanicolaou Staining method. Papanicolaou OG – 6 is a multichromatic cytological stain.

Summary

PAP stain is a polychromatic staining method containing multiple dyes to differentially stain various components of the cells which is used to differentiate cells in smear preparations of various bodily secretions. PAP stain is a very reliable technique. Papanicolaou Stains are used in conjunction with Hematoxylin nuclear stains in the diagnosis of malignant cytological disease. As such it is used for cervical cancer screening. Cancer cells may be found by the smear technique in imprints of puncture biopsy material and in smears of cervical cells, vaginal secretion, prostatic secretion, urine, gastric contents, bronchial aspirations, cavity fluids and sputum. A diagnosis of malignancy made from stained smears should be considered tentative and should be checked by tissue sections.

Principle

The classic form of PAP staining method involves five dyes in three solutions: A nuclear stain, hematoxylin, is used to stain cell nuclei. First OG-6 counterstain. The Orange G is used to stain keratin. Its original role was to stain the small cells of keratinizing squamous cell carcinoma present in sputum. Second EA (Eosin Azure) counterstain, comprising of three dyes; the number denotes the proportion of the dyes, eg. EA-36, EA-50, EA-65. This group of reagents provides excellent cytoplasmic staining of gynecological and non-gynecological samples. EA-36 and EA50 are used in conjunction with OG-6 for gynecological staining. EA-65 is used with OG-6 for non-gynecological staining. The wide range of formulations available allows the end user to select from various color intensities and hues. Eosin Y stains the superficial epithelial squamous cells, nucleoli, cilia, and red blood cells. When performed properly, the stained specimen should display hues from the entire spectrum: red, orange, yellow, green, blue, and violet. The chromatin patterns are well visible, the cells from borderline lesions are easier to interpret and the photomicrographs are better. The staining results in very transparent cells, so even thicker specimens with overlapping cells can be interpreted. On a well-prepared specimen, the cell nuclei are crisp blue to black. Cells with high content of keratin are yellow, glycogen stains yellow as well. Superficial cells are orange to pink, and intermediate and parabasal cells are turquoise green to blue. Metaplastic cells often stain both green and pink at once.

Reagents / Contents

Papanicolaou EA – 36

Light green 45.0 g

Bismark brown 10.0 g

Eosin Y 45.0 g

Phosphotungstic acid 0.20 g

Lithium carbonate, saturated aqueous solution 1 drop

Appearance: Dark blue solution.

OR

Papanicolaou EA – 65

Eosin Y, 0.23%,

Bismarck brown, 0.05%,

Fast green FCF, 0.01%,

Phosphotungstic acid, 0.2%, in denatured alcohol

Appearance: Bluish violet solution.

OR

Papanicolaou OG – 6

Orange G-6 Certified 0.3 g

Phosphotungstic acid 0.015 g

Denatured alcohol 100.0 mL

Appearance: Orange colored solution.

Procedure

Fixation:

Do not allow smears to dry and fix immediately in 95% alcohol for 5-15 min. The smears may be left in the fixative for 3 days if necessary, but prolonged fixation affects the staining reaction.

1. Rinse in 70% alcohol, 50% alcohol and distilled water.
2. Stain in Haematoxylin Harris (without acetic acid) for 5 -10 minutes.
3. Rinse in distilled water.
4. Rinse 3 or 4 times in 0.5% aqueous solution of hydrochloric acid.
5. Rinse thoroughly in water.

6. Leave for 1 minute in a weak solution of lithium carbonate (3 drops saturated aqueous solution / 100 mL water) or in Scott's tap water. Rinse thoroughly in water.
7. Rinse in distilled water, 50% alcohol, 70% alcohol, 80% alcohol and 95% alcohol.
8. Stain for 1 minute in the Papanicolaou Orange G-6 solution.
9. Rinse 5-10 times in each of two jars containing 95% alcohol.
10. Stain in Papanicolaou EA-36 for 2 minutes.
11. Rinse 5-10 times in each of three jars containing, 95% alcohol (not the same alcohol that was used after orange G-6 solution).
12. Rinse in absolute alcohol, then in a mixture of equal parts of absolute alcohol and xylene and allow drying.
13. Dip in xylene and allow drying.
14. Mount in DPX and observe under microscope.

Interpretation of results

Nuclei: Blue

Cytoplasm: Pink to pale pink

Acidophilic cells: Red

Basophilic cells: Blue Green

Erythrocytes: Orange-red

Keratin: Orange-red

Superficial cells: Pink

Intermediate & Parabasal Cells: Blue Green

Eosinophil: Orange Red

Candida: Red

Trichomonas: Grey green

Crystal violet staining

Principle

Gram's Method uses retained crystal violet dye during solvent treatment to amplify the difference in the microbial cell wall. The cell walls for gram-positive microorganisms have higher lipid content than gram-negative cells. First, crystal violet ions penetrate the cell wall of both types of cells. Then, iodine is added to form a complex that makes the dye difficult to remove, in a step referred to as "fixing" the dye. Following iodine, the cells are treated with decolorizer, a mixture of ethanol and acetone, which dissolves the lipid layer from the gram negative cells, and dehydrating the thicker gram-positive cell wall. As a result, the stain leaches from gram-negative cells and is sealed in gram-positive cells. With expedient removal of the decolourizer, cells will remain stained. The addition of a safranin counterstain to dye the gram negative cells with a pink colour for easier observation under a microscope. Thus, gram-positive cells will be stained purple and gram-negative cells will be stained pink.

Preparation of solutions

Gram Crystal Violet Solution:

- Dissolve 20 g of crystal violet in 100 ml of ethanol to make a crystal violet stock solution.
- Similarly, dissolve 1 g of ammonium oxalate in 100 ml of water to make an oxalate stock solution.
- The working solution is obtained by mixing 1 ml of the crystal violet stock solution with 10 ml of water and 40 ml of the oxalate stock solution. Store the working solution in a drop bottle.

Methylene Blue Solution:

- Dissolve 1 g of methylene blue, 90% dye content, in 100 ml of ethanol, this is solution A.
- Mix 0.03 g of KOH in 300 ml of water, this is solution B.
- Mixing solutions A and B yields the working solution.
- **Gram Iodine Solution:**
- Dissolve 1 g of iodine, 2 g of potassium iodide and 3 g of sodium bicarbonate in 300 ml of water.
- **Gram Decolorizer Solution:**
- Mix equal volumes of 95% ethanol and acetone.

Gram Safranin Solution:

- Dissolve 2.5 g of Safranin O in 100 ml of 95% ethanol to make a stock solution.
- Working solution is obtained by diluting one part of the stock solution with five parts of water.

Procedure

1. Add about 5 drops of crystal violet stain over the fixed culture. Let stand for 60 seconds. Note that a clothes pin is used to hold the slide during the staining procedure to avoid staining one's hand.
2. 2. Pour off the stain and gently rinse the excess stain with a stream of dH2O. Note: The objective of this step is to wash off the stain, not the fixed culture.
3. 3. Add about 5 drops of the iodine solution on the smear, enough to cover the fixed culture. Let stand for 30 seconds
4. 4. Pour off the iodine solution and rinse the slides with running water. Shake off excess water from the surface.
5. Add a few drops of decolorizer so the solution trickles down the slide. Rinse it off with water after 5 seconds. Stop when the solvent is no longer colored as it flows over the slide. Note: Leaving the decolorizer on for longer than 5 seconds will cause excess decolorization in the gram-positive cells, and proper staining will not occur.
6. Counterstain with 5 drops of the Safranin solution for 20 seconds.
7. Wash off the red Safranin solution with water. Blot with bibulous paper to remove any excess water. Alternatively, the slide may be shaken to remove most of the water and air-dried.
8. Liberally wash off any spilled stain immediately with water to avoid leaving permanent marks on the sink, lab bench or glassware.
9. Examine the finished slide under a microscope.