

TEXT BOOK OF
BIOCHEMISTRY

For 2nd year Medical Laboratory Technician Course
(INTERMEDIATE VOCATIONAL)

Author :

S. Srinivasa Rao, B.Pharmacy,
Jr. Lecturer in M.L.T.,
Alluri Sri Rama Raju Govt. Junior College,
Shanthi Nagar, Khammam.

Editor :

S. Kamalakar Rao, M. Pharmacy
Senior Lecturer in Pharmacy,
Department of Biotechnology,
Govt. institute for post diploma courses
in engineering and technology,
Ramanthapur, Hyderabad.

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I - INSTRUMENTAL METHODS OF BIOCHEMICAL ANALYSIS

Quantitative determination of substances in body fluids and tissues is needed for diagnosis of clinical condition, progress of therapy / pharmacotherapy. This is also facilitated by using instruments basing on the properties of the substances like absorption of radiation, flame emission / flame absorption, fluorescence, scattering of light etc. Often instrumental methods include chemical steps. Flame photometry depends on absorption / emission of radiation in flame. Fluorimetry depends on the phenomenon of fluorescence. Nephelometry depends on scattering of light by suspended particles in fluids. Potentiometry depends on change of potential at the end point of a chemical reaction.

Instrumental methods have certain advantages over other methods. They are -

1. Small quantity of sample being analysed is enough.
2. Determination can be highly sensitive.
3. Results are reliable.
4. Analysis is fast.
5. Complex samples can also be handled easily.
6. Time economy factor will help more samples to be analysed in less

time.

Instrumental methods have certain limitations also. They are -

1. Calibration of the instrument is needed in the beginning or continuously.
2. Investment cost is high.
3. Special training is needed for the lab personnel.
4. Instruments require sufficient space to instal.
5. Care and maintenance of instruments should be perfect.
6. Sensitivity and accuracy depend on the quality of instrument and also on the principle on which instrument works.

1 - FLAME PHOTOMETRY

Flame photometry or Flame emission spectroscopy is the quantitative method of analysis based on determination of intensity of light emitted by a metallic compound in flame. Determination of concentration of metallic compound in solution of unknown concentration is based on comparison of its flame emission

intensity with that of standard solution.

Principle and theory of Emission Flame photometry :

When a solution containing a metallic compound is aspirated into a flame, vapour containing atoms of metal is formed on evaporation of solvent. Some of these gaseous atoms of metal get excited and emit radiation characteristic of that metal. Intensity of radiation emitted by an element in flame is proportional to the concentration of that element. For ex: Sodium emits characteristic yellow light.

However, much larger number of atoms remain in ground state. These ground state atoms absorb radiation of their specific wave length. Wave length of the radiation absorbed is that, the ground state atoms would emit if they are excited..

Relationship between Flame emission intensity and concentration: Relation between flame emission intensity and concentration is direct.

i.e. if the concentration is higher, F.E.I. is higher,
lower the concentration, lower the F.E.I..

Relationship between detector response and concentration is given by-

$E \propto C$ (Flame emission intensity is directly proportional to concentration of metallic compound)

and $E \propto a$

Where E = Flame emission intensity

C = concentration

a = efficiency of atomic excitation

From this relation, following equation can be derived.

$E = K a C$

Where K = Constant.

Flame temperature: Temperature of flame lies between 1000°C and 3000°C.

S.No.	Name of the fuel	Temperature in air	Temperature in oxygen
1)	Methane	2000 ⁰ C	2700 ⁰ C
2)	Propane	1925 ⁰ C	2800 ⁰ C
3)	Acetylene	2000 ⁰ C	3050 ⁰ C

Construction of Flame Photometer :

Flame photometers consist of the following parts:

- 1) Pressure regulators and flow meters for the fuel gases and the flame source.
- 2) Atomizer
- 3) Filter / Mono chromator
- 4) Photo cells
- 5) Electrical circuit for measuring the intensity of radiations

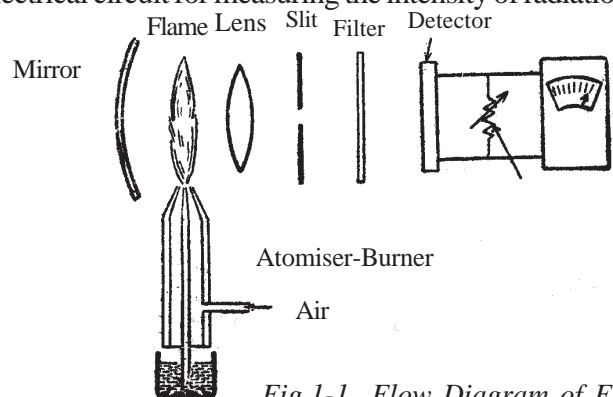


Fig.1-1 Flow Diagram of F.P.M

1) Pressure regulators and flow meters:

Regulation of pressure of fuel gas and oxygen/air is needed to achieve steady emission reading. Pressure of fuel gas can be regulated by a double diaphragm pressure regulator (10lb/in²). Pressure of oxygen air can be regulated by 20 lb/in² gauge. Rotameter should be installed in the line between fuel gas cylinder to the burner. Flame source consists of gas flow regulator, atomizer and a burner.

2) Atomizer: Atomizer is a device which introduces sample solution into the flame in the form of fine spray.

3) Filter / Monochromator:

Filter or monochromator can be used to isolate lines / bands emitted from flame. Instruments containing filter for isolation of flame emission line / bands are called as flame photometers, where as the instruments containing monochromator for the purpose are called as flame spectrophotometers. Flame sepctrophotometers use prism or grating as monochromator. Flame photometers use glass, gelatin and interference filters.

4) Photo cell : It is a device which detects flame emission intensity. Vaccum tubes or photo multiplier tubes are used for the purpose.

5) Electrical circuit: It measures the intensity of radiations emitted in flame.

Types of Flame photometers based on number of light paths:

1) Single beam instruments: Instruments containing one light path are called as single beam instruments.

2) Double beam instruments: Instruments containing two light paths are called as double beam instruments.



Fig.1-2 Flame photometer

Operation of flame photometer :

- 1) Put on the main switch
- 2)Regulate the air pressure.
- 3)Introduce glass distilled water through atomiser.
- 4) Supply the fuel gas and regulate the flame.

- 5) Adjust the filters.
- 6) Set the instrument to zero reading.
- 7) Introduce different standards and read the flame emission intensities.
- 8) Now introduce the sample and read the flame emission intensity.
- 9) Calculate by plotting calibration curve of flame emission intensity Vs. concentration.

Applications of Flame Photometer:

It is applied in analysis of alkali metals and alkaline earth metals in different samples. It has-

- 1) General applications
- 2) Clinical applications

1) General applications:

- 1) Water : Water contains sodium, calcium, magnesium and iron as bicarbonates, chlorides, hydroxides, nitrates and sulphates etc. Flame photometer can be used for the analysis of these constituents in water.
- 2) Glasses : Determination of sodium and potassium in the glass can be done by grinding the glass, dissolving in perchloric acid, and hydrofluoric acid, and diluting the residue with water.
- 3) Cements : Flame Photometer can be used to determine sodium and potassium in raw materials used in cement manufacture.
- 4) Petroleum products: f.p.m. can also be used to determine tetraethyl lead and manganese in petroleum products accurately.
- 5) Metallurgical products: Flame Photometer can be used for determination of alkali and alkaline earth metals in number of metallurgical products.
- 6) Alloys: Copper, manganese and iron in aluminium alloys can be determined by flame photometer.
- 7) Agronomical materials:
Using flame photometer, sodium and potassium can be determined in agronomy. Calcium can also be determined.

2) Clinical applications :

- 1) It can be used in pharmaceutical analysis - for analysis of Na, K etc. a) in drugs b) clinical studies.
- 2) FPM can be used to determine Na, K, Ca and lithium in samples as blood, serum, urine etc.

Flame photometer is ideally suited for clinical applications for determinations of concentrations in biological fluids.

Analysis of sodium in biological fluids can be carried out by diluting the fluid properly and atomizing on cool air gas flame. Though, effect of interfering substances like proteins, sugars etc. is minimum, in case of sodium, it has to be avoided by appropriate treatment. Cool air gas flames are preferred in case of potassium also because of minimum ionisation.

In case of tissues and bones, preliminary ashing is needed. Ashing is two types.

- 1) Dry ashing
- 2) Wet ashing

In dry ashing, specimen is reduced to ash in an electrical muffle furnace and the grey ash is further treated and diluted with suitable solvent. In wet ashing, specimen is treated with 12 M HCl, 15 M HNO₃ and digested on hot plate. Digestion is repeated twice or thrice.

Determination of calcium is difficult because of binding of calcium to proteins is complex and barrier for its determination. Hot oxyacetylene flame is used.

Determination of serum sodium and potassium :

Clinical significance:

Hyponatraemia : It is a clinical condition in which serum sodium levels are below normal.

Hyponatraemia occurs in-

- 1) Prolonged diarrhoea and vomiting

2) Salt losing nephritis

3) Addison's disease

Hypernatraemia : It is a clinical condition in which serum sodium levels are increased above normal.

Hypernatraemia occurs in-

1) Severe dehydration

2) Diabetes insipidus

3) Cushing's syndrome

4) Salt poisoning

5) Post renal conditions obstructing the flow of urine

Normal range: 133-148 m eq/L

Hypokalaemia : It is a clinical condition in which serum potassium levels are below the lower limit of normal range.

Hypo kalaemia is observed in-

1) Cushing's syndrome

2) Renal tubular damage

3) Metabolic alkalosis

4) Malnutrition

Hyperkalaemia : It is a clinical condition in which serum potassium levels are increased above the upper limit of the normal range.

Hyperkalaemia is observed in-

1) Addison's disease

2) Renal glomerular disease

3) Anuria

4) Oliguria

Normal range: 3.8 - 5.6 m Eq/L.

Requirements:

1) Test tubes

2) Dispenser

3) Bulbs

4) 50 or 100 mL push button pipette

5) F.P.M.

Reagents:

1) Sodium stock standard solution (1000 meq/L)(Stock standard -1)

Composition:

Sodium Chloride analar - 5.85 g

Glass distilled water upto - 100 ml.

Preparation: 5.85 g of sodium chloride is weighed accurately, dissolved in about 75 ml. of glass distilled water and diluted to 100 ml. with glass distilled water in a volumetric flask.

2) Potassium stock standard solution (100 m eq/L) (Stock standard-2)

Composition : Potassium chloride analar - 0.7 g

Glass distilled water upto - 100 ml.

Preparation :

0.74 g of potassium chloride analar is accurately weighed, dissolved in about 75 ml. of glass distilled water and diluted to 100 ml. with glass distilled water in a volumetric flask.

3) Sodium-Potassium mixed working standards :

Solutions	120 /2 mEq/L	140 / 4mEq/L	160/6 mEq/L
Stock standard -1	12 ml	14 ml. 1	6 ml.
Stock standard-2	2 ml.	4 ml.	6 ml.
Glass distilled water	upto 100 ml.	up to 100 ml.	up to 100 ml.

Specimen : Serum or Heparinised plasma

Procedure: 1) Take four test tubes and label them as T, S₁, S₂ and S₃

2) Pipette reagents as follows.

	T	S ₁	S ₂	S ₃
a) Glass distilled water	10 ml.	10 ml.	10 ml.	10 ml.
b) Heparinised plasma/serum	0.1 ml.	-	-	-
c) Standard-1	-	0.1 ml.	-	-
d) Standard-2	-	-	0.1 ml.	-
e) Standard-3	-	-	-	0.1 ml.

3) Mix and transfer to bulbs for flame photometric determination.

4) Determine flame emission intensities.

5) Prepare a calibration curve.

6) Determine the conc. of unknown from calibration curve.

Determination of Urinary Sodium :

Requirements : Same as for serum sodium/potassium determination

Procedure: Same as for serum sodium / potassium determination

Specimen : Urine

Calculation :

Qty. of sodium excreted in urine in 24 hours =

$$\frac{\text{Volume of 24 hours urine X conc. of sodium in 24 hrs. urine}}{1000}$$

Normal range: Average 120 meq/L.

Determination of Urinary Potassium :

Requirements : Same as for serum sodium/potassium determination

Procedure: Same as for serum sodium / potassium determination

Specimen: Diluted urine (1:10)

Calculation:

Multiply the reading with 10.

$$\frac{\text{Potassium excreted in urine in 24 hrs}}{\text{Volume of 24 hours urine X Conc. of urine potassium}} = \frac{\text{Reading}}{1000}$$

Normal range: 40 meq/L.

2 - FLUORIMETRY

Quantitative method of analysis based on measurement of intensity of fluorescence is called as fluorimetry.

Fluorescence , principle and theory , construction of Fluorimeter , General and clinical applications :

Photoluminescence : Phenomenon of absorption of radiation and re-emission of some of radiation is called as photo luminiscence.

Fluorescence: When re-emission of radiation after absorption is instantaneous, the phenomenon is called as fluorescence.

Phosphorescence: When re-emission of radiation takes place after some time lag, the phenomenon is called as phosphorescence.

Principle and theory of Fluorescence : Relation between intensity of fluorescence and concentration can be explained by Lambert-Beer's Law.

$$I_e / I_o = e^{-kct}$$

where
 I_e = Intensity of emitted light
 I_o = Intensity of incident light
 e = exponential
 k = constant
 c = concentration
 t = length of the light path through the solution.

If the magnitude of K_{ct} is small, equation reduces to

$$F = Kc$$

Hence, relation between concentration and intensity of fluorescence is direct.

Construction of fluorimeter : Instruments used for fluorimetric analysis are called as fluorimeters or fluorophotometers. In this method of analysis, intensity of light emitted by the substance at right angles to the incident light is measured to avoid interference from direct beam from the source.

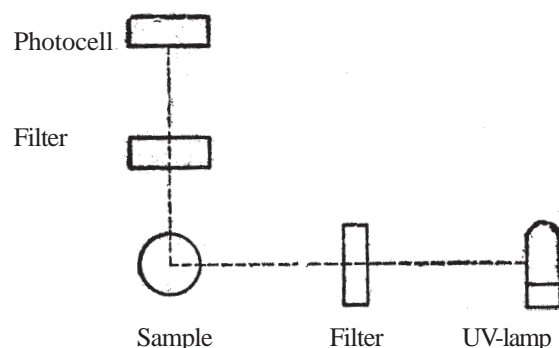


Fig.1-3 Flow Diagram of Fluorimeter

Construction of fluorimeters:

- 1) Light source
- 2) Filters/monochromators
- 3) Cells
- 4) Detectors

1) Light source :

Mercury vapour lamp is the widely used light source in fluorimetry. Sodium arc lamp is also employed. Tungsten lamp may also be used. Hydrogen arc lamp or deuterium arc lamps are not employed.

2) Filters / Monochromators:

Interference and absorption filters are used. Grating chromators are used in spectroflurometers.

3) Cells : Cylindrical and rectangular cells are used. They may be made of either

silica or glass. Cells are to be designed to minimise the scattering of radiation.

4) Detectors: Photomultiplier tube is used as detector.

General and clinical applications : It has applications in chemical analysis and pharmaceutical analysis and for diagnostic purposes :

- 1) Inorganic analysis -
 - Cations
 - Anions
- 2) Clinical applications : Some of the applications are-
 - a) Determination of Thiamine (vit B₁) in drugs and serum
 - b) Determination of Riboflavin (vitamin - B₂) in drugs and serum
 - c) Determination of Tetracycline in drugs and serum

3) - NEPHELOMETRY

When light is passed through a suspension, part of it is dissipated by absorption, reflection and refraction. Remaining portion is transmitted. Measurement of intensity of light transmitted is related with concentration of dispersed phase.

Method of analysis for measurement of concentration based on measurement of intensity of transmitted light is called TURBIDIMETRIC ANALYSIS. The term scattered light is used instead of reflected light since reflection is irregular and diffuse. This scattered light accounts for opalescence or cloudiness. Method of analysis for measurement of concentration based on measurement of intensity of scattered light (light at right angles to incident light) is called NEPHELOMETRIC ANALYSIS. Nephel means cloud in Greek. Nephelometric analysis is most sensitive for very dilute suspensions. Technique of turbidimetry resembles filter photometry and that of nephelometry resembles Fluorimetry.

Suspensions of reasonably uniform character should be produced. For this, following conditions should be controlled. They are - 1. Concentrations of ions

combining to form precipitate .2 . Manner of mixing 3. Amounts of other substances present 4 . Temperature

Nephelometers : Nephelometers are instruments used in Nephelometry .Nephelometers are of two types . 1) Visual nephelometers (Comparator type)

2) Photo electric type nephelometers

Visual nephelometers : Visual nephelometers are superseded by photoelectric type. Duboscq colorimeter can be used for nephelometric work . Cups of this instrument must be replaced by clear glass tubes with opaque bottoms for nephelometric work . This is because nephelometric work involves measurement of intensity of scattered light . Light shall enter at right angles to the cups . This light entering at right angle must be regulated so as to obtain equal illumination on both sides . Standard suspension is placed in one cup and unknown is placed in another cup. Dividing line between two fields will disappear when the two fields match .

Photo electric type nephelometers : Nephelometers containing photocell for detection of intensities of scattered light are called photoelectric nephelometers . Fluorimeters can be adapted for use in nephelometry .

Construction of photoelectric nephelometer : EEL nephelometer is discussed for understanding of construction of a nephelometer .Essential part of nephelometer is nephelometer head .

Parts of nephelometer are -

- 1) Light source
- 2) Filter
- 3) Sample holder
- 4) Metal cap
- 5) Reflector
- 6) Photo cell
- 7) Galvanometer

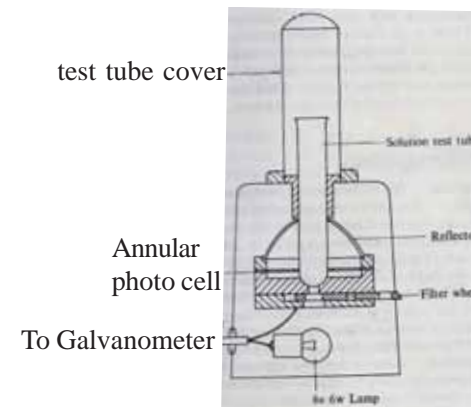


Fig.1-4 Diagram of photo electric nephelometer

1) Light source : A six volts six watt lamp is fit within rhe base of this unit . It will shine light vertically through orifice of annular photocell onto the hemispherical base of a test tube .A stabilising transformer is incorporated to supply power to nephelometer lamp .

2) Filter : A tricolour filter wheel is interposed between light and photocell . It contains filters OB2, OR1 and OGR1. It is provided with a position for white light measurement . While doing a determination , a filter of colour of the solution being determined should be selected .

3)Sample holder : A test tube is used as sample holder . It is the means of holding the standard or unknown solution while measuring the intensity of scattered light . Standard size of a matched test tube is 1.5 cm (diameter) x 15cm (length).

4) Metal cap :A metal cap is provided to fit over solution test tube when instrument is in use and it is meant for excluding extraneous light . When this cap is removed, a microswitch is operated to disconnect photocell from galvanometer and this arrangement is meant for preventing damage to the suspension by large current resulting from sudden entry of external light .

5) Reflector : A reflector is mounted above the photocell to collect light scattered by multiple reflections from particles of the suspension in the solution test tube . It is then directed onto the photocell .

6) Photocell : Photocell is for detecting the intensity of light scattered .While eye

is used for comparison of intensities of light scattered by standard and unknown in visual nephelometry by matching the two fields, Photocell is used for the purpose of comparison of intensities in photoelectric nephelometry.

7) Galvanometer: Current generated in photocell due to incidence of light scattered by the particles of suspension in solution test tube is fed to a sensitive galvanometer by means of a flexible lead and plug. This is of taut-suspension mirror type. A large plastic knob is present which protrudes through the top of galvanometer casing. It provides a smooth zero setting.

Operation of a photoelectric nephelometer:

- 1) Adjust zero control knob of galvanometer to zero reading. Connect the nephelometer head and galvanometer.
- 2) Remove cap cover and place standard in position. Replace the cap.
- 3) Select the required filter so that colour is same as that of the solution.
- 4) Adjust the sensitivity control of galvanometer so as to get a reading of one hundred divisions on the scale.
- 5) Remove standard and place blank (distilled water). Adjust galvanometer reading to zero using zero control knob of galvanometer.
- 6) Check the reading of standard. Check the full scale deflection and zero settings.
- 8) Note the readings of various dilutions of standard.
- 9) Draw the calibration curve with galvanometer readings against concentrations of different dilutions of standard.
- 10) Place unknown in position and note the galvanometer reading.
- 11) Find the concentration of unknown from standard calibration curve.

Applications of nephelometry: 1) Determination of Sulphate ion content

2) Determination of Phosphate ion content

1) Determination of Sulphate ion content: Nephelometric method of determination of sulphate ion content involves conversion of sulphate ion into Barium sulphate. It is done by reacting solution of sulphate ion with Barium chloride. Intensity of scattered light is compared with that of Potassium sulphate standard solution by preparing calibration curve. Sodium chloride and Hydrochloric acid are added before precipitation to prevent growth of microcrystals of Barium sulphate. Glycerol-Ethanol solution stabilises turbidity.

2) Determination of Phosphate ion content: By this method, 1 part of phosphorous per 300 million parts of water can be detected. It is done by formation of strychnine phosphomolybdate, which is white in colour. Strychnine phosphomolybdate is formed by reaction of phosphate ion with Molybdate-Strychnine reagent. Scattered light intensity is compared with that of Standard solution of Potassium dihydrogenphosphate by preparing calibration curve.

4-BASIC PRINCIPLES AND APPLICATIONS OF POTENTIOMETRY

Potentiometry is method of quantitative determination by measuring e.m.f.

When a metal M is immersed in a solution containing its own ions M^{n+} , value of electrode potential established is given by Nernst equation.

$$E = E^\circ + \frac{RT}{nF} \ln a_M^{n+}$$

E° is standard electrode potential of metal and is constant.

E is electrode potential established and can be measured by combining the electrode with a reference electrode (commonly a saturated calomel electrode) and measuring e.m.f. of resultant cell.

a_M^{n+} is metal ion activity and it can be calculated knowing E_r (and thus deducing the value of E) and E° .

Direct potentiometry : Direct potentiometry is procedure of using single measurement of electrode potential to determine the concentration of an ionic species in the solution.

Indicator electrode : Electrode whose potential is dependent on the concentration of ion to be determined is called indicator electrode .

Reference electrodes : A reference electrode is one , having constant potential .

Electrode of the first kind : When ion to be determined is directly involved in the electrode reaction ,electrode is said to be the electrode of the first kind .

Electrode of the second kind : When ion to be determined is not involved in the electrode reaction , Electrode is said to be electrode of the second kind .

An example of it is silver - silver chloride electrode which is formed by coating a silver wire with silver chloride .Silver wire can be regarded as silver electrode and potential is given by the equation -

$$E = E_{Ag}^{\circ} + (RT/nF) \ln a_{Ag^{+}}$$

Silver ions involved are derived from silver chloride . By the solubility product principle , activity of these ions are governed by the chloride ion activity .

$$a_{Ag^{+}} = K_s(AgCl) / a_{Cl^{-}}$$

Electrode potential can be expressed as -

$$E = E_{Ag}^{\circ} + (RT/nf) \ln K_s - (RT/nF) \ln a_{Cl^{-}}$$

It is clearly governed by the activity of chloride ions. In Nernst equation , the term RT/nF involves known constants and introducing factor for converting logarithms to logarithms to base 10 the term has a value of 0.0591 V at 25°C when n is equal to 1.

An element of uncertainty is introduced into e.m.f. measurement by liquid junction potential . Liquid junction potential is established at the interface between two solutions of which one is pertaining to reference electrode and other is pertaining to indicator electrode .This liquid junction potential can be overcome by replacing the reference electrode by electrode containing standard solution of same cation

as that in the unknown solution together with a rod of the same metal as in the indicator electrode .

Activity of metal ion in the test solution can be measured by the following formula -

$$E_{cell} = (RT/nF) \ln \frac{\text{(activity) known}}{\text{(activity) unknown}}$$

Null point potentiometry : Null point potentiometry is method of potentiometry in which e.m.f. will be zero when the two solutions have the same concentration

Potentiometric titration : It is a method of titration procedure in which potentiometric measurements are carried out in order to detect end point .

Reference electrodes : A reference electrode is one , having constant potential .Reference electrodes used are Hydrogen electrode , Calomel electrode , Silver-Silver chloride electrode etc.Potentials of all electrodes are quoted with reference to standard hydrogen electrode. A typical hydrogen electrode consists of a piece of platinum foil , coated electrolytically with platinum black and immersed in a solution of hydrochloric acid containing hydrogen ions at unit activity .

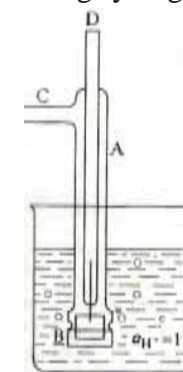


Fig.1-5 Hydrogen electrode

Some of the examples of hydrogen electrodes are Hilderband bell type electrode , Lindsey hydrogen electrode etc . Calomel electrode is the most widely used electrode due to its ease of preparation and constant nature of potential .A calomel half cell is one in which mercury and calomel are covered with potassium chloride of definite concentration . Silver-silver chloride electrode is of next importance to calomel electrode . This electrode consists of a silver wire or a silver plated

platinum wire, which is coated electrolytically with a thin layer of silver chloride dipping into a standard solution of potassium chloride.

Indicator electrodes: An indicator electrode is one, the potential of which is a function of concentration of the ion to be determined. Hydrogen electrode, antimony electrode, glass electrode etc. are used as indicator electrodes. In addition to its function as reference electrode, Hydrogen electrode also acts as indicator electrode. It can be used to measure pH of solutions and also in potentiometric neutralisation titrations. Hydrogen electrode cannot be used in solutions containing oxidising agents. It is also unsatisfactory in presence of salts of noble metals. Antimony electrode is antimony-antimony trioxide electrode. This electrode is prepared by casting a stick of antimony in air. A wire is attached to one end of antimony rod and other end is inserted in solution. Potential is measured against a reference electrode. Glass electrode is the most widely used hydrogen ion sensitive electrode. When a glass membrane is immersed in a solution, a potential is developed. This potential developed is a linear function of the hydrogen ion concentration of the solution.

Ion sensitive electrodes: Ion sensitive electrodes are alkali ion sensitive electrodes, solid membranes, liquid membrane electrodes etc. Glass electrodes used for measurement of pH, which are constructed with lime soda glass are subjected to alkaline error. It is due to sodium ion in the composition of glass. If sodium in the glass is replaced by lithium, alkaline error is eliminated.

Measurement of e.m.f. of a cell: Measurement of e.m.f. of a cell can be done by -1. Poggendorff's compensation method

2. Tinsley general utility potentiometer

Poggendorff's compensation method is the most satisfactory method for measurement of e.m.f. of a cell. Principle of this method is to balance unknown e.m.f. against a known e.m.f.. Current will not flow through galvanometer placed in the circuit when the two e.m.f.s are exactly equal. In Tinsley general utility potentiometer, balancing is effected upon a main dial having 18 steps of 0.1 volt and a calibrated circular slide wire range of

- 0.005 to + 0.01. It can be read to 0.0001 volt by estimation.

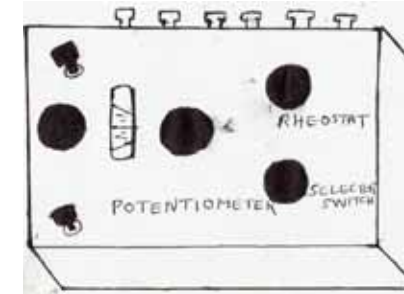


Fig.1-6 Potentiometer

pH meters: Potentiometers with glass electrode were used for determining pH of solutions. Hence they were called as pHmeters. pH meters were classified as

1. Direct reading pHmeters
2. Potentiometric type instruments

In direct reading type, scale was calibrated in units of pH. In potentiometric type instruments, potentiometric circuit was employed.

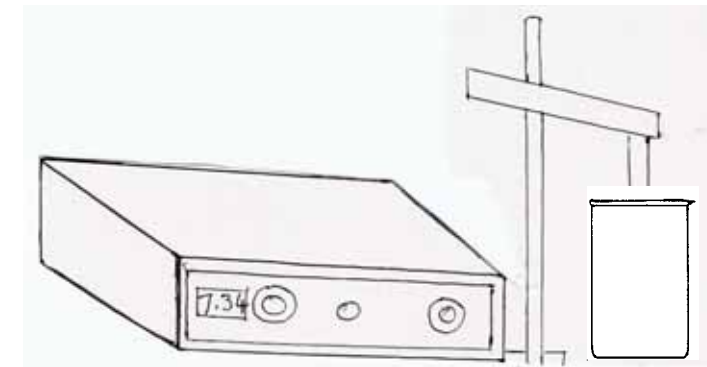


Fig.1-7 pH meter

Selective ion meters: Direct reading meters suitable for use with specific ion electrodes are referred to as ion activity meters. Most of them can be used as pH meters and by virtue of extended range of applications, circuitry required is more complex.

Direct potentiometry: Direct potentiometry involves measurement of e.m.f. between indicator electrode and reference electrode. Measurement of hydrogen ion concentration or some other ion concentration in a solution is direct potentiometry is an example of direct potentiometry. Normal procedure to

measure pH of a given solution is to use a glass electrode along with a saturated calomel reference electrode and to measure e.m. f. of the cell with a pH meter.

Potentiometric titrations : Titrations involving measurement of e.m.f. between indicator electrode and reference electrode for detection of end point are called Potentiometric titrations. In potentiometric titrations, absolute potentials are not required. Measurements are made while titration is in progress. End point is determined by a sudden change in potential in the plot of e.m.f. readings against volumes of titrating solution. One electrode must be maintained at a constant potential. Other must act as indicator electrode. Solution must be stirred during titration. To measure e.m.f., system should be connected to a potentiometer.

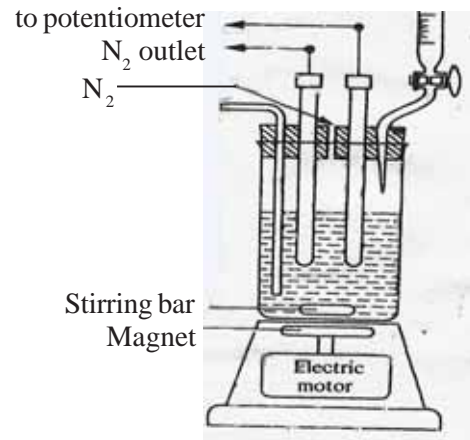


Fig.1-8 Potentiometric Titration

Reactions in Potentiometric titrimetric analysis : Reactions in Potentiometric titrimetric analysis can be broadly classified into

1. Neutralisation reactions (Acidimetry & alkalimetry) -
2. Complexometric reactions
3. Precipitation reactions
4. Oxidation - reduction reactions

Applications of potentiometry : Potentiometry is useful in determining the concentrations of metal ions in solutions. Potentiometric titrations are useful in detecting end point in titrations. Determination of fluoride can be done by potentiometry. Potentiometric titrations can be useful to titrate mixture of acids differing greatly in strengths like mixture of hydrochloric acid and acetic acid. Potentiometric titrations can be used to detect end point in titrations involving oxidation reduction reactions ex- Iron with potassium dichromate or permanga-

nate etc. They can be used to detect end point in precipitation reactions. ex-titration of silver ions with a halide. They can also be used in precipitation reactions ex - titration of potassium cyanide with silver nitrate.

Summary

Method of analysis using flame photometer is called as flame photometry. Flame Photometer is an instrument useful for determination of electrolytes in body fluids in clinical studies. This method of quantitative analysis is based on comparison of flame emission intensity of the unknown with that of standard to determine the concentration of unknown. Relation between conc. and FEI is direct.

Parts of flame photometer are 1) Pressure regulators and flowmeters for fuel gas and oxidant gas.

- | | |
|--------------|---------------------------|
| 2) Atomizer | 3) Filter / monochromator |
| 4) Photocell | 5) Electrical circuit |

It has diverse applications for chemical, clinical pharmacy, pharmaceutical analysis, diagnostic, agronomical and other purposes.

Fluorimetry : Absorption and Instantaneous re-emission of radiation by some chemical substances is called fluorescence. Measurement of fluorescence forms the basis of fluorimetry.

Fluorimeter consists of-

- | | |
|-----------------|---------------------------|
| 1) Light source | 2) Filter / monochromator |
| 3) Cell | 4) Detectors |

Fluorimetry finds applications in clinical pharmacy studies, diagnostics, chemical analysis and pharmaceutical analysis.

Nephelometry : It is a method of volumetric analysis of measurement of concentrations by measuring intensity of scattered light and comparison with that of standard solution.

Two methods of nephelometry are visual and photoelectric types. Parts of nephelometer are - 1) Light source 2) Filter 3) Sample holder 4) Metal cap 5) Reflector 6) Photocell 7) Galvanometer

Nephelometer is operated by checking zero and full deflection, determining scattered light intensity of unknown and evaluating from standard calibration curve obtained using various dilutions of standard. Some of its applications are -

- 1) Determination of Sulphate ion content and 2) Determination of Phosphate ion content.

Potentiometry is method of quantitative determination by measuring e.m.f. A reference electrode is one, having constant potential. An indicator electrode is one, the potential of which is a function of concentration of the ion to be determined. Measurement of e.m.f. of a cell can be done by - 1. Poggendorff's compensation method 2. Tinsley general utility potentiometer. Potentiometers with glass electrode used for determining pH of solutions are called as pHmeters. Direct reading meters suitable for use with specific ion electrodes are referred to as ion activity meters. Direct potentiometry involves measurement of e.m.f. between indicator electrode and reference electrode. Titrations involving measurement of e.m.f. between indicator electrode and reference electrode for detection of end point are called Potentiometric titrations.

Essay Questions :

- 1) Write the principle, construction and operation of f.p.m.
- 2) How do you determine serum sodium by Flame Photometry?
- 3) Write about determination of serum potassium by Flame Photometry.
- 4) How do you determine urinary sodium by Flame Photometry?
- 5) Give the principle and procedure of determination of urinary potassium by flame photometry.
- 6) What are the diverse applications of Flame photometer?
- 7) Give the principle, construction and applications of Fluorimeter.
- 8) Write a note on nephelometry.
- 9) Write briefly about potentiometry.

Short Answer Questions

- 1) Define Flame photometry.
- 2) What are different types of Flame photometry?

- 3) Give the principle of Flame Emission Photometry.
- 4) What is the relation between conc. and F.E.I.?
- 5) Give the range of Flame temperature.
- 6) Exemplify some fuel gases.
- 7) Differentiate between oxidant gases air and oxygen with respect to the capacity for combustion of fuel gases.
- 8) Mention diagnostic applications of Flame Photometer.
- 9) What is Hypokalaemia?
- 10) Define fluorimetry and fluorescence.
- 11) Name the parts of fluorimeter.
- 12) Write the filters used in fluorimetry.
- 13) Write any two applications of fluorimetry.
- 14) Differentiate between turbidimetric analysis and nephelometric analysis.
- 15) Mention the types of nephelometers.
- 16) Write about light source in nephelometer.
- 17) Give two applications of nephelometry.
- 18) Define potentiometry and write Nernst equation.
- 19) Write the definitions of reference electrode and indicator electrode.
- 20) What is meant by electrode of first kind and electrode of second kind?
- 21) What is null point potentiometry?
- 22) Write the definition of potentiometric titrations.
- 23) Mention the types of reactions in potentiometric titrations.
- 24) What are different types of pHmeters?

II - SEPARATION TECHNIQUES

Separation means to divide a homogeneous mixture into its component substances. Separations are made by different methods based on the physical and chemical properties of the component substances of the homogeneous mixture. Separation is needed for purification, qualitative identification, quantitative determination etc. Separation of homogeneous mixtures can be done using different methods like filtration, distillation, sublimation, precipitation, centrifugation, extraction, crystallisation, floatation, dialysis, chromatography, electrochromatography (also called as electrophoresis) etc. Of these, chromatography has a unique position.

1 - CHROMATOGRAPHY

Definition, Basic principles, Different types and their techniques, General and Clinical applications :

Definition: Chromatography is a method of analysis by which components of a mixture are separated by redistribution of the molecules of the mixture between two or more phases. Complex mixtures, isomers and unstable substances can also be isolated by this technique. It is employed for purification, separation and preparative purposes.

Chromatographic experiment was carried out by David Day, a geologist and Mikhail Tswett, Botanist and Physical chemist. The term chromatography and its principles were first discovered by Mikhail Tswett in 1906. In his experiment, he separated various pigments from a leaf extract sample in petroleum ether passing through a column of CaCO_3 . Richard Kuhn and his co-workers applied adsorption chromatography as a preparative method for separation of carotene into its components in 1931. Partition chromatography was proposed by Martin and Synge in 1941. They were awarded Nobel prize in 1952 for this discovery. Conden, Gordon and Martin discovered Paper chromatography in 1944. Reversed phase paper chromatography was discovered by Kirtchovesky and Tiselius. Martin and James introduced gas chromatography in 1952. Martin and Synge applied partition to Gas chromatography. Thin layer chromatography was described by Inmailov and Sehraiber.

Phases in chromatography:

The two phases in a chromatographic method of analysis are

- 1) Stationary phase.
- 2) Mobile phase.

Stationary phase : The phase in a chromatographic method which acts as support for the mobile phase for separation to take place is called as stationary phase.

Mobile phase : The phase in a chromatographic method which moves over stationary phase to affect separation is called as mobile phase.

Types of Chromatography based on states of matter of the phases :

- 1) Solid-liquid chromatography:

Chromatographic method in which stationary phase is in solid state and mobile phase is in liquid state is solid-liquid chromatography.

- 2) Liquid-Liquid chromatography :

Chromatographic method in which stationary phase is in liquid state and mobile phase is also in liquid state is liquid-liquid chromatography.

- 3) Liquid-gas chromatography:- Chromatographic method in which stationary phase is in liquid state and mobile phase is in gaseous state is Liquid-Gas chromatography.

- 4) Solid-gas chromatography : Chromatographic method in which stationary phase is in solid state and mobile phase is in gaseous state is called Solid-Gas chromatography.

Types of chromatography based on principle: Following are the types of chromatography based on their principles. They are-

- 1) Adsorption chromatography
 - 2) Partition chromatography
 - 3) Exclusion chromatography
 - 4) Ion exchange chromatography
- 1) Adsorption chromatography: It is the method of chromatography in which separation of the components of a mixture takes place by differential

adsorption caused by intermolecular forces between surface atoms of the solid and molecules of the external solute.

- 2) Partition chromatography: It is the method of chromatography in which components of a mixture separate by partitioning between the two phases. Partition between the two phases takes place due to differential solubilities of the components in the two phases.

In partition methods, stationary liquid phase is held on a solid inert support. This solid may be an adsorbant. In adsorption method, mobile liquid phase retaining over the surface of stationary phase contributes to partition. Thus, chromatography is neither purely adsorption nor partition.

- 3) Exclusion chromatography : Chromatographic method in which exclusion is used for separation of components of the mixture is called as exclusion chromatography. In this process, separation of components takes place according to molecular size. Gel permeation and sieving separation are recently developed techniques in exclusion chromatography. In gel permeation, separation is affected according to the size of the solute molecules. Gel permeation is widely used for separation of sugars, proteins, butyl rubbers and many other molecules. In sieving separations, natural and synthetic zeolites are widely used.

- 4) Ion exchange chromatography : Chromatographic method in which ion exchange causes separation of components of mixture is called as ion exchange chromatography. Cation exchangers exchange cations while anion exchange resins exchange anions.

Cation exchangers-zeolites, clays etc.

Anion exchangers-dolomite, heavy metal silicates.

Different chromatographic methods in general use are :

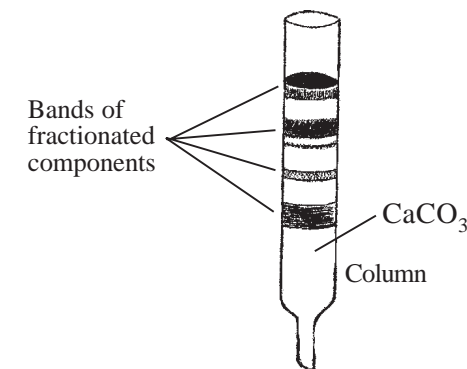
- 1) Column Chromatography
 - a) Column adsorption chromatography.
 - b) Column partition chromatography.

- 2) Thin layer Chromatography
 - a) Thin layer adsorption chromatography.
 - b) Thin layer partition chromatography.
- 3) Paper Chromatography
 - a) Paper adsorption chromatography.
 - b) Paper partition chromatography.
- 4) High-pressure liquid chromatography.
- 5) Ion exchange chromatography.
- 6) Exclusion chromatography.
- 7) Electrochromatography.
- 8) Gas chromatography
 - a) Gas solid chromatography
 - b) Gas-liquid chromatography.

1) COLUMN CHROMATOGRAPHY :

Chromatographic method, using column for conducting the separation of components of a mixture is called as column chromatography.

Column:- Column for chromatographic use is usually cylindrical with axial length much greater than diametric length. The tube is usually made of glass. Accessories are attached at the top and bottom of the column. Porous septum is present at the bottom.



Types of column chromatography :

Two types of column chromatography are there. They are-

- a) Column adsorption chromatography.
- b) Column partition chromatography.

Fig.2-1 Column chromatography

a) Column adsorption chromatography:- This method of chromatography was first demonstrated by Tswett in 1906. He separated plant pigments by pouring the ether extract of plant pigments over vertical column of CaCO_3 and irrigating with

alcohol. Bands are formed by the components in the descending order of adsorbability.

Principle : Components of the mixture separate by differential adsorption. The most strongly adsorbed component forms upper most band. Least strongly adsorbed component forms lowest band. The bands in between are formed by the components in descending order of adsorbability.

Requirements :

- 1) Column.
- 2) Adsorbant.
- 3) Solvent.
- 4) Cotton.

1) Column : Already described above.

2) Adsorbant : Adsorbant is stationary phase.

Requirements of Adsorbant :

- a) It should not be soluble in the solution under analysis.
- b) It is required to be colourless.
- c) It should not react chemically with mixture under analysis.

Types of adsorbants:

There are three types of adsorbants. They are-

- a) Weak adsorbants Ex:-Sucrose, Starch.
- b) Intermediate adsorbants ex:- CaCO_3 , $\text{Ca}_3(\text{PO}_4)_2$, $\text{Ca}(\text{OH})_2$ and $\text{Mg}(\text{OH})_2$.
- c) Strong adsorbants ex:-Alumina, Charcoal, Fuller's earth.

Solvent:- Solvent is mobile phase. Solvent helps in bringing about resolution of the components of the mixture into bands. It is also called as eluting agent.

Requirements of a solvent :

- 1) Solvent should have viscosity suitable to run through the column.
- 2) It should be pure enough.
- 3) It should have enough stability.
- 4) It should provide maximum resolution.

Solvents are three types :

- 1) More polar solvents : Ex: Benzene.
- 2) Polar solvents:- Water, Ethyl alcohol etc.

3) Non-polar solvents:- Petroleum, Ether, CCl_4 etc.

Procedure:- A wad of cotton is placed over the porous septum. Column is built above the cotton wad by the adsorbant. Solution of the substance is poured over the adsorbant column. Irrigating solvent is poured over the column. This process of irrigation by the solvent is also called as elution. Elution of the packed column by the solvent causes desorption of the components of the mixture. After reasonable resolution has occurred, column is drained, extruded carefully and bands are separated by cutting with knife or scalpel. Scrappings are extracted with a solvent and resulting substance is estimated by suitable method. Alternatively, continuous elution with solvent can be done to get one component after the other. Each component is received in a separate container.

b) Column Partition-chromatography :

Martin and Synge proposed partition chromatography in 1941. In this chromatography, stationary phase is a liquid held on an inert porous solid such as cellulose. One of the liquids of the stationary phase is a liquid held on an inert porous solid such as cellulose. One of the liquids of the stationary phase is frequently water. Mobile phase can be liquid mixture or gas. More than one solvent is always present in a liquid mobile phase.

Principle :

Separation of the components of the mixture takes place by partitioning between two phases due to differential solubilities of the components between the two phases.

Requirements :

- 1) Column.
- 2) Supports for liquid stationary phase.
- 3) Solvents.

1) Column: Described in column adsorption chromatography.

- 2) Supports for liquid stationary phase: Since the stationary phase is a liquid, it needs a support to be retained over. Common supports for column partition chromatography are glass powder, rubber powder, organic polymers etc.
- 3) Solvents: Solvents include both stationary and mobile phase liquids. Stationary phase liquid is retained by the support whereas mobile phase liquid runs over it.

Common solvents for column partition chromatography include light petroleum, Cyclohexane, Carbon tetrachloride, Toluene, Benzene, Chloroform, Ether, Ethyl acetate, Acetone-Propanol, Ethyl alcohol, Methyl alcohol, Water, Pyridine, Organic acids, Inorganic acids and bases etc.

Ex:- Solvent systems for Column partition chromatography:

<u>Stationary phase</u>	<u>Mobile phase</u>
Water	Butanol, Isobutanol
Water + Acid	Benzene, Toluene, Hexane etc.

In reversed phase partition, Mobile phase liquids act as stationary phase and stationary phase liquids act as mobile phase.

Procedure : A Wad of cotton is placed over the porous septum in the column. Powdered silicagel is filled in the column. Stationary liquid phase is introduced on to the support prior to the packing of column. A closely fitting disc or filter paper is placed at the top of the column. Solution of substance under analysis is now added to the column. Solvent of mobile-phase is allowed to move downwards. Components of the mixture are separated into series of bands progressing down the column.

Detection of the components :

Different methods of detection of components are :

- 1) Visual detection for coloured components
- 2) Detection with U.V. lamp for colourless components
- 3) Detection by chemical development for colourless components
Ex:-Phenols with Ferric chloride.
- 4) Detection by physical properties such as viscosity, density and

refractive index.

- 5) Detection by radio isotopic methods for colourless substances.

Applications of column chromatography :

- 1) It is used to determine amino acid contents of protein hydrolysate.
- 2) It is used in separation of urinary 17-keto steroids.
- 3) It is used in separation of 17 keto steroids glucuronides.
- 4) It is used in separation of plasma cortisol etc.
- 5) It is used in the purification of Sudanred.

2) THIN LAYER CHROMATOGRAPHY (TLC) :

Izmailov and Shraiber first discovered thin layer chromatography in 1938. It was further developed by Meinhard and Hall in 1949, Kirchner, Miller and Keller in 1951, Mottier in 1952 and Reitsema in 1954. In 1958, Precise work was carried out by E.Stahl.

Definition:- Chromatography using thin layers of an adsorbant held over a supporting medium like glass plate is called as

Thin layer chromatography.

Types of T.L.C based on principles :

- 1) Adsorption T.L.C.
- 2) Partition T.L.C.
- 3) Ion exchange T.L.C.
- 4) Thin layer electrochromatography.

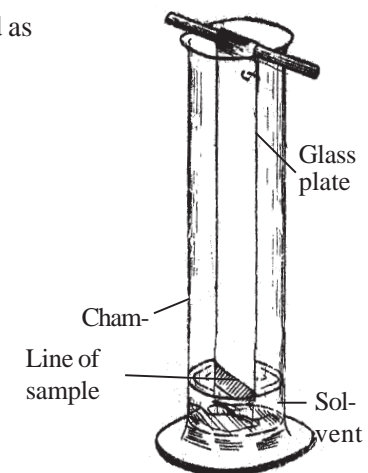


Fig.2-2 Thin layer chromatography

Principles:- In adsorption T.L.C., components of the mixture separate by differential adsorption by the adsorbant. In partition, components of the mixture separate by differential solubilities between the stationary and mobile phase liquids. Thus, components of the mixture separate between the two phases.

Requirements:-

- 1) Glass plates/Microscope slides/Plastic sheets/Aluminium foils.
- 2) Materials for producing thin layer of adsorbant/ support for stationary liquid phase.
- 3) Applicator (Stahl's applicator)
- 4) Developing chamber, capillaries, micro pipettes or micro syringes.
- 5) Solvents.
- 6) Detecting reagents.

1) Glass plates :

For producing a thin layer, a support is needed. It is provided by a glass plate. Previously coated sheets are also commercially available. Previously coated sheets are coated microscope slides or glass plates or plastic sheets or Aluminium foils.

2) Material for producing thin layer:

Common adsorbants used in T.L.C are silicagel, alumina, kieselguhr and cellulose powder, Plaster of Paris (Calcium Sulphate) is used as binding agent.

3) Applicator: Applicator is a device used to produce a thin layer of the adsorbant material over the plate. Some of the several types of commercial applicators available are

- 1) Stahl's original applicator.
- 2) Stahl's model 11-S applicator.
- 3) Stahl's G.M applicator.
- 4) Sample applicator.

Capillaries, Micropipettes or Micro syringes are used to apply sample on chromatoplates.

4) Developing chamber : Developing chamber can be a cylindrical chamber made of glass - beaker etc. Different jars are proposed by Geiss Schlitt, Ritter, Weimar, Brenner and Niederwieser, Weil and Rybicka and others. Baby food jars or glass beakers covered with aluminium foil can also be used.

5) Solvent : Almost all the solvents used in paper chromatography and column chromatography can be used in T.L.C. ex : Petroleum ether, Benzene, Chloroform, Ether, Acetone, Ethyl alcohol, Methyl alcohol, Water, Glycerine etc.

6) Detecting reagents : Physical and chemical properties can be useful for detection of the spots fractionated from mixture.

U.V. light can also be used in some cases.

Procedure :

Steps involved in T.L.C are-

- 1) Production of thin layers on plates.
- 2) Application of sample on the plates.
- 3) Development.
- 4) Detection.
- 5) Calculation of R_f Values.

1) Production of thin layers on plates :

A slurry of coating material is prepared and thin layers can be prepared with or without special equipment.

With Use of Special Equipment :

With Stahl's original applicator, 0.25 mm thick layers can be prepared. 0.25-2 mm thick layers can be prepared with Stahl's 11-S applicator. Gradient layers can be prepared with Stahl's G.M. applicator.

Methods of production of thin layers are:-

- 1) Spreading.
- 2) Pouring.
- 3) Dipping.
- 4) Spraying.

2) Application of sample on chromatoplates :

0.1 to 1% solutions of the samples are applied as single spots about 2cm from the edge by means of capillaries, micropipettes or microsyringes etc. Amount of sample to be applied depends upon-

- 1) Thickness of layer.
- 2) Principle of chromatography used.

Ex:-50-500 mg sample can be applied on 25 mm thick layer.

- 3) Development: There are different methods of development by which components of the mixture are fractionated in T.L.C. They are -
 - a) Ascending / descending development
 - b) Horizontal development
 - c) Multiple development
 - d) Step wise development
 - e) Gradient development
 - f) Continuous development
 - g) Two dimensional development

Ascending development is normally used. In this method, sample is spotted at one end. Plates are placed vertically in a container saturated with developer vapour. They are placed with the sample line just above the solvent level. Solvent moves upwards causing fractionation of the components of mixture. Process of development is also called as elution. After the development is completed, plate is removed from the solvent and dried. It is called as chromatoplate.

- 4) Detection: Detection of the fractionated components can be visual if they are coloured. Their physical properties can also be made use of for detecting. Chemical method of detection can be done by spraying with a locating reagent. Auto radiography can also be used.
- 5) Calculation of R_f values : R_f values can be determined by measuring the distances traveled by fractionated components and solvent front and substituting in the formula -

$$R_f = \frac{\text{distance moved by component}}{\text{distance moved by solvent front}}$$

Determination of R_f value can be useful for qualitative identification.

R_f values of standards can be useful for quantitative determination

Applications :

T.L.C is used in

- 1) Drug analysis.
- 2) A nalysis of natural products.
- 3) Detection of trace pesticides in water.
- 4) Identification of metabolites of drugs in excreta.
- 5) Analysis for presence of poisons.
- 6) Separation of vitamins.
- 7) Qualitative and quantitative analysis of biological fluids for the diagnosis of disease.

3) PAPER CHROMATOGRAPHY :

Paper chromatography was first introduced by schonbein in 1961. It was popularised by Consden, Gorden, Martin and Synge in 1964.

Paper chromatography can be defined as technique in which separation of mixture is carried out on specially designed filter paper.

Types of paper chromatography :

- 1) Paper Adsorption chromatography
- 2) Paper partition chromatography

Paper adsorption chromatography is separation technique in which adsorbant impregnated over paper acts as stationary phase. Paper partition chromatography is a separation technique in which paper acts as inert support, one liquid acts as stationary phase and another liquid acts as mobile phase.

Principle :

Paper adsorption chromatography is based on differential adsorption of components of the mixture by the adsorbant material impregnated over the paper. Paper partition chromatography is based on partitioning of the components between the stationary phase held over the paper and mobile phase. Principle of the paper chromatography is based on the fact that solutes have the capacity to migrate through filter paper at different rates as the solution of the sample is drawn into strips of paper by capillary action.

Requirements :

- 1) Paper.
- 2) Apparatus.
- 3) Solvent for making solution of the sample.
- 4) Pencil.
- 5) Sample applicator.
- 6) Solvents for stationary and Mobile phases.
- 7) Requirements for detection.

1) Paper: Whatmann No.1 filter paper is used for this purpose.

Composition: a - Cellulose-98.99%

b - Cellulose-0.3 to 1%

Pentosans=0.4 to 0.8%

Ether soluble matter = 0.015 to 0.02%

Ash-0.07 to 0.01%

2) Apparatus :

It is cylindrical chamber made of glass, polyethylene, stainless steel or porcelain can also be used.

Requirements of apparatus :

- a) Material of the apparatus should not be soluble in the solvent.
- b) Apparatus should be vapour tight.
Glass rod and clips can be used to hang the paper strips.

3) Solvent for making solution of the sample :

A suitable solvent is used to make solution of the sample. A volatile solvent is used since it evaporates leaving the mixture to be resolved at the spot of application.

4) Sample applicator :

Suitable device is used for applying the sample on the pencil line. Microsyringes, Micropipettes, Capillary tubes, Wireloop etc. are used for this purpose.

5) Pencil for drawing a line :

Pencil can be used for drawing the line on which sample is applied with the help of sample applicator.

6) Solvents for stationary and Mobile phases:-

When the method is paper adsorption chromatography, mobile phase is the liquid. When it is paper partition method, stationary and mobile phases are solvents. Stationary phases liquids are classified into :

a) Aqueous stationary phase: Ex: water

b) Hydrophilic stationary phase: Ex: Methanol, formamide, glycol, glycerol etc.

c) Hydrophobic stationary phase: Ex: dimethyl formamide, kerosene etc.

Mobile phase liquids are mixtures of two or more solvents

ex : 1) Isopropanol-Ammonia- Water mixture in the ratio of 9 : 1 : 2

2) N-butanol-Acetic acid-Water mixture in the ratio of 4 : 1 : 5.

7) Requirements for detection :

When the components of the

mixture are not coloured

, requirements for the

detection depend on the

method employed. Different

methods employed are U.V.

method, physical and chemical

methods. In U.V. method U.V.

lamp is needed. For chemical

methods chemical reagents

are required and they depend on the component fractionated. For example, ninhydrin

solution is used for detection of amino acids in serum.

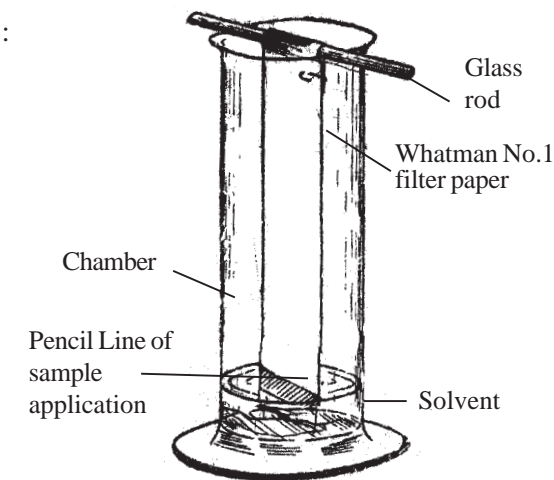


Fig.2-3 Paper chromatography

Procedure: Different steps in the separation of components of a mixture by paper chromatography are :

- 1) Selection, modification and preparation of paper.
 - 2) Preparation of sample.
 - 3) Application of sample on the paper.
 - 4) Development.
 - 5) Drying of paper
 - 6) Detection.
 - 7) Calculation of R_f values.
- 1) Selection, modification and preparation of paper: Whatmann No.1 filter paper is the paper of choice in paper chromatography. It provides the quality required for resolution of components of the mixture. If the cellulose paper used in paper chromatography is not of suitable quality, it has to be impregnated with diatomaceous earth, alumina, silicagel, and ion exchange resins. Paper can also be impregnated by 1) Buffer treatment, Silicone treatment. Glass fiber type paper is suitable for elution with corrosive agents.
- After selecting the paper of desired quality, it has to be cut into suitable size and shape. Rectangular, circular as the case may be. It should be washed before use.
- 2) Preparation of sample :
- Mixture to be resolved should be dissolved in a suitable solvent. Volatile solvent can be used for a mixture in solid form. Quantity of solution should be minimum to avoid diffusion.
- 3) Application of sample on the paper :
- Sample is applied as spots or bands. Amount of sample to be applied depends on.
- 1) Capacity of the solvent.
 - 2) Optimum concentration required for.
 - 3) Time required for development.
- 4) Development : Suitable solvent/Solvent mixture is selected, sufficient quantity

is taken in the chamber and rectangular paper is hung vertically from glass rod so that its edge below the pencil line dips in the solvent mixture taken in the cylindrical chamber. Paper can also be rolled into cylindrical shape and kept in the solvent in circular paper chromatography. Chamber atmosphere also must be saturated with solvent. Development causes fractionation of components of mixture. The process is also called as elution.

- 5) Drying of the paper : After the mobile phase liquid has travelled the length of the strip, paper is removed and dried. It is done by keeping in a hot air oven or over a hot plate for few minutes. It can also be done by using a fan or hair drier. Drying is needed for evaporating the solvent. The finished, dried paper after completion of elution is called as paper chromatogram.
- 6) Detection : Positions of the fractionated components on the paper chromatogram can be located by different methods. Coloured substances can be spotted visually. When the fractionated substances are colourless, suitable methods have to be followed. Ultraviolet lamp can be used if the substance is not visible in the ordinary light. Radio active substances can also be used. Physical and chemical methods can be also made use of for locating. Chemical methods involve using of chemical reagents. Ninhydrin solution is used as detecting /locating reagent for amino acids. Metallic ions of II and IV group elements can be located by H₂S.

Locating reagents are applied by

- 1) Spraying method.
- 2) Dipping method.

In spraying method, solution of the reagent is sprayed over the paper chromatogram uniformly using an atomiser and dried. In dipping method, paper chromatogram is dipped in the solution of the reagent and dried. It is followed when solvent used for dissolving the locating reagent is volatile.

- 7) Calculation of R_f Values : Distance moved by the component from the pencil line and distance moved by the solvent front shall be measured. Rate of flow of a component can be determined by using the formula.

$$R_f = \frac{\text{Distance moved by the component}}{\text{Distance moved by the solvent}}$$

Applications : It can be used for qualitative identification and quantitative determinations. For quantitative applications, colour intensities of spots got with unknown have to be compared with the colour intensities of spots got with standard solution.

Paper chromatography is successfully used for fractionation of amino acids in a mixture. Clinically, it is used for the fractionation of serum amino acids. Fractionation of serum amino acids can be done by using serum as sample.

4) HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC):

Liquid chromatography: Liquid chromatography is the method in which mobile phase is a liquid. There are two types of liquid chromatography

- 1) Solid-liquid chromatography.
- 2) Liquid-liquid chromatography.

HPLC: Liquid chromatography using high pressure to force the liquid through column is called as High pressure/High performance liquid chromatography (HPLC). Difference between- HPLC and older technique is speed. Older technique of liquid chromatography (LC) is of slow speed where as the newer technique is of increased speed. This method was used by Hadden in 1967 for separating mixture of nucleosides containing Uridine, Guanosine, Adenosine and Cytidine using 10-20 μ Si_g and it took 60 minutes for fractionation.

Principle : Differences in partition coefficients causes separation.

Construction of HPLC apparatus :

HPLC apparatus consists of

- 1) Reservoirs, degassing chambers and mixing valve
- 2) Precolumn
- 3) Pumps
- 4) Columns
- 5) Detectors

Reservoirs, degassing chambers and mixing valve : HPLC apparatus is provided with two reservoirs each with a degassing chamber. Degassing chamber will expel dissolved air. Mixing valve mixes the two liquid phases.

Precolumn:- Precolumn is loaded with same solid support and stationary liquid phase as in the main column.

Pumps:- Pumps are used to apply high pressure to the liquid to force through column. Motor driven pumps are widely used for producing high pressure. It is either reciprocating or single stroke pump. High pressure can also be got by pressure transfer from a cylinder of compressed Nitrogen.

Columns: Columns are usually of 1-6mm inside diameter. They are made of stainless steel or glass. Solid support used in Liquid-Liquid chromatography can be porous diatomaceous earth. Alumina or silicagel is used in Solid-Liquid chromatography.

Detectors: Different detectors used are

- 1) Photometric detectors.
- 2) Refractive index detectors.
- 3) Moving wire Flame ionisation Ledetectors.
- 4) Reduction detectors.
- 5) Conductometric detectors.

Photometric detectors depends on adsorption of visible or U.V. light. Source of U.V. light is a low pressure mercury lamp. Refractive index detectors measure difference in refractive index. Moving wire flame ionization LC detector is used in the Pye chromatograph. In reduction detector, liquid stream from the column is mixed continuously with a stream containing a reagent. It produces coloured or fluorescent product with sample components.

Conductometric detector is based upon measurement of electrolytic conductivity.

Procedure :

Steps involved are -

- (1) Degassing of liquid phases
- (2) Mixing of degassed liquids
- (3) Passing the mixed liquid into chromatograph

- (4) Injection of sample
- (5) Elution
- (6) Detection

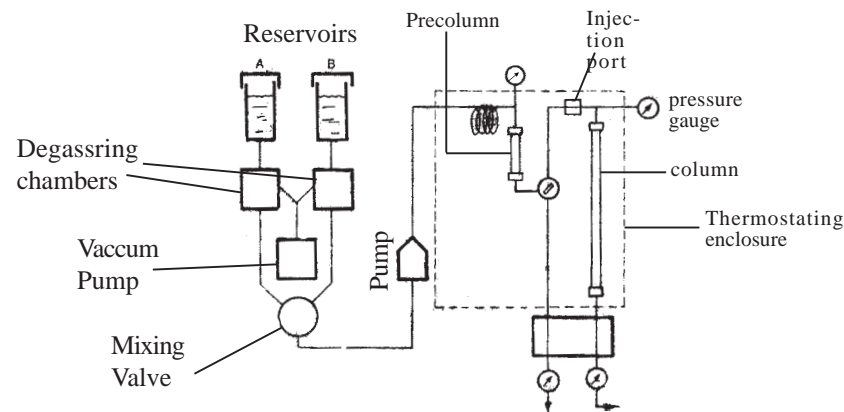


Fig. 2-4 Flow diagram of HPLC

Procedure :

- (1) Degassing of liquid phases :

Two liquid phases of the HPLC present in the two reservoirs are degassed in degassing chambers separately. Degassing step is needed to expel air dissolved in liquid phases.

- (2) Mixing of the degassed liquids : Mixing valve is positioned properly to mix the two liquids from the reservoirs after expulsion of dissolved air in the degassing chambers. Mixing valves can be programmed with a geared down motor drive. It is possible to change the proportions of two solvents by programming during chromatographic process.
- (3) Passing the mixed liquid into chromatograph proper : Mixed liquid is pumped at high pressure in to chromatograph proper. Mixed liquid is first allowed to pass through a coil of tubing which it is brought to working temperature. Then it is passed through a precolumn with pressure which is loaded with the same solid support and stationary phase liquid as the main column. Mobile phase liquid gets saturated with stationary phase liquid in the pre column. This prevents removal of stationary phase form the column.

- (4) Introduction of the sample :

Sample is introduced between Precolumn and column by using a syringe or a valve.

- (5) Elution : Gradient elution or solvent programming causes separation of the components due to differences in the partition coefficients.
- (6) Detection : Separated components of the sample are detected by making use of detector. It was introduced by Small, Stevens and Baumann in 1975.

5) ION EXCHANGE CHROMATOGRAPHY :

Chromatographic method by which ions of similar properties are separated due to ion exchange is called as ion exchange chromatography. Ion exchange is the process of exchange of ions of like sign between a solution and ion exchange resin. Ion exchange resins consist of beads of highly polymerised, cross linked organic materials. These materials consist of large number of acidic or basic groups. Many ohter natural and synthetic substances also cause ion exchange. Anion exchangers exchange anions and cation exchangers exchange cations.

Properties of ion exchangers:- Colour, Density, Mechanical strength, Particle size, Cross linking, Swelling , Porosity, Surface area, Chemical resistance etc.

Requirements of a good resin :

- 1) It must be sufficiently cross linked.
- 2) It must be sufficiently hydrophilic.
- 3) It must be chemically stable.
- 4) Number of accessible ion exchange groups contained must be sufficient.
- 5) Density of swollen resin must be greater than 1.

Types of ion exchange resins : They are two types.

I - cation exchangers II-Anion exchangers

I) Cation exchangers :

- 1) Natural Inorganic anion exchangers: Examples are zeolites and clay.
- 2) Natural organic cation exchangers: Examples are peat, natural

sulphanated coal and wood.

- 3) Synthetic Inorganic cation exchangers:- MgO , SiO_2 , Al_2O_3 , $SiO_2-Al_2O_3$.
- 4) Synthetic organic cation exchangers: Polymeric resin matrix containing acidic exchange sites.

II) Anion exchangers :

- 1) Natural Inorganic anion exchangers :

Ex: Dolomite.

- 2) Synthetic Inorganic anion exchangers :

Ex: Heavy metal silicates.

- 3) Synthetic organic anion exchangers :

Ex: Polymeric resin matrix containing basic exchange sites.

Types of ion exchange resins on the basis of functional groups contained :

There are two important types of Ion exchange resins.

- 1) Acid Resins. (Cation exchange resins)
- 2) Basic Resins. (Anion exchange resins)

- 1) Acid resins: Acid resins contain acid functional groups. They give rise to common functional groups as $-SO_3H$, $-COOH$, $-OH$, $-SH$ and $-PO_3H_2$. They are further divided into

a) Strong acid resins b) Weak acid resins.

a) Strong acid resins: These are prepared by nuclear sulphonation of cross linked polystyrene. They are effective over extra range of pH in both acid and salt forms. They contain sulfonic acid groups as ionisable groups. They are useful for fractionation of amino acids, vitamins, lanthanoids etc.

b) Weak acid resins: They are polymers of methacrylic acid. They possess carboxyl groups. They are effective in the pH range of 5 to 14. They are useful for fractionation of transition elements, antibiotics etc.

- 2) Basic Resins : They contain basic functional groups. They give rise to common functional groups as $-NH_2$, $-NHR$, $-NR_2$. Anion exchange resins are further divided into

a) Strong basic resins

b) Weak basic resins.

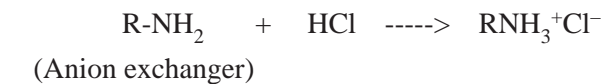
Strong basic resins:- They are resins with positively charged quaternary ammonium groups attached to cross linked polystyrene frame. They are ionised. They offer wide range of pH. i.e. - 0 - 12.

Ex:- Trimethyl ammonium groups. They are useful in fractionation of halogens, alkaloids, B-complex vitamins, fatty acids etc.

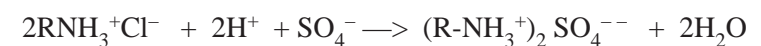
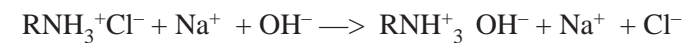
(b) Weak basic resins: Tertiary amine resins and polyamine polystyrene resins belong to this class. Polyamine polystyrene resins are mixtures of primary, secondary and tertiary groups on the polystyrene network. Tertiary amines are effective between 0-9 pH. Polyamine resins are effective between 0-7 pH. They are useful in the fractionation of anionic complexes of metals, anions of different valencies, vitamins and amino acids.

Principle: Separation of the components of a mixture takes place by Ion exchange. Ion exchange takes place between ion exchange resins and solutions of ionised material.

An anion exchange resin contains amine or quaternary ammonium groups as part of the resin and also anions like Cl^- , SO_4^{2-} , OH^- etc in equivalent amount. When an anion exchange resin is treated with hydrochloric acid, substituted ammonium cations are produced.

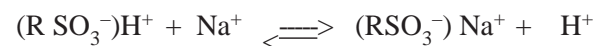


When it is further treated with solution of any ionisable material, ion exchange takes place as follows.



A cation exchange resin contains sulphonic, or carboxylic or phenolic groups as the part of the resin and also cations in equivalent amount. When a cation exchange resin is treated with 5% HCL, resin is converted into acid form (RSO_3^-)

) H⁺. When NaCl is passed through hydrogen form of the resin H⁺ ions will be replaced by Na⁺ ions.



For each equivalent of sodium ion one equivalent of Hydrogen ion is freed.

Equilibrium position depends on a) Concentration of Na⁺ ions in solution which are in contact with resin. b) Acidic strength of resin.

Procedure: Steps in ion exchange chromatography are

- 1) Packing of the column
- 2) Application of sample
- 3) Operation of ion exchange
- 4) Analysis of elute

1) Packing of the column:

Column is packed by pouring the slurry of the resin into the vertically held column. Care should be taken to avoid air bubbles. Slurry is added in parts. Resin is allowed to settle down between each addition. Resin is brought to equilibrium with the solvent before packing of the column by slurry of the resin. After completion of packing of the column, eluent is passed through the column for certain time to ensure uniform flow rate of eluent over the entire cross section of the column. Level of the liquid is adjusted to be below the top of the resin bed.

2) Application of sample :

Sample solution to be fractionated is poured on the top of the resin in the column. Sample application is made by using a micro pipette or micro syringe.

3) Operation of ion exchange chromatography:-

Ion exchange chromatography can be operated by elution, frontal analysis or displacement analysis.

High rate of ion exchange is affected through-

- a) Resin of low cross linking and small particle size
- b) Ions of smaller size
- c) High temperature
- d) Concentration of the solution

4) Analysis of the elute:- Fractionated components of the mixture are collected into automatic fraction collectors. Analysis of the elute is made by-

- a) Determination of pH.
- b) Determination of Refractive index
- c) Determination of light absorptive power
- d) Spectro Photometric method
- e) Polarographic method
- f) Conductometric method
- g) Radio chemical method

Applications: Some important applications of ion exchange chromatography are -

- 1) Determination of sodium and potassium in the mixture
- 2) Separation of interfering ions
- 3) Concentration of traces of an electrolyte
- 4) Separation of transition metals
- 5) Conversion of fats to acids or bases
- 6) Demineralisation of water
- 7) Softening of hard water
- 8) Removal of carbonates from NaOH
- 9) Separation of Isotopes etc.

6) EXCLUSION CHROMATOGRAPHY :SEE TYPES BASED ON PRINCIPLES

7) ELECTROCHROMATOGRAPHY: It is also called as electrophoresis. This topic is dealt as separate chapter.

8) GAS CHROMATOGRAPHY : Gas chromatography was originally suggested by Martin and synge in 1941.

Definition : Gas chromatography is a method of chromatographic analysis in which mobile phase is a gas.

Principle : In this technique, components of a vaporised sample are separated due to partition between mobile gaseous phase and a stationary phase held on the column.

Types of Gas chromatography:

There are two types of gas chromatography based on stationary phase.

- 1) Gas-Solid chromatography.
- 2) Gas liquid chromatography

When stationary phase is a solid, the method is called as Gas solid chromatography. When it is a liquid, it is called as Gas liquid chromatography. Of these two techniques, gas-liquid chromatography is the widely used one.

Construction of GLC apparatus :

GLC apparatus consists of

- (1) Tank of Mobile phase gas (Carrier gas)
- (2) Injection port for sample application
- (3) Column
- (4) Detector

(1) Tank of Mobile phase :

A high pressure gas cylinder is used as a reservoir for carrier gas. It is attached to a pressure regulator to regulate the gas flow through the separation column. Soap bubble meter can also be used for accurate regulation of the rate of mobile phase gas.

(2) Injection port :

Injection port is the place from where sample is introduced. It is located at a place so that the sample is introduced directly into the carrier gas. It is constructed with a material that can be maintained at high temperature. It contains a pliable septum through which samples are injected.

(3) Column : Column is the heart of the chromatography. Column is the site where components of the sample are fractionated. There are two types of columns, commonly employed in GLC. They are-

- (1) Capillary column.
- (2) Packed column.

Capillary column is fabricated from capillary tubing. These columns are available in lengths up to 200' with 1/16' or less diameter. Column for packed

column is made of stainless steel or copper packed with stationary phase solid in Gas solid Chromatography and solid support in Gas Liquid chromatography. Glass tubes can also be used as columns.

(4) Detector : Detector is the part which detects a fractionated component on the basis of properties of that component like thermal conductivity, flame emission, differential adsorption etc. Different types of detectors used are-

- (1) Thermal conductivity detectors
- (2) Flame Ionisation detectors
- (3) Flame Photometric detectors
- (4) Electron capture detectors

Requirements :

- 1) Mobile phase gas
- 2) Solid supports
- 3) Stationary phase liquid

I. Mobile phase gas : Mobile phase gas is also known as carrier gas. It is usually Helium or Nitrogen. Carbon dioxide and Hydrogen are also tried. Hydrogen is explosive. Hence it is a disadvantage.

Requirements of a carrier gas :

- 1) It should be chemically inert.
- 2) It should be available at cheaper rate.
- 3) It should allow detector response.

2) *Solid supports :* Since the stationary phase in GLC is liquid, it should be provided support by an inert solid. Examples are Kieseguhr, Powdered teflon, Alumina etc.

Requirements of solid support:

- 1) It should be porous.
- 2) It should provide large surface area. Particles should be small, uniform and spherical.
- 3) It should be mechanically strong.
- 4) It should be chemically inert.

5) It should be easily wettable by the stationary phase liquid.

3) *Stationary phase liquid* : Examples are Squalene, Silicone oil, Silicon rubber gum, carbowax etc.

Requirements of stationary phase liquid :

- 1) It should be a good solvent for the sample.
- 2) It should be thermally stable.
- 3) It should be chemically inert.
- 4) It should be very less volatile.
- 5) Boiling point should be at least 200°C higher than the maximum operating temperature of the column.
- 6) Solubilising power of the stationary phase liquid should be differential for components of the sample mixture.

4) *Sample injector* :

Syringe is used for injecting sample. 0.1-100 ml capacity syringe is used for introducing liquid sample. Gas light syringe of 0.5-10 ml capacity is used for injecting gaseous samples. Special syringe can be used for solid samples.

Procedure:

Different steps involved in Gas chromatography are :

- (1) Column packing
- (2) Column conditioning
- (3) Column thermostating
- (4) Sample injection
- (5) Elution

(1) Column packing:

Column packing is very important since it provides the base for separation process. Column is packed with stationary phase solid in GSC and solid support in GLC.

Column packing in G.S.C. (Gas Solid Chromatography): It is prepared by plugging one end of the column with glass wool and filling the column with substrate with the help of funnel and vibrating. Other end is now plugged and tube is coiled

Column packing in G.L.C.(Gas-liquid Chromatography): Stationary phase liquid is dissolved in a volatile solvent. Inert dry solid is mixed in enough quantity and volatile solvent is removed by evaporation. This freely flowing solid coated with stationary phase liquid is packed in to the column by tapping or vibrating. Tube is now coiled or folded loosely.

(2) Column conditioning: Column is conditioned by baking at prescribed temperature while mobile phase gas is flowing. This will remove foreign material. Conditioning is carried out at highest permissible temperature for a period of 6-12 hours during which carrier is flushed.

(3) Column thermostating: Column temperature should be maintained constant to a few tenths of a degree. Circulating air baths, electrically heated metal blocks and vapour Jackets are used to provide column thermostating.

4) Sample injection : Sample is injected directly into the injection port. It is introduced into carrier gas. Liquid and gaseous samples are injected by syringe through silicon rubber diaphragm in the injection port. Liquids are introduced as solution with a syringe of 0.1 - 100 mL capacity. Gases are injected with a gas light syringe of 0.5 -10 ml capacity. Sample loop can also be used to inject gaseous samples. It is done by allowing gaseous sample into a chamber of known volume and inserting into carrier gas flow. It can be done using valve. Solid samples can be dissolved suitable solvent and injected with a syringe. A special syringe can also be used to inject solid samples directly. Samples which can not be vapourised at the operating temperature should not be injected. Such samples can clog the port and can cause damage to column.

(5) Elution: A temperature slightly greater than average boiling point of sample gives reasonable sample elution. If the components of the sample have boiling points in broad range, temperature is to be increased as the elution proceeds. It can be carried out continuously or step wise. Elution period will be 10-30 minutes. Optimum resolution is achieved by minimal temperature.

(5) Detection : Detection of the fractionated components is achieved by using

detectors. Detection of a component is based upon the physical properties of the components.

Applications of G.C. :

- (1) Clinical applications: It is used for determining body components. Blood gases, Oestrogens, Homovanillic acid, Vanilylmandelic acid, Hydroxy corticosteroids etc. can be determined and analysed.
- (2) General applications: It is also used in -
 - a) Petroleum industry
 - b) Food industry
 - c) Cosmetics and perfumes industry
 - d) Plastic industry e.t.c.

2 - ELECTROPHORESIS

Definition ,Various types,Methods ,Serum protein and lipoprotein electrophoresis:

Definition: Electrophoresis is the phenomenon of migration of individual ions as well as colloidal particles in a solution towards either positive or negative electrode under the influence of an electrical field. They behave as electrically charged w.r.t. dispersion medium. It is a powerful analytical tool for fractionating the components of a mixture.

Purposes of Electrophoresis :

- 1) Purification
- 2) Preparation
- 3) Determination

Factors influencing the rate of travel of the particle:

- 1) Particle size
- 2) Molecular weight
- 3) Properties of electrical field
- 4) Temperature
- 5) Nature of suspending medium

Free solution method: Free solution method of electrophoresis is carried out in the absence of a supporting or stabilizing medium. It was first proposed by Picton and Linder in 1892. It was fully developed by Tiselius in 1937 for which work, he was awarded Nobel Prize. Number of improvements were later made by Stevenson, Longworth in 1939 and Philpot in 1948.

In free solution Electrophoresis, sample is introduced as a band at the bottom of a 'U' tube which has been filled with unstabilized buffer solution. Capillary tube side arm is used to introduce sample to the bottom of the tube under the buffer. Electrical field is applied by means of electrodes located at the ends of the tube. Separation takes place as a result of differential mobilities.

Zone Electrophoresis : Zone electrophoresis or electro chromatography is carried out in the presence of a supporting or stabilizing medium.

Types of supporting or stabilising media :

Examples of supporting media include filter paper, cellulose acetate strips, starch powder, cellulose powder, starch gel, agarose gel etc. Electrophoresis carried out with filter paper as supporting medium is called Paper Electrophoresis. Electrophoresis carried out with Agarose gel as supporting medium is called as Agarose gel electrophoresis.

Types of paper used in paper electrophoresis :

Various types of filter papers can be used for paper electrophoresis. Some of them are What man 1,2,3, Eaton- Dikeman 301-85, 320 and 352, Munketells 20/50 and Schleicher and Schull 2040 A and B. If paper reacts with sample, paper made from fibres of borosilicate glass as Whatman GF/B is suited.

Electrodes used in Electrophoresis : Graphite rods,Stainless steel,Silver chloride, Platinum have been recommended as materials for electrodes. Platinum is the best choice for electrodes. Platinum electrodes are available in various forms ranging from fine wires to sheets of foil. Platinum electrodes do not deteriorate and need no replacement.

Source of current used in electrophoresis: Source of D.C. is a rectifier delivering between 100-300 V.

voltage applied for electrophoresis depends on the sample to be fractionated.

Location of components:- Various methods are used for location of components.

<u>Substance</u>	<u>Method of location</u>
1. Proteins	Staining
2. Enzymes	Specific activities
3. Ions	Colour reactions
4. Amino acids	Ninhydrin
5. Radio active Substances	Autoradiography or Scanning

Electrophoretic chamber :

Requirements:

- 1) It should be made of Insulating material.
- 2) Voltage drop between the ends of electrophoretic bed should be measurable.
- 3) It should be provided for preventing evaporation of buffer during electrophoresis.
- 4) It should be provided to adjust buffer levels in the electrode compartments.
- 5) It should be designed to adjust electrolyte in the electrode chamber to equal level.
- 6) Strip or sheet must be suspended so as to avoid sagging.
- 7) Destruction of buffer at the electrodes should be prevented or minimised.
- 8) Continuous mixing and recycling of buffers in the chamber should be provided to neutralize decomposition products.

Applications of Electro chromatography:

This technigue is widely used in clinical diagnosis for analysis of serum,urine, cerebrospinal fluid, gastric juices and other body fluids. Diagnosis of clinical

condition is useful for treatment and also for therapeutic evaluation of drugs in clinical pharmacy studies.

It is a useful technique for separation of serum protiens and lipoproteins.Electrophoresis is used also for the fractionation of nucleic acids, vitamins, natural pigments, amino acids, organic acids, and carbohydrates etc. Inorganic ions can also be separated by this technique.Thus this technique is a tool of significance in Phramaceutical analysis, Chemical analysis and Biochemical analysis.

Fractionation of serum proteins :

Clinical significance :

Plasma contains 1) Albumin - 4.7 - 5.7%

2) Globulin- 1.3 - 2.5%

3) Fibrinogen - 0.2 - 0.4%

4) Prothrombin etc.

Serum contains Albumin and Globulin only as serum is the fluid part of blood after clotting. Fibrinogen and prothrombin being the proteins responsible for coagulation of blood, they are not present in serum. Serum globulin is four types.

1) a-1 - globulin

2) a-2 - globulin.

3) b- globulin

4) g- globulin

Albumin - 3.3-4.8%.

a-1-globulin - 0.2-0.4%

a-2-globulin - 0.4-0.8%

b-globuliln - 0.6-1.2%

g-globulin - 1.0-1.8%

Patterns of proteins in related diseases are as follows :

- 1) In nephrotic syndrome,
 - a) Albumin is decreased.
 - b) α_1 - globulin is normal .
 - c) α_2 - globulin and β -globulin are increased.
 - d) γ - globulin is decreased.
 - e) Additional band is absent.
- 2) In cirrhosis,
 - a) Albumin is decreased.
 - b) α_1 globulin, α_2 globulin and β -globulin are normal.
 - c) γ -globulin is normal, or slightly increased.
 - d) Additional band is absent.
- 3) In multiple myeloma ,
 - a) Albumin, α_1 - globulin, α_2 -globulin, β -globulin and γ -globulin are normal.
 - b) Additional band is present.
- 4) In Agammaglobulinaemia ,
 - a) Albumin, α_1 -globulin, α_2 -globulin and β -globulin are normal.
 - b) γ -globulin and additional band are absent.

Electrophoretic method of fractionation of serum proteins:

Serum proteins can be fractionated by -

- 1) Paper electrophoresis.
- 2) Agarose gel electrophoresis.

Fractionation of serum proteins by paper electrophoresis :

Principle:- Serum proteins, being colloidal in nature are separated by migration towards anode as they behave like anions. Sequence of migration of different serum proteins in the decreasing order is as follows.

- 1) Serum Albumin – fastest.
- 2) α_1 -globulin.

- 3) α_2 -globulin.
- 4) β -globulin.
- 5) γ -globulin.

Differential migration pattern is due to differences in molecular weights and particle sizes of the components.

Specimen: Serum

Reagents :

- 1) Veronal buffer.
- 2) Staining solution.
- 3) Destaining solution.
- 4) Fixative .
- 5) Eluting solution.

Requirements :

- 1) Whatmann no.1 or 3 filter paper strips.
- 2) Electrophoresis apparatus.
- 3) Beakers.
- 4) Test tubes.
- 5) Micropipette or Hb pipette.
- 6) Hot air oven or incubator.
- 7) Pencil.

1) Veronal buffer: (pH-8.6, 0.075M) :

Composition:

Sodium barbitone	–	42.2g
Barbituric acid	–	7.52g.
Sodium azide	–	0.4g.
Distilled water up to	–	2,670ml.

Method of preparation :

- 1) Dissolve Barbituric acid in about one litre of water by heating.

- 2) Cool and dissolve sodium barbitone.
- 3) Now add sodium azide and mix.
- 4) Dilute to 2,670 ml with water.

Storage: Store at 2-8°C.

2) Staining solution:

Composition :

Bromophenol blue-1 g.

95% v/v ethanol saturated with mercuric chloride up to 100ml.

Storage: Store in amber coloured bottle at 25°C ± 5°C.

3) Destaining solution :

2% V/V acetic acid.

Storage : Store at 25°C ± 5°C.

4) Fixative :

Composition:

Sodium acetate – 2g.

10% v/v acetic acid – upto 100ml.

Storage: Store at room temperature (25°C ± 5°C)

Eluting solution:- (N/100 Sodium hydroxide)

Composition:

Sodium hydroxide:-0.4g.

Distilled water up to – 1litre.

Method of preparation:- 1) Dissolve 0.4g of sodium hydroxide in enough quantity of water.

2) Dilute to 1litre with water.

3) Standardise and adjust normality if necessary .

Storage: Store at Room temp(25°C ± 5°C).

Requirements :

1) Whatman No.1 filter paper strips :

Whatman No-1 or 3 filter paper is cut into strips of 3cm width and length depending on the buffer tank. It is the supporting medium for carrying out electrophoresis.

2) Electrophoresis apparatus: Electrophoresis apparatus consists of power supply unit and buffer tank.

3) Micropipette: Micropipette is used for pipetting serum sample and applying it along the pencil line drawn on the strip.

4) Incubator or Hot air oven: Temperature required for drying the paper strip after electrophoretic elution is provided by Incubator/oven.

5) Pencil : Pencil is used for drawing the pencil line on the paper strip.

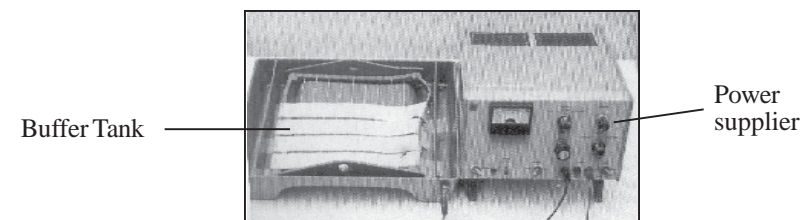


Fig.2-5 Paper Electrophoresis

Procedure:

- 1) Set up the apparatus on level surface.
- 2) Fill the electrode chambers with veronal buffer.
- 3) Adjust the buffer levels in the two electrode chambers to be equal.
- 4) Wet the paper strip by dipping in buffer mixture.
- 5) Remove the excess buffer on the paper strip by laying it on a sheet of filter paper.
- 6) Place the paper strip in horizontal position with out delay.

- 7) Allow the paper to stand for an hour.
- 8) Enclose the entire apparatus excluding power supply in an air tight chamber to prevent excessive evaporation of buffer.
- 9) Apply 5mL of serum uniformly along the pencil line.
- 10) Turn on the power. Apply potential difference of 100-1000 volts across the electrodes. Allow to run electrophoresis for 16 to 18 hours.
- 11) Remove the paper strips from the apparatus and dry at about 80°C for half an hour.
- 12) Stain the paper strip by rolling the dried strip placing it in a 250ml beaker and adding sufficient quantity of staining solution.
- 13) Keep at room temperature for an hour.
- 14) Destain the strips by occasional rinsing in the destaining solution until background of paper is clear.
- 15) Fix the paper strips in fixative solution for 10 minutes.
- 16) Dry the paper strips at about 80°C for 10 to 15 minutes.

Fractionation of serum proteins by Agarose gel electrophoresis :

Principle: Same as for paper electrophoresis.

Reagents :

- 1) Veronal buffer.
- 2) Staining solution.
- 3) Fixative solution.
- 4) Destaining solution.
- 5) Dehydrating solution.
- 6) Agarose.

Requirements :

- 1) Whatmann no.1 filter paper.(used to connect glass slides)
- 2) Electrophoresis apparatus.

- 3) Glass slides.
- 4) Petridishes.
- 5) Capillary tubes.

1) Veronal buffer : Composition, method of preparation and storage same as in paper electrophoresis.

2) Staining solution:

Composition:

Amidoblack-1g.

7% V/V acetic acid upto-100ml.

Method of preparation : Dissolve 1g amido black in 75ml of 7%v/v acetic acid and dilute to 100ml with 7%v/v acetic acid solution.

3) Fixative solution:

Composition:

Isopropanol - 75parts.

Distilled water - 25parts.

Glacial acetic acid - 5parts.

4) Destaining solution :

7% v/v acetic acid.

5) Dehydrating solution :

Acetone - 90parts.

Distilled water – 10 parts.

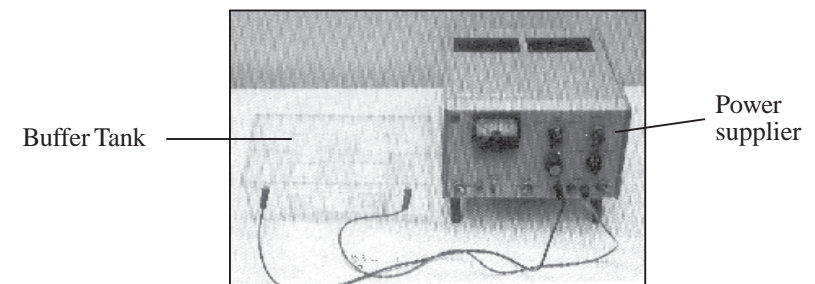


Fig.2-6 Agarose gel electrophoresis

Procedure :

- 1) Melt 200mg of agarose in 20ml of veronal buffer by boiling for 2-3 minutes.
- 2) Pour 2.5ml of molten agarose on the glass slides to form the layers of gel.
- 3) Cut slots on the layer across the middle of the slides.
- 4) Apply 2-3 ml of serum on the sample line by means of capillary tube.
- 5) Keep the slides in the buffer tank.
- 6) Add 100ml of veronal buffer in anode and cathode compartments.
- 7) Connect the slides by means of filter paper strips.
- 8) Put on the power supply.
- 9) Set the voltage of 200v for five slides.
- 10) Put off the power supply after 3hours.
- 11) Fix the slides in fixative solution for half an hour.
- 12) Dehydrate them in dehydrating solution for 4 hours.
- 13) Keep the slides in incubator overnight.
- 14) Stain the slides in staining solution for 1hour.
- 15) Destain in destaining solution until background becomes clear.
- 16) Dry at room temperature.
- 17) Determine protien fractions using densitometer.

Fractionation of serum lipoproteins by Agarose gel electrophoresis:

Clinical significance: Lipoproteins are-

- 1) Chylomicrons
- 2) VLDL (Very low density lipoproteins)
- 3) LDL (Low density lipoproteins)
- 4) HDL (High density lipoproteins)

Hyper lipidaemia or Hyper lipoproteinaemia:-

In hyper lipoproteinaemia, serum lipids are increased above normal. There are two types of hyper lipoproteinaemia.

- 1) Primary dyslipoproteinaemia.
- 2) Secondary dyslipoproteinaemia.

Primary dyslipoproteinaemia:

Primary dyslipoproteinaemia is due to inherited defective lipoprotein metabolism.

These conditions are classified into -

<u>Type</u>	<u>Class</u>
a) Hyper chylomicronaemia.	I, V
b) Hyper low density lipoproteinaemia.	II
c) Hyper low density dyslipoproteinaemia. and very low density dyslipoproteinaemia.	III
d) Hyper very low density lipoproteinaemia.	IV

Secondary dyslipoproteinaemia :

Secondary dyslipoproteinaemias are result of some underlying disorder. They manifest as increased plasma triglyceride level, increased serum cholesterol level or increase in both . HDL may be normal or decreased. Some of secondary conditions are -

- 1) Diabetes miletus.
- 2) Alcoholism.
- 3) Hypothyroidism.
- 4) Renal failure.
- 5) Nephrotic syndrome.
- 6) Liver disease etc.

According to Fredrickson, hyper lipoprotienaeimias are divided into six phenotypes.

Type	Serum	Clinical conditions
Type-I .	Milky with Heavy chylomicron band	Xanthomas, Hepatosplenomegaly
Type-IIa	Clear Heavy betaband, high Cholesterol.	Xanthomas, increased incidence of atherosclerosis.
Type-IIb	Clear Heavy beta, Slightly increased prebeta & T.G.	Xanthomas, increased incidence of atherosclerosis
Type-III	Turbid and may be at surface broad beta & Prebeta. Increased cholesterol & triglyceribes.	Impaired glucose tolerance, Atherosclerosis
Type-IV	Turbid, Nochylo microns Increased prebetaband.	Xanthomas, Increased Uric acid and impared glucose tolerance
Type-V	Turbid , Nochylomicrons Increased Prebetaband.	Hepatosplenomegaly Acute relapsing pancreatitis-

Principle:- Same as for electrophoesis of proteins.

Requirements: Same as for fractionation of serum proteins by agarose gel elctrophoresis.

Reagents: 1) Veronal buffer:- Same as in electrophoresis of proteins.

2)Staining solution:

Composition:

Sudan black-1g.

60% v/v ethanol- up to 100ml.

Method of preparation: Dissolve 1g of sudan black in 75ml of 60% v/v ethanol and make up to 100 ml. with 60% V/V ethanol.

3) Destaining solution: 50% v/v ethonol.

4)Fixative solution:

Composition:

Isopropanol-70parts..

Distilled water-25parts.

Glacial acetic acid-5parts.

5)Dehydrating solution:

Composition:-

Acetone-90parts.

Distilled water-10 parts.

6) 40% sodium hydroxide solution:

Composition:

Sodium hydroxide-40g.

Distilled water up to-100ml.

Method of preparation :

Dissolve 40g of sodium hydroxide in 75ml of distilled water and dilute to

100ml with distilled water.

7) Agarose

Procedure: up to 10th step, same as for fractionation of serum proteins by agarose gel electrophoresis (up to electrophoretic run).

- 11) Fix the slides in fixative for 30 minutes.
- 12) Keep the slides in dehydrating solution for 4 hours.
- 13) Dry in incubator at 37^o C.
- 14) Stain in staining solution for 1 hour. Add 1 drop of 40% NaOH in stain before use.
- 15) Destain in destaining solution for about 10 minutes and dip in tap water till back ground is clear.

Summary

Chromatography is a method of analysis by which components of a mixture are separated by redistribution of molecules of mixture between two or more phases. It is used for purification, separation and preparation purposes. Phases in chromatography are Stationary phase and Mobile phase. One of the methods of classification of chromatographic techniques is based on state of matter of stationary and mobile phases. Another method of classification is based on principles. General classification contains different well known methods.

Different principles of chromatography are adsorption, partition, ion exchange, exclusion. Diagnostically important method of chromatography is paper chromatography, which is used for separation of serum amino acids. Other methods are also clinically useful.

Electrophoresis is the phenomenon of migration of colloidal particles towards positive or negative electrode under the influence of electrical field. Based on the presence or absence of stabilising medium, these methods are classified into-

- 1) Free solution method.
- 2) Zone electrophoresis

Examples of stabilising media are paper, starch, agarose etc. Electrophoresis is used for fractionation of proteins and lipoproteins for clinical purposes. Types of serum proteins are – Albumin, α_1 globulin, α_2 globulin, β -globulin and γ globulin. Types of lipoproteins are Chylomicrons, LDL, VLDL and HDL.

Essay questions

- 1) Define chromatography. Add note on different methods of classification.
- 2) Write about column chromatography.
- 3) Write about different aspects of thin layer chromatography.
- 4) Explain paper chromatography.
- 5) Describe HPLC.
- 6) Define Gas chromatography. Explain different aspects of Gas chromatography.
- 7) Classify electrophoretic methods and write about stabilising media.
- 8) Write the clinical significance, principle, requirements & procedure of fractionation of serum proteins by paper electrophoresis.
- 9) Explain in detail about fractionation of serum proteins by agarose gel electrophoresis.
- 10) Write about fractionation of serum lipoproteins by agarose gel electrophoresis including clinical significance.

Short answer questions

- 1) Define chromatography.
- 2) What are the phases in any chromatographic procedure?
- 3) What is adsorption chromatography?
- 4) Describe the column used in column chromatography.
- 5) Name the two methods in paper chromatography.
- 6) Exemplify some adsorbents used in column chromatography.
- 7) What are different classes of adsorbents used in column chromatography?
- 8) Name different types of TLC based on principles.
- 9) Mention different applicators used in TLC.

- 10) Give the formula for calculation of R_f value.
- 11) Write the composition of paper used in paper chromatography.
- 12) Classify stationary phase liquids in paper partition chromatography.
- 13) What are applications of paper chromatography?
- 14) Give full forms of a) HPLC b) TLC c) GLC d) GSC
- 15) What are the requirements of carrier gas in Gas chromatography?
- 16) Define electrophoresis.
- 17) Mention the factors influencing electrophoresis.
- 18) What are the methods of electrophoresis?
- 19) What is free solution method of electrophoresis?
- 20) Define zone electrophoresis.
- 21) Mention any 4 supporting (stabilising) media.
- 22) How do you locate following components
 - a) Proteins
 - b) Radio active substances.
- 23) Write the clinical application of electrophoresis.
- 24) Mention plasma proteins and their normal ranges.
- 25) What are serum proteins ?
- 26) Write the pattern of proteins in Nephrotic syndrome.
- 27) What is the pattern of proteins in multiple myeloma?
- 28) Write the sequence of serum proteins in descending order of fastness after paper electrophoresis.
- 29) Mention the classes of lipoproteins.
- 30) What is primary dyslipoproteinaemia?
- 31) Define secondary dyslipoproteinaemias.
- 32) What are the supporting media used in
 - a) paper electrophoresis
 - b) agarose gel electrophoresis
- 33) Write the composition of fixative solution used in electrophoresis of serum lipoproteins by Agarose gel electrophoresis.

III - IMMUNO ASSAYS

DEFINITION , BASIC PRINCIPLES OF IMMUNOCHEMICAL REACTIONS AND IMMUNO ASSAYS

Immunoassays are specialised techniques of analysis based on antigen - antibody reaction . Radioimmunoassay and Enzyme linked immunosorbent assays are used as techniques of diagnosis . RIA is based on immunological reaction involving use of radio isotopes/ radiopharmaceuticals . RIA is based on setting up competition between radioactively labelled substance and unlabelled substance to bind with specific antibody developed for the purpose. There is inverse relation existing between quantities of labelled and unlabelled substances.ELISA is based on immunological reaction involving use of enzyme . In ELISA antigen antibody reaction takes place and enzyme labelled specific antiglobulin is added which attaches to antigen - antibody complex . Unattached antiglobulin is washed away . Enzyme substrate is then added which is then acted upon by attached enzyme, which produces a colour whose intensity can be read colorimetrically . Peroxidase or phosphatase can be used as enzyme in ELISA in common . Intensity of colour produced is proportional to amount of enzyme attached and thus to the amount of antibody in test serum .

1 - RADIO IMMUNO ASSAYS (RIAs)

Introduction to radio activity :

Radioactivity was discovered in 1896 by Henry Becquerel, a french Physicist. The discovery was very accidental. Radio activity finds application in Medicine. Radio isotopes are used in diagnostic procedures as well as in treatment. Radioactive substances used in diagnostic procedures and therapeutic purpose are called as radio pharmaceuticals. Radiation emitted by radio pharmaceuticals is also used for sterilisation purposes. In India, Bhabha Atomic Research Centre (BARC), located at Mumbai is in the task of producing, quality control, storage in bulk, packing the the isotopes into ready usable dosage forms. Its ancillary distributing centre is established at Kolkata.

Radioactivity: Radio activity is defined as spontaneous emission of radiation by elements with atomic number more than 83. There are about 40 radioactive

elements which are arranged into families like Uranium series, Thorium series and Actinium series.

Radioactive transformation : Radioactive transformation is the process of transformation of an unstable nucleus into another nucleus by disintegration of the original nucleus.

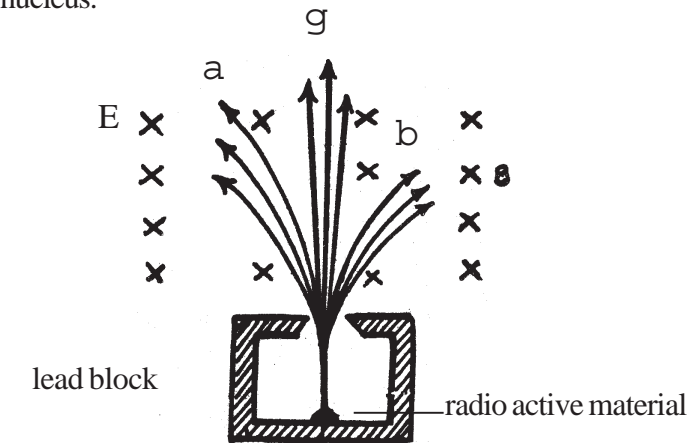


Fig. 3-1 Deflection of radio active radiations in magnetic field

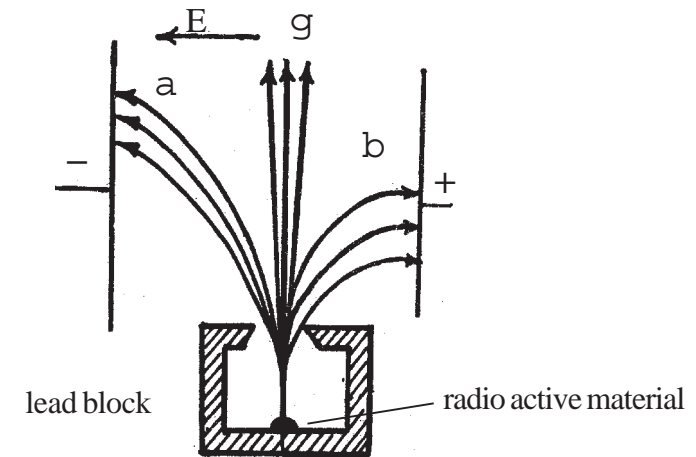


Fig.3-2 Deflection of radio active radiations in electrical field

Classification of radiations from a radioactive substance:

Radiations emitted from a Radioactive substance are three types.

- 1) Positively charged α - particles.
 - 2) Negatively charged β - particles.
- and
- 3) Uncharged γ rays.

This classification is based on the behaviour of these radiations in the magnetic and electrical fields.

Curie : Curie is the unit of radioactivity which is the amount of any radio active substance undergoing 3.7×10^{10} disintegrations per second. It is denoted by 'Ci'.

Kilocurie : Kilocurie is the amount of any radioactive substance undergoing 3.7×10^{13} disintegrations per second. It is denoted by 'KCi'.

Millicurie : It is the amount of any radio active substance undergoing 3.7×10^7 disintegrations per second. It is denoted by 'mCi'.

Micro Curie : It is the amount of any radioactive substance undergoing 3.7×10^4 disintegrations per second. It is denoted by 'mCi'.

MeV : It is the unit of radiation energy. (Million electron volt).

Rad : Rad is the unit of sterilising dose.

It is the amount of ionising radiation from any source delivering 100 ergs of energy per gram of absorbing material.

Half life period : Time taken for disintegration of 50% of the total atoms present in a radio pharmaceutical substance to take place is called as its half life period. It is denoted by 'T' and it differs from substance to substance.

- ex:
- 1) Half life period of ^{60}Co is 5.25 years.
 - 2) Half life period of ^{137}Cs is 30 years.

Decay constant : Decay constant is another characteristic parameter to describe the activity of a radioactive element. It is denoted by ' λ ' and relation between half life period and decay constant is inverse.

$$\lambda = 0.693 / T$$

Biological half life :

Time taken for 50% of the administered radio pharmaceutical to be eliminated from the body is called as biological half life.

Isotopes : Isotopes are the atoms of same element with same atomic number but differing mass numbers.

- ex:
- 1) ^1_1H , ^2_1H , and ^3_1H
 - 2) $^{20}_{10}\text{Ne}$, $^{21}_{10}\text{Ne}$ and $^{22}_{10}\text{Ne}$

Isobars : Isobars are the atoms of different elements with same mass number. They have differing atomic numbers.

- Ex: $^{40}_{19}\text{K}$, and $^{40}_{20}\text{Ca}$

Isotones : Isotones are the atoms with nuclei having same number of neutrons, but differing number of protons.

- Ex: Silicon and Phosphorous have same number of neutrons (19) but differing number of protons (Si-14 and P-15).

Measurement of Radio activity:

Radio activity is measured using different instruments.

- 1) Ionisation chambers
- 2) Proportional counters
- 3) Geiger-Muller counters
- 4) Scintillation counters
- 5) Auto radiograph

Radio Pharmaceuticals :

Any nuclide, which is not radio active in nature is stable. Radioactive nuclides / Isotopes do not have stability and undergo spontaneous nuclear change in order to attain stability by emitting radiation. They are of two types. 1) Natural radio nuclides 1) Artificial radio nuclides. Natural radio nuclides are high atomic weight elements of Uranium series, Thorium series and Actinium series. Artificial radionuclides are prepared by irradiation of suitable material with neutrons in a nuclear reactor.

Ex: ^{60}Co , ^{32}P and ^{131}I

Both natural and artificial radionuclides can be used for labelling other compounds. Each radio nuclide disintegrates at a characteristic electromagnetic radiation. Disintegration rate, half life and type of radiation emitted are characteristic of radionuclide.

Requirements of radiopharmaceuticals :

- 1) They should have relatively short half-life.
- 2) They should emit γ -rays rather than α or β -particles.
- 3) Energy of a radio isotope should be between 30 to 300 KeV.
- 4) They should have organ specificity.
- 5) They should follow first order kinetics.
- 6) They should be easily eliminated.
- 7) They should be easily available.
- 8) They should be cheap.
- 9) They should be easily produced.

Applications of radioisotopes:

Radio isotopes have applications in medicine for-

- 1) Diagnostic procedures
- 2) Therapeutic purpose
- 3) Sterilisation

1) Radio Isotopes used in diagnostic procedures:

Radio Isotopes on introduction into body emit radiation, which is subsequently measured, thus acting as tracers.

Radio isotopes in diagnosis can be further classified into:

- a) Radio Isotopes used in the diagnosis of liver functioning tests : These are used in the diagnosis of liver functioning.

Ex: $^{99\text{m}}\text{Tc}$ sulfur colloid, ^{131}I , $^{99\text{m}}\text{Tc}$ disofenin,

$^{99\text{m}}\text{Tc}$ Sulfur colloid (Sc) : Its biological half life is 6 hours. On intravenous injection of $^{99\text{m}}\text{Tc}$ Sc in the usual dose (adults) of 5 to 8 mCi locates mainly in reticuloendothelial cells of liver, spleen and bone marrow. It accumulates mainly in tumours and other lesions.

Iodine 131 (^{131}I) : Sodium rose bengal, a reactive dye labelled with ^{131}I on I.V. administration accumulates in polygonal cells of liver. Its half life is 8.06 days. Its usual dose is 2-3 m Ci/Kg. Its renal excretion indicates impaired liver functioning. Normal route of its excretion is faeces.

$^{99\text{m}}\text{Tc}$ disofenin : It is useful in diagnosis of acute cholecystitis associated with cystic duct obstruction. Its usual dose is 2 to 15 mCi.

b) Radio isotopes used in the diagnosis of Renal functioning:

These are used in the diagnosis of kidney functioning.

Ex: $^{99\text{m}}\text{Tc}$ DMSA (Dimercapto succinic acid), $^{99\text{m}}\text{Tc}$ glucoheptonic acid, $^{99\text{m}}\text{Tc}$ DTPA (Diethylene triamine penta acetic acid), ^{131}I orthoiodohippurate sodium.

$^{99\text{m}}\text{Tc}$ DMSA : It is used to assess renal parenchyma. It will distinguish between normal anatomy and abnormal anatomy. Renal tumours and cysts can be identified with this compound. It should be used within 30 minutes after preparation and usual adult dose is 2 to 6 mCi.

$^{99\text{m}}\text{Tc}$ Glucoheptonic acid : On I.V. injection it is useful in assessing shape, size and position of kidney and identification of kidney lesions. on I.V. injection, it concentrates in renal parenchyma. Its usual adult dose is 10 to 20mCi.

$^{99\text{m}}\text{Tc}$ DTPA (Diethylene triamine penta acetic acid)

It is used in evaluation of renal vascular integrity. Unperfused regions indicate renal cysts. Its usual adult dose is 3 to 5 mCi.

^{131}I Ortho iodo hippurate sodium :

It is useful in diagnosis of renal functioning by measurement of concentration of ^{131}I in each kidney after injection. Abnormal functioning

of kidneys is indicated by inequalities in concentration. Average dose is 3 to 5 mCi/Kg where as maximum dose is 350 mCi.

c) Radio Isotopes used in the diagnosis of cardiac functioning :

They are used to evaluate cardio vascular functioning. These studies are two types - static and dynamic. Static scan localises the site of myocardial infarction where as dynamic scan evaluates cardiac functioning.

Ex: $^{201}\text{TlCl}$ (Thallous chloride 201), $^{99\text{m}}\text{Tc}$ pyrophosphate etc.

$^{201}\text{TlCl}$: Its half life is 73 hours. It is used for diagnosis of myocardial ischaemia. Its dose is 1 to 2 mCi.

d) Radio Isotopes used in the diagnosis of pulmonary functioning :

Two types of scans are conducted for evaluation of pulmonary functioning with use of radio isotopes. They are-

- 1) Lung perfusion scans.
- 2) Ventilation scans.

Lung perfusion scans are useful in the diagnosis of pulmonary embolism while ventilation scans are useful in differentiation of pulmonary embolism from chronic obstructive pulmonary disease (COPD).

Ex: TC macro-aggregated albumin (MAA), ^{133}Xe (Xenon 133).

$^{99\text{m}}\text{Tc}$ MAA : It contains spheres of denatured human albumin labelled with $^{99\text{m}}\text{Tc}$. For lung perfusion scan, particles enter the lungs carried through pulmonary arteries. Impaired lung perfusion is indicated by abnormal distribution patterns. Half life period is 14 to 15 hours. Usual dose is 1 to 4 mCi of 60,000 to 1,20,000 particles.

^{133}Xe (Xenon 133) Gas:

Xenon 133 is administered with the help of spirometer or a special breathing apparatus. In COPD. There is delay in ventilation and radio active gas trapping. Its half life period is 5.3 days. Usual adult dose is 10 to 15 mCi.

e) Radio -isotopes used in the scans of skeletal system:

Bone scans are helpful in diagnosis of cancer with metastases. Bone scans are more sensitive than radiography.

Ex : $^{99\text{m}}\text{Tc}$ methylene diphosphate ($^{99\text{m}}\text{Tc}$ MDP). Its usual dose is 10 to 20 mCi.

f) Miscellaneous:

Na ^{123}I (Sodium Iodide 123) : It is used in thyroid functioning studies, imaging studies of thyroid, liver, brain and lungs. Half life is 13.2 hours. Usual adult dose is 100 to 400 mCi.

^{67}Ga (Gallium citrate - 67) :It is used in detection of lymphomas, hepatoma etc. Its half life is 78 hours. Usual dose is 2 to 10 mCi.

^{111}In Oxy Quinoline : It is used in labelling of blood cells. Half life is 2.8 days, Its usual dose is 1 mCi.

2) Radio-isotopes used for therapeutic purpose : Radio pharmaceuticals have applications in medicine for treatment of cancers, hyperthyroidism etc. These substances on administration into the body give out radiation which cause ionisation in the cells. It causes disruption in the cellular metabolism and ultimately leading to cell destruction.

Ex: Na ^{131}I , Sodium phosphate ^{32}P , Radio Gold (^{198}Au)

Na ^{131}I (Radio Iodine) : It is used in Thyroid Carcinoma in the oral dose of 100-200 mCi. Follicular carcinoma can be destroyed by this therapy. Therapy with radioiodine combined with surgery gives excellent results.

Sodium Phosphate ^{32}P : It emits β -particles. It is used in the treatment of polycythaemia vera alone or combined with phlebotomy. Its oral dose is 5-6 mCi and I.V. dose is 2.5 mCi. Its half life is 14.3 days.

Radio Gold (^{198}Au) : It emits low energy β - and γ radiations. Radio Gold is used in treatment of malignant pleural and peritoneal effusions in the dose of 100-150 mCi. It is also used in prostatic and pelvic cancers.

3) Radio Isotopes used for sterilisation :

Radio isotopes are also used for sterilisation due to emission of radiations

therapeutic purpose, diagnostic purpose and sterilisation. Precautionary measures have to be taken to prevent harmful effects of radiation. α and β particles, γ rays, protons and neutrons can cause damage. Damage caused by these radiations depend on different factors such as susceptibility of tissues, dose and penetrating power. Radio sensitivity differs from tissue to tissue. It also differs from species to species. Following irradiation, cell death is caused by mitotic inhibition and failure of reproduction.

Damage caused by irradiation can be divided in to two types.

- 1) Somatic effect : It affects cells of skin, mucous membranes of intestines, liver, bone marrow etc.,
- 2) Genetic effect : which is concerned with hereditary patterns of species.

Effects of radiation can be acute if the radiation dose is very high and chronic if the radiation dose is low.

Acute effects of Radiation :

LD_{50} for man is expected to be about 450 - 500 rads .

LD_{50} : A dose which can kill 50% of same population is LD_{50} . Large doses cause death due to injury to brain.

Delayed effects of Radiation:

They are -

- 1) Ageing effect: Life span may be shortened.
- 2) Carcinogenicity: Cancers may be caused.
- 3) Gonadal effect: Sterility, azo spermia or hypospermia may be caused.
- 4) Genetic damage: In cells, genetic damage may be caused.

Precautionary measures to be taken to safe guard from the effects of Radiation: Precautionary measures have to be taken to safe guard the personnel involved in storage, dispensing, administration, diagnosis and any other concerned with radio pharmaceuticals from their hazardous biological effects, which are mentioned in the above paras. In a hospital, or elsewhere, undertaking use of radio pharmaceuticals, regulations to be followed have been framed by Govt. of India. They are similar to those framed by atomic energy commission of America. These regulations in India are being imposed through Atomic energy commission

by them. Mode of action is both excitation and ionisation.

ex: ^{60}Co , ^{137}Cs

^{60}Co : It emits γ radiation which is useful for sterilisation . γ -rays emitted by ^{60}Co have the energies of 1.33 and 1.17 MeV. Its half life period is 5.25 years.

^{137}Cs : This is a product of disintegration of uranium. It is the major component of spent fuel rods from nuclear reactors. It has a half life period of 30 years. γ -rays emitted by ^{137}Cs have the energy of 0.66 MeV.

Effectiveness of γ -rays over organisms:

- a) Gram negative organisms are more sensitive than gram positive organisms.
- b) Sporing bacteria are most resistant.

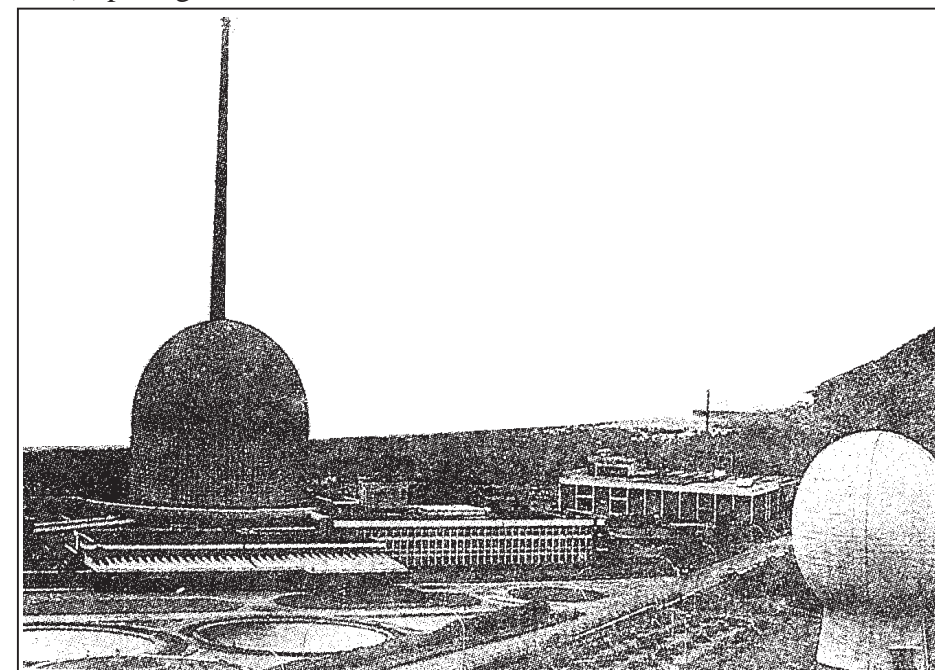


Fig.3-3 Bhabha Atomic Research Centre, Trombay, Mumbai.

Safety and precautions :

Radiations have hazardous effects if proper care is not taken. Great care should be taken in storage and handling of radiopharmaceuticals used for

and isotopes division of BARC, Trombay, Mumbai.

In hospitals using radio pharmaceuticals, radiopharmaceutical committee has to be formed including radiation physicist, radiologist, internist, haematologist, a surgeon and other members as decided by the hospital. This committee will look into the matters regarding decisive matters on administration of radio isotopes radio diagnosis, safe handling of radio isotopes and remedial measures in case of breach of precautionary measures. A radio isotope administration sheet is also placed in the medical record of the patient by the doctor prescribing radio therapy . It will consist of precautions to be followed.

Precautions to be taken during Radio therapy :

- 1) More than one patient undergoing radiotherapy should not be cared at one time.
- 2) Patient should be instructed to do bedside working himself/herself.
- 3) Gloves should be worn while handling patients belongings or touching patient.
- 4) Plastic apron should be worn during bedside nursing.
- 5) Utensils containing patients excreta should be marked symbol for radioactive.
- 6) Such utensils have to be flushed with plenty of water.
- 7) Any draining wound of the patient should be intimated to the doctor concerned immediately.
- 8) Linen, dressings, tissues etc. related to the patient have to be placed in yellow coloured dust bin with lid. It should be marked with radio active marker and should be shifted to decay area.
- 9) Visitors should not be allowed for first two weeks. Children and pregnant women should not be allowed to visit at any time.

Precautions to be taken during storage and handling of radio Pharmaceuticals:

- 1) Never touch radio isotope with hands. Use forceps.
- 2) Wear protective clothing / shielding during handling.
- 3) Keep radiopharmaceuticals in containers shielded by lead bricks and label

suitably.

- 4) Check the radio activity regularly.
- 5) Dispose the radio active materials with great care.
- 6) Receive the instructions from radiation safety officer.

Precautions in a laboratory conducting radio diagnosis :

- 1) Wear protective clothing.
- 2) Wear protective shoes.
- 3) Take measures for respiratory protection.
- 4) Use remote pipetting devices.
- 5) Wear gloves for hands.
- 6) Label the equipment using radioactive materials.
- 7) Place the waste in proper container and label.
- 8) Check the working areas regularly.
- 9) Maintain good lab practice procedures (GLP).
- 10) Report any spillages to the radiation safety officer.
- 11) Do not allow smoking, drinking and eating in the laboratory.
- 12) Perform decontamination procedures as directed by the radiation safety officer.

Hormone assays

Radio Immuno Assay : RIA is a specialised technique among quantitative analytical procedures and based on immunological reaction, i.e. antigen-antibody reaction which involves use of radio isotopes. Kits for determination of various substances are prepared and supplied by BARC.

Advantages offered by RIA : Advantages of RIA are -

- 1) Specificity
- 2) Sensitivity

- 3) Precision and accuracy
- 4) Simplicity
- 5) Less time required for assay
- 6) Direct determination of hormone instead of its excretory products
- 7) Use of blood specimen instead of urine

Applications of RIA :

Radioimmunoassay is useful in the field of medicine for diagnosis and clinical studies. It finds application in Pharmacy for Biopharmaceutical analysis and clinical studies for - determination of hormones, vitamins, cardiac glycosides etc.

- 1) This technique is useful for determination of various hormones like Thyroxine (T_4), TriIodothyronine (T_3), Human chorionic gonadotrophin (HCG), Human placental lactogen, Insulin etc.
- 2) It is useful for determination of vitamins like Cyanacobalamine
- 3) It is useful for determination of cardiac glycoside Digitoxin.

Principle of RIA :

RIA is based on setting up competition between radioactively labelled substance and unlabelled substance to bind with specific antibody developed for the purpose. There is inverse relation existing between quantities of labelled and unlabelled substances.

RIA of Thyroxine (T_4) :

Clinical Significance:

Determination of thyroxine is significant in assessment of thyroid functioning.

Hyperthyroidism : It is clinical condition in which thyroid hormones are secreted above normal.

Hypothyroidism: It is a clinical condition in which thyroid hormones are secreted below normal.

Elevated levels of thyroxine are observed in Hyperthyroidism, Acute thyroiditis and sub-acute thyroiditis. Low levels of thyroxine are observed in hypothyroidism, chronic thyroiditis, cretinism and myxoedema.

Requirements :

- 1) Test tubes
- 2) Pipettes
- 3) Centrifuge
- 4) Gamma counter
- 5) Incubator

Re agents / solutions in the RIA kit for T_4 :

- 1) Thyroxine (T_4) labelled with ^{125}I (antigen)
- 2) Antibody for T_4
- 3) Control sera
- 4) Poly ethylene glycol
- 5) Buffer solution

Specimen : Serum

- Procedure:
- 1) Take 24 mL of serum in a test tube.
 - 2) Add 0.4 ml. of antiserum and mix.
 - 3) Add 0.1 ml. of radioactive labelled thyroxine and mix.
 - 4) Cover and incubate for 1 hour at $37^{\circ}C$.
 - 5) Add 2ml. of poly ethylene glycol and mix.
 - 6) Centrifuge for about 10 minutes.
 - 7) Decant supernatant and count using Gamma counter.
 - 8) Perform similarly for different concentrations of control sera and

count.

9) Determine ratios of Bound hormone (B) to Free hormone (F).

10) Prepare graph with B/F ratios on Y-axis and thyroxine concentrations on X-axis.

11) Read the concentration of unknown from the graph.

Normal range: 48-115 mg/ml.

Interpretation :

Determination of thyroxine is significant in assessment of thyroid functioning.

Hyperthyroidism : It is clinical condition in which thyroid hormones are secreted above normal.

Hypothyroidism: It is a clinical condition in which thyroid hormones are secreted below normal.

Elevated levels of thyroxine are observed in Hyperthyroidism, Acute thyroiditis and sub-acute thyroiditis. Low levels of thyroxine are observed in hypothyroidism, chronic thyroiditis, cretinism and myxoedema.

RIA of Triiodo thyronine (T_3) :

Clinical significance: Determination of TriIodothyronine is useful in the diagnosis of hyper thyroidism. It is however, not useful for diagnosis of hypothyroidism.

Requirements:

Same as for Thyroxine determination.

Reagents/ Solutions : RIA kit for TriIodo thyronine supplied from BARC.

Specimen: Serum

Procedure:

1) Take serum in a test tube.

2) Add antiserum and mix.

3) Add radio active labelled hormone and mix.

4) Incubate .

5) Add adsorbant .

6) Centrifuge.

7) Decant and count with Gamma counter.

8) Perform similarly for different concentrations of control sera and count.

9) Determine B/F ratios.

10) Plot with B/F on Y axis and T_3 conc. on X-axis.

11) Read the concentration of unknown from graph.

Normal range 0.7 -2 ng/ml.

Interpretation : Determination of TriIodothyronine is useful in the diagnosis of hyper thyroidism. It is however, not useful for diagnosis of hypothyroidism.

RIA of Human Chorionic Gonadotrophin (HCG):

Clinical Significance: 1) It is a hormone produced by placenta of pregnant women.

2) It is secreted by gestational, trophoblastic tumours derived from placenta. High values of this hormone are associated with this condition.

3) Small quantities of HCG are also present in blood of normal non pregnant women. Significance is unknown.

4) Its determination is significant in monitoring treatment in men with non seminomatous germinal cell tumours.

Requirements:

Same as in Thyroxine determination.

Reagents / Solutions :

RIA kit for HCG

Specimen : Serum

Procedure : Same as in T₄ and T₃ determinations with the reagents/ solutions in RIA kit for HCG.

RIA of human prolactin (HPRL) :

Normal range: 5-18 ng/ml (male) 6-22 ng/ml (female)

- 1) Main function of prolactin is initiation and maintenance of lactation. Its levels are increased during pregnancy and post partum.
- 2) Increased levels are observed after exercise and stress.
- 3) It is possibly involved in causing mammary cancer.
- 4) Its determination is significant in diagnosis of tumours of hypothalamus and pituitary.

Requirements: Same as for above RIAs.

Reagents: RIA kit for determination of human prolactin.

Specimen : Serum

Procedure:

Same as in above methods with RIA kit for prolactin.

RIA of Human Placental Lactogen:

Reagents: RIA kit for HPL.

Specimen : Serum

Procedure: Same as above methods with RIA kit for HPL.

Normal range:

5-27 weeks -- below 4.6 mg/ml.

28-31 weeks -- 2.4 to 6.1 mg/ml.

32-35 weeks -- 3.7 to 7.7 mg/ml.

36th week -- 5.8 - 8.6 mg/ml.

Interpretation : Its determination is significant in management of complicated pregnancies. Decrease in HPL is observed five to ten days before abortion

RIA of Insulin :

Clinical significance:

Insulin is a hormone produced by b-cells of Islets of langerhans of pancreas. It is concerned with carbohydrate metabolism. It enhances utilisation of glucose by cells thus decreasing the glucose levels in blood. Deficient and absent secretions of this hormone causes raised blood sugar levels. When this crosses 180mg/100ml, glucose gets excreted in urine. Condition of raised blood sugar levels above normal is called as Hyper glycaemia and appearance of glucose in urine is called as Glycosuria. Blood sugar level of 180 mg% above which glycosuria occurs is called as renal threshold for blood sugar. Hyperglycaemia and glycosuria occur in diabetes mellitus. Excessive secretion of Insulin causes lowered blood sugar levels below normal called as Hypoglycaemia. Determination of serum insulin levels is significant in diagnosis of diabetes mellitus and insulinoma. Normal ranges of post glucose insulin levels.

S.No.	Time interval	Insulin levels
1	0 hr .	4-24 mcU/ml.
2	1/2 hr.	25-231 mcU/ml.
3	1 hr.	18-276 mcU/ml.
4	2 hrs.	16-166 mcU/ml.
5	3 hrs.	4 - 38 mcU/ml.

Reagents / Solutions :

RIA kit for determination of insulin

Specimen : Serum

Procedure: Same as in above methods with RIA kit for insulin.

Clinical interpretation :

Insulin is a hormone produced by β -cells of Islets of Langerhans of pancreas. It is concerned with carbohydrate metabolism. It enhances utilisation of glucose by cells thus decreasing the glucose levels in blood. Deficient and absent secretions of this hormone causes raised blood sugar levels. When this crosses 180mg/100ml, glucose gets excreted in urine. Condition of raised blood sugar levels above normal is called as Hyper glycaemia and appearance of glucose in urine is called as Glycosuria. Blood sugar level of 180 mg% above which glycosuria occurs is called as renal threshold for blood sugar. Hyperglycaemia and glycosuria occur in diabetes mellitus. Excessive secretion of Insulin causes lowered blood sugar levels below normal called as Hypoglycaemia. Determination of serum insulin levels is significant in diagnosis of diabetes mellitus and insulinoma. Normal ranges of post glucose insulin levels.

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4	2 hrs.	16-166 mcU/ml.
5	3 hrs.	4 - 38 mcU/ml.

RIA of digitoxin :

Digitoxin is a cardiac glycoside obtained from the leaves of digitalis purpurea belonging to family Scrophulariaceae.

Requirements : Same as in above methods.

Reagents/ solutions : RIA kit for digitoxin.

Specimen: Serum

Procedure:

Same as in above methods with RIA kit for digitoxin.

Effective therapeutic levels in serum: 1-2 ng/ml.

Clinical significance: Digitoxin is cardiactonic. It is used in congestive cardiac failure and other cardiac disorders. Its determination in blood is useful for monitoring the therapy. Its bio-pharmaceutical analysis gives the cardiologist with very useful clinical data for effective cardiac care.

RIA of cyanocobalamin (B₁₂) :

Cyanocobalamin is a water soluble, crystalline substance and red in colour. It contains metalloporphyrin nucleus and cobalt. Its molecular weight is about 1500. Its determination in serum is useful in clinical studies, quality assurance and progress of vitamin B₁₂ therapy in deficiency conditions.

Daily requirement: 1 mg/day

Reagents / Solutions:

RIA kit for cyanocobalamin.

Specimen: Serum.

Procedure : Same as in above procedures with RIA kit for cyanocobalamin.

Clinical significance :

- 1) It is essential for formation and maturation of red blood cells. Its deficiency causes pernicious anaemia.
- 2) It plays important role in the synthesis of nucleic acid. Its deficiency causes disturbed DNA metabolism.
- 3) Its deficiency also causes hyperglycaemia and its administration can correct hyperglycaemia caused by its deficiency.
- 4) Its deficiency also causes atrophy of mucosal membranes, inflammation of tongue, mouth etc.

2 - ENZYME LINKED IMMUNO ASSAYS ,

description of instruments used in these assays

Definition : Enzyme linked immunosorbent assay is a method of qualitative identification and quantitative determination based on antigen - antibody

reaction . It is used for serological diagnosis in microbiology and quantitative determinations in biochemistry.

Advantages of ELISA : 1. It provides more sensitivity than other serological and quantitative methods .

2. It provides more specificity than other serological and quantitative methods .

2. It can be done using a single serum dilution .

3. It can be automated .

Applications of ELISA : Enzyme linked immunosorbent assay is used for serological and quantitative determinations. It can be used in biochemistry , serology , parasitology and for several purposes requiring more sensitivity and specificity over other methods . Hormones determined by ELISA are T_3 , T_4 , TSH , LH , FSH , Prolactin , hCG and hGH etc . Enzymes determined by ELISA are Lipase , Amylase etc. It is also useful in diagnosis of toxoplasma , rubella , herpes simplex virus , HIV , HBsAg , tuberculosis , Rabies virus , Measles virus , Mumps virus , parasitic diseases like amoebiasis , echinococcus etc . It is also used to identify tetanus toxin antibodies , meningitis , rotavirus , brucella , candida albicans etc . It is also used to identify autoimmune diseases .

Instrumentation : ELISA technique depends on determination of absorbance readings . It can be done using spectrophotometer or ELISA reader . These instruments are constructed with light source , filter / monochromator , cuvette , detector and galvanometer .

Determination of T_4 by ELISA technique :

Principle : Peroxidase labelled thyroxine competes with thyroxine hormone in binding with thyroxine specific antibodies . Amount of antibody - thyroxine - POD complex is a measure of thyroxine concentration of the sample . This complex on addition of hydrogen peroxide and chromogen forms a colored complex whose colour intensity is directly proportional to the concentration thyroxine of hormone . Comparison of absorbance in the test sample with that in the control serum by plotting a calibration curve using different dilutions of control serum gives the

value of thyroxine hormone in the specimen .

Requirements : 1. Pipettes

2 . Test tubes

3. Centrifuge

4. Spectrophotometer/ ELISA reader

5. Incubator

6. ELISA kit containing thyroxine - POD conjugate , substrate buffer and control sera

Procedure : 1. Add control sera to vials coated with antibody.

2. Add patient's serum to another vial coated with antibody .

3. Incubate the vials for 2 hours at 20 - 25⁰ C. 4.

Aspirate vial contents . Discard and rinse vials with tap water

5. Add substrate buffer solution in each vial .

6. Mix and incubate for one hour at 20 - 25⁰ C.

7. Set the absorbance to zero against blank .

8. Take the readings of absorbance of test and standards . 9.

Plot standard graph with absorbances of standards on Y axis and concentrations on X axis .

10. Take the value of T_4 in test serum from standard graph .

Summary

Radio activity is spontaneous emission of radiation by elements with atomic number more than 83. Different radiations emitted by radio active substance are α -particles, β -particles and γ -rays. Radio isotopes are used for 1) Diagnostic procedures 2) Therapeutic purpose and 3) Sterilisation

Radio immunoassay is a specialised technique among quantitative analytical procedures based on immunological reaction (antigen - antibody reaction), which involves use of radio isotopes. Radio immunoassay is useful in diagnosis and clinical studies.

Precautionary measures have to be taken to prevent harmful effects of radiations. Precautions have to be taken during radio therapy, storage and handling of radio pharmaceuticals and during radio diagnosis.

Enzyme linked immunosorbent assay is used for serological and quantitative determinations. Enzyme linked immunosorbent assay is used for serological and quantitative determinations. It can be used in biochemistry, serology, parasitology and for several purposes requiring more sensitivity and specificity over other methods.

Essay Questions

- 1) Define Radio activity and different units related to Radio activity.
- 2) What are the applications of radio isotopes? Write about their applications in diagnostic procedures.
- 3) Mention requirements of radiopharmaceuticals.
- 4) Write about the applications of radio-isotopes in therapeutics and sterilisation.
- 5) Define radio immunoassay. Write the advantages, applications and principle of RIA.
- 6) Describe RIA of Thyroxine (T_4) hormone.
- 7) Write about determination of Triiodothyronine (T_3) by RIA.
- 8) Give a short note on biological effects of radiation.
- 9) Write about the precautionary measures to be taken during radio therapy.
- 10) Discuss the precautions to be followed during storage and handling of radioisotopes and radio diagnosis.
- 11) Write the ELISA of T_4 .

Short Answer Questions

- 1) Define Radio activity.
- 2) What is meant by Radio active transformation?
- 3) Mention different radiations emitted by radio active substances.

- 4) Define Curie.
- 5) What is half life period?
- 6) Define MeV.
- 7) What is decay constant?
- 8) What is meant by biological half life?
- 9) Define isotopes and exemplify.
- 10) What are radio isotopes?
- 11) Write the definitions of a) Isobars b) Isotones
- 12) Exemplify some radio isotopes used in diagnosis of hepatic functioning
- 13) Give a few examples of radio isotopes used in the diagnosis of renal functioning.
- 14) Mention a few radio isotopes used in therapeutics.
- 15) Mention radio-pharmaceuticals used in sterilisation.
- 16) Write the advantages of RIA.
- 17) Write the principle of RIA
- 18) Define a) Hyper thyroidism b) Hypo thyroidism
- 19) What are the normal ranges of
a) Thyroxine (T_4) b) Tri iodothyronine (T_3).
- 20) Write the clinical significance of RIA of Human placental lactogen.
- 21) Mention normal ranges of postglucose values of insulin.
- 22) What is cyanacobolamin?
- 23) What are different instruments used to measure radioactivity?
- 24) Name the agencies imposing regulations regarding radioactive substances in India.
- 25) Write the full forms of RIA and ELISA.
- 26) Write the principle of ELISA of T_4 .

IV - METABOLISM

Metabolism : Metabolism of a substance is defined as series of biochemical reactions occurring within the living organism from the time of incorporation until its excretion .Metabolism consists of anabolism and catabolism .

Anabolism : Anabolism or synthesis is defined as series of biochemical reactions involved in the process of synthesis of larger molecules from smaller ones

Catabolism : Catabolism or analysis is defined as series of reactions involved in the process of breakdown of larger molecules into smaller ones for supply of energy .

Energy metabolism : There will be continuous exchange of energy between living body and its surrounding environment . It is in accordance with the principles of thermodynamics as applied to physical and chemical processes in non living things . This energy exchange is based on first law of thermodynamics which states that energy is neither gained nor lost when it is converted from one form to another form .These forms are chemical, electrical , mechanical , thermal etc .

Unit of energy : It is mainly calorie .It is defined as amount of heat energy which can raise the temperature of 1 g. of water from 15°C to 16°C. Amount of heat can also be expressed as joule .

1 kilocalorie = 4.19 kilojoules

Uses of energy in body : Energy obtained from food sources is used in body for several purposes such as -

1. Synthesis of protoplasmic constituents like D.N.A., R.N.A. , phospholipids etc.
2. Functioning of different organs like heart , kidneys etc.
3. Maintenance of body temperature
4. Generation of electrical potentials in CNS , ANS , heart etc.
5. Transport of substances against concentration gradient
6. Growth and maintenance

Basl metabolism : Energy produced as by-product of cellular metabolism is essential for sustnace of life . Although there is variation in rquirement of amount of energy , rate of production of energy in body remains more or less constant under some standard conditions known as basal metabolism .

Basal metabolic rate : The rate of production of energy at basal conditions per hour per metre² of body surface area is called basl metabolic rate .Its normal value in adult male is 40 calories per hour per metre² and 37 calories per hour per metre² in adult female .

1.CARBOHYDRATE METABOLISM -GLYCOLYSIS AND TCA CYCLE

Carbohydrates are converted to monosacharides in the process of digestion in the digestive system. Mainly they are glucose, fructose and galactose. They are absorbed from the small intestines into portal circulation. Major function carbohydrates is release of energy by undergoing oxidation. Carbohydrates are mainly used by cells in the form of glucose. Especially brain cells use glucose only. Fructose and galactose are also converted to glucose in liver.

Different metabolic processes undergone by carbohydrates in the body are

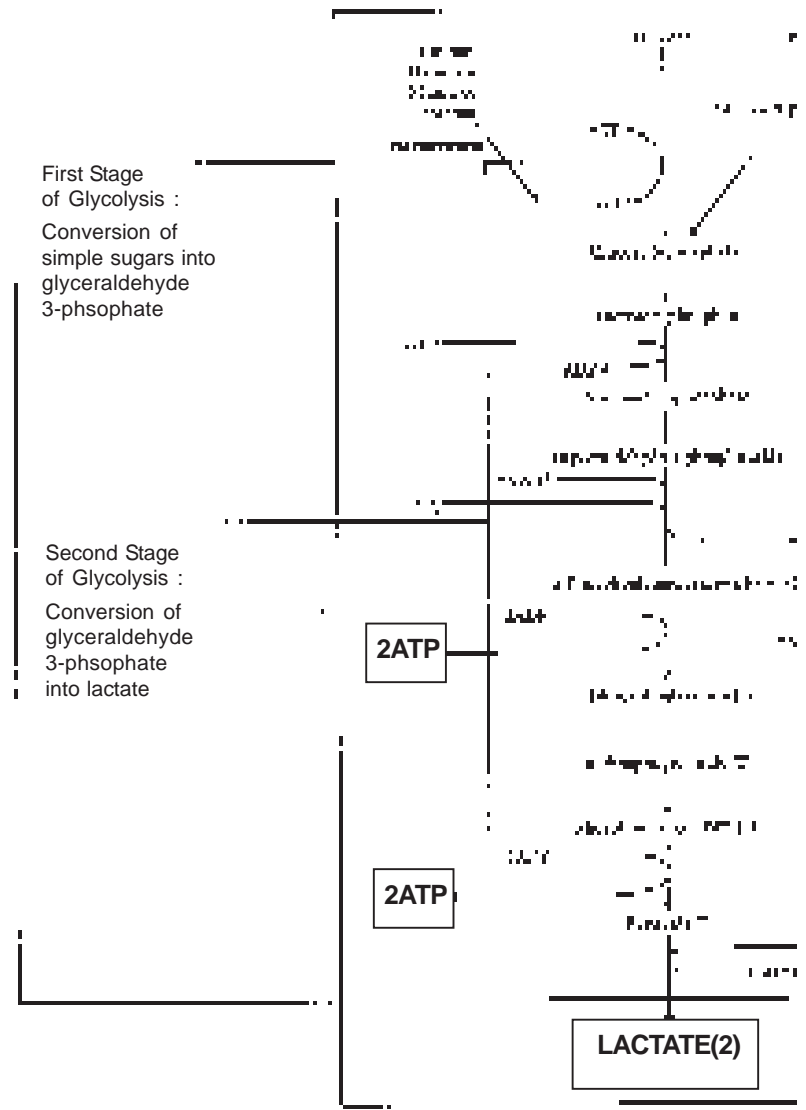
1. Glycogenesis : Glycogenesis is the synthesis of glycogen from glucose.
2. Gluconeogenesis : Gluconeogenesis is formation of glucose from non-carbohydrates as glycerol.
3. Glycogenolysis : Glycogenolysis is the conversion of glycogen to glucose.
Glycogen -----> glucose
4. Glycolysis : Glycolysis is the oxidative path way of glycogen or glucose.
5. Citric acid cycle : It is also oxidative pathway of glucose.
6. Hexosemonophosphate shunt is alternative to glycolysis and citric acid cycle for oxidation of glucose.

Significance of Glycolysis:

Glycolysis is also known as embden - meyer hoff pathway. Glycolysis is anaerobic degradation of glucose to yield lactic acid. Many organisms extract energy by this process in the absence of molecular oxygen. It is the most ancient type of mechanism for obtaining energy from nutrient molecule-glucose.

Most higher organisms retained this anaerobic degradative mechanism and it is preparatory pathway in aerobic glucose catabolism-citric acid cycle. However it is selected as an important mechanism for yielding energy in the most of the

animals when oxygen is not available.



Diagrammatic representation of GLYCOLYSIS

Obligate or strict anaerobes are the organisms which survive in the absence of oxygen only. Ex: Denitrifying bacteria of the soil, Clostridium perfringens and Clostridium botulinum use this process for energy. Facultative anaerobes use this process in absence of oxygen. In presence of oxygen they use this process followed by oxidation of the products of glycolysis. Facultative anaerobes are

the organisms which survive in absence of oxygen and also in the presence of oxygen.

Stages of Glycolysis: There are two stages in glycolysis. In the first stage, glucose is converted to glyceraldehyde 3-phosphate, a three carbon compound. In the second stage, glyceraldehyde 3-phosphate is converted into pyruvate. This pyruvate enters citric acid cycle in aerobic conditions. In anaerobic conditions pyruvate is converted to lactate.

Steps in Ist stage of Glycolysis:

Hexokinase

1) Glucose $\xrightarrow[\text{ATP} \rightarrow \text{ADP}]{} \text{Glucose 6-Phosphate}$

In this step, phosphorylation of D-Glucose to Glucose 6-Phosphate by ATP takes place. It is catalysed by enzyme hexokinase.

Isomerase

2) Glucose 6-Phosphate $\xrightarrow{} \text{Fructose 6-Phosphate}$

In this step, isomerisation of Glucose 6-phosphate to fructose 6-phosphate takes place. It is catalysed by enzyme isomerase.

Hexokinase

3) Fructose 6-Phosphate $\xrightarrow[\text{ATP} \rightarrow \text{ADP}]{} \text{Fructose 1,6-Di-Phosphate}$

Aldolase

4) Fructose 1,6-Di-Phosphate $\xrightarrow{} \text{Dihydroxy acetone phosphate} + \text{Glyceraldehyde 3-phosphate}$

In this step, Fructose 1,6-Di-Phosphate is broken down to Dihydroxy acetone phosphate and Glyceraldehyde 3-phosphate (3 carbon compounds). This reaction is catalysed by enzyme Aldolase.

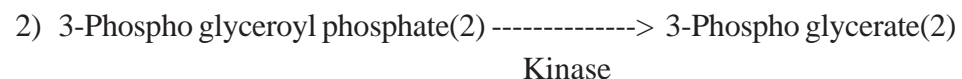
Steps in IInd stage of Glycolysis:

Dehydrogenase

1) Glyceraldehyde 3-phosphate(2) + NAD⁺ + Pi $\xrightarrow{} \dots$

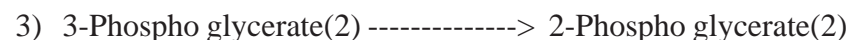


In this step, oxidation of Glyceraldehyde 3-phosphate(2) to 3-Phospho glyceroyl phosphate(2) takes place. It is catalysed by enzyme Glyceraldehyde 3-phosphate Dehydrogenase.

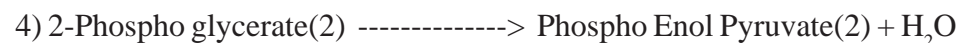


In this step, glyceroyl phosphate(2) to 3-Phospho glycerate(2) takes place. It is catalysed by enzyme Kinase.

In this step, 2ATP molecules are produced by transfer of phosphates from 2 molecules of 3-phospho glyceroyl phosphate.



In this step, conversion of 3-Phospho glycerate(2) to 2-Phospho glycerate(2) takes place. It is catalysed by enzyme Mutase.

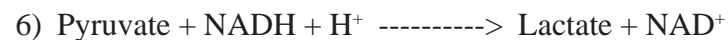


In this step, conversion of 2-Phospho glycerate(2) to Phospho Enol Pyruvate(2) takes place. It is catalysed by enzyme Enolase.



In this step, conversion of Phospho Enol Pyruvate(2) to Pyruvate(2) takes place. It is catalysed by enzyme Kinase.

2 ATP molecules are produced by transfer of phosphates from Phospho Enol Pyruvate.



In anaerobic conditions, pyruvate is converted to lactate by enzyme lactate

dehydrogenase (LDH). In aerobic conditions, pyruvate enters citric acid cycle.

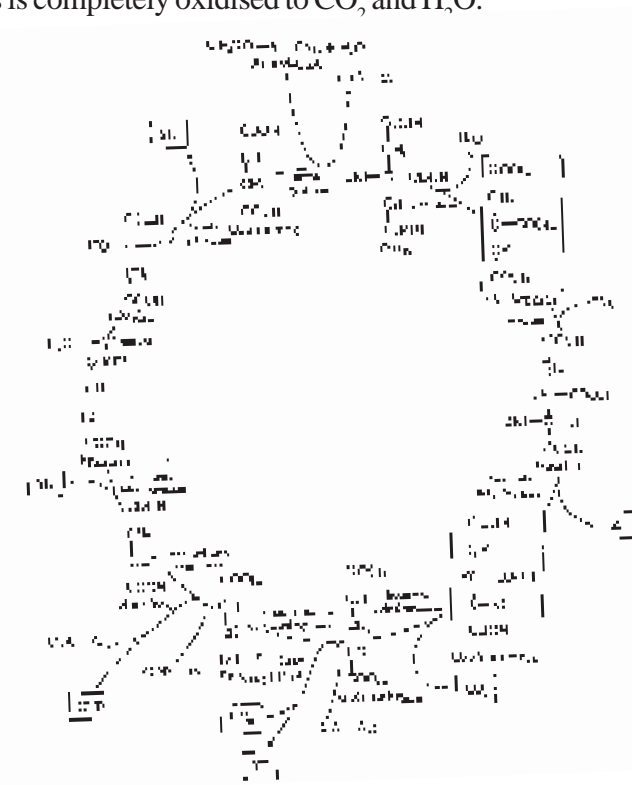
Energy Balance in Glycolysis : 10 ATPs are produced.

2 ATPs are consumed.

Net yield of ATP is 8.

Significance of Tricarboxylic acid cycle :

TCA cycle is a cyclic sequence of reactions in which pyruvate formed in glycolysis is completely oxidised to CO₂ and H₂O.



Diagrammatic representation of TCA CYCLE

It was postulated by H.A. Krebs in 1937. Its original name is Citric Acid Cycle.

TCA cycle is the common pathway for degradation of two carbon acetyl residues derived not only from carbohydrates but also from fatty acids and amino acids. TCA cycle actually takes place in intact cells. It accounts quantitatively for oxidation of carbohydrates, fatty acids and amino acids.

Glycolysis releases only a fraction of energy present in glucose molecule. More energy is released when glucose molecule is completely oxidised to CO₂ and H₂O, which occurs in TCA cycle.

TCA cycle is both catabolic and anabolic pathway. It also generates precursors for anabolic pathways. α-keto glutarate and oxaloacetate serve as precursors of amino acids. α-ketoglutarate and oxaloacetate are intermediates of this cycle. Citrate can be removed from this cycle and can be used as precursor of extra mitochondrial acetyl-CoA for fatty acid biosynthesis. Succinyl-CoA can also be removed from the cycle for biosynthesis of heme.

Steps in TCA Cycle :

- 1) Citrate synthetase

$$\text{Acetyl -CoA} + \text{Oxaloacetate} + \text{H}_2\text{O} \text{ -----} > \text{Citrate} + \text{CoA}$$

In this step, condensation of acetyl co-A with oxaloacetate takes place. It is catalysed by Citrate Synthetase forming Citrate.
- 2) Aconitase Aconitase

$$\text{Citrate} \text{ -----} > \text{Cis-Aconitate} \text{ -----} > \text{Isocitrate}$$

In this step, conversion of Citrate to Iso citrate takes place. These catalysed by enzyme Aconitase.
- 3) Isocitrate + NAD⁺ (NADP⁺) -----> α-Keto glutarate + CO₂ + NADH (NADPH) + H⁺

Oxidation of Isocitrate to α-Keto glutarate takes place in this step. It is catalysed by Isocitrate de-hydrogenase.
- 4) α-Keto glutarate + NAD⁺ + CoA ----> Succinyl CoA + CO₂ + NADH + H⁺

In this step, oxidation of α-Keto glutarate to succinyl CoA takes place. It is catalysed by α-Keto glutarate dehydrogenase.
- 5) Succinyl CoA synthetase

$$\text{Succinyl CoA} + \text{Pi} + \text{GDP} \text{ -----} > \text{Succinate} + \text{GTP} + \text{CoA-SH}$$

In this step, deacylation of succinyl CoA to succinate takes place. It is catalysed by succinyl CoA synthetase.

- 6) Succinate dehydrogenase.

$$\text{Succinate} + \text{E-FAD} \text{ -----} > \text{Fumarate} + \text{E-FADH}_2$$

Oxidation of succinate to fumarate takes place in this step. It is catalysed by enzyme succinate dehydrogenase.
- 7) Fumarase.

$$\text{Fumarate} + \text{H}_2\text{O} \text{ -----} > \text{L-malate}$$

In this step, hydration of fumarate to L-maleate takes place. It is catalysed by Fumarase.
- 8) L-malate dehydrogenase

$$\text{L-malate} + \text{NAD}^+ \text{ -----} > \text{Oxalo acetate} + \text{NADH} + \text{H}^+$$

Oxidation of malate to oxalo acetate takes place in this step. It is catalysed by L-malate dehydrogenase.

Oxalo acetate can combine with another molecule of acetyl Co-A to start another cycle.

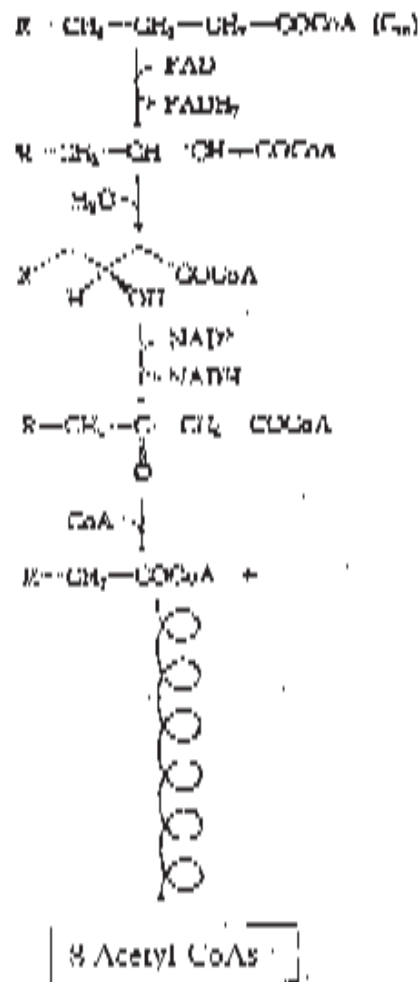
Energy Balance in TCA cycle :

TCA Cycle : 30 ATPs are produced.

2 - LIPID METABOLISM - b OXIDATION OF FATTY ACIDS

Lipid is extremely important component of diet. Dietary lipids are hydrolysed to fatty acids and glycerol. Fatty acids are required for energy production as well as synthesis of phospholipids. Glycerol can enter glycolysis. Free fatty acid enters mitochondria and undergoes beta-oxidation of fatty acids.

Significance of Beta-Oxidation of fatty acids :



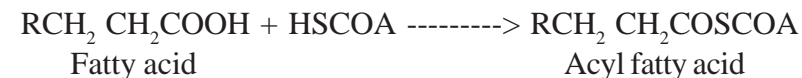
Diagrammatic representation of β -OXIDATION OF FATTY ACIDS

Fatty acids play an extremely important part as energy rich fuel in higher animals and plants. Triglycerides or tri acyl glycerols have high energy content- i.e. 9 K cal./g; where as glycogens and starch can yield only 4 K cal./g. Fatty acids are providing about 40% of energy requirement in man of normal diet. Fatty acids provide means of energy source in fasting conditions. For hibernating animals and migrating birds, fatty acids are the sole source of energy. Free fatty acids are liberated from triacyl glycerols by the action of hormonally controlled lipases.

They, then bind to serum albumin, and in this form are carried to other tissues for oxidation. They undergo enzymatic activation in cytoplasm and then enter mitochondria for oxidation. They are oxidised to CO_2 and H_2O in nearly all tissues except brain. Some tissues as cardiac muscle procure most of the energy requirement from oxidation of fatty acids.

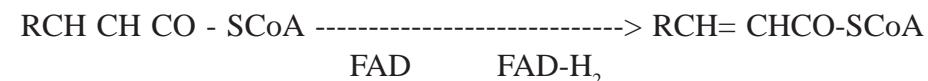
Steps of the cycle : It is a stepwise degradation of long chain fatty acids. It is an oxidative pathway.

1) Activation of fatty acid : Thiolase



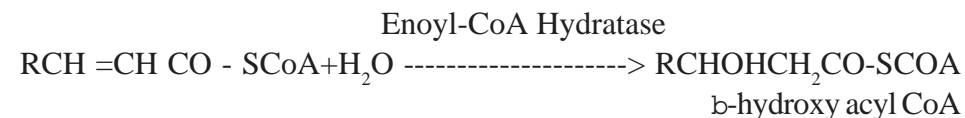
In this step, conversion of fatty acids to acyl fatty acid takes place. It is catalysed by enzyme Thiolase.

2) Formation of Unsaturated Acyl- CoA : Acyl CoA Dehydrogenase



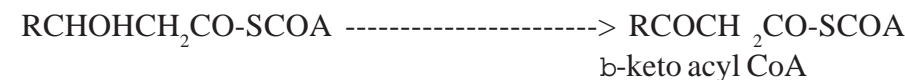
In this step, conversion of acyl fatty acid to α,β unsaturated acyl CoA takes place. It is catalysed by enzyme Acyl CoA Dehydrogenase. In this process of dehydrogenation, hydrogen molecules are taken by FAD forming FADH_2 .

3) Formation of β -hydroxy-Acyl CoA :



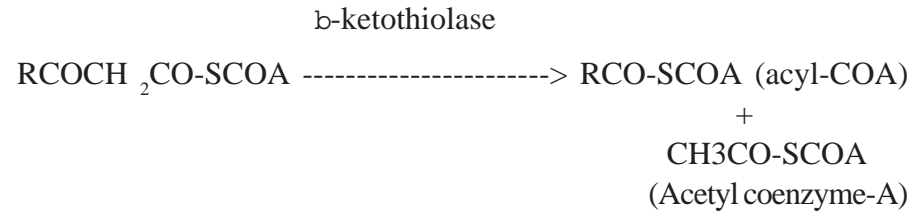
In this step, conversion of unsaturated acyl CoA to β -hydroxy acyl CoA takes place by the process of hydration which is catalysed by enzyme Enoyl-CoA Hydratase.

4) Formation of β -keto-Acyl CoA : Hydroxy Acyl-CoA Dehydrogenase



In this step, conversion of β -hydroxy acyl CoA to β -keto acyl CoA takes place by the process of dehydrogenation which is catalysed by enzyme Hydroxy Acyl-CoA Dehydrogenase. Hydrogen molecules are taken by NAD^+ .

5) Thiolytic cleavage of β -keto acyl CoA :



In this step, conversion of β -keto acyl CoA to acyl-CoA and Acetyl coenzyme-A takes place by the process of thiolytic cleavage, which is catalysed by enzyme β -ketothiolase.

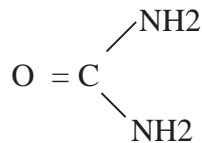
Energy Balance in β -oxidation of fatty acids:

Net yield of ATP molecules per molecule of palmitate is 129.

3 . PROTEIN METABOLISM - UREA CYCLE

Significance of Urea Cycle :

Urea is principal excretory product of protein catabolism. It is water soluble and has the chemical structure.



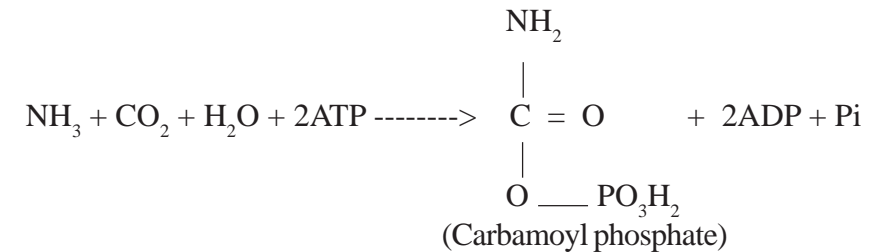
Most of the higher organisms re-use ammonia derived from the catabolism of amino acids. However some part is excreted by terrestrial vertebrates in the form of urea. Urea is a nontoxic compound. This process takes place in the liver of ureotelic organisms. Organisms which excrete amino nitrogen in the form of urea will be called as ureotelic organisms. Aquatic animals excrete amino nitrogen in the form of ammonia. Ammonia is a toxic compound. Organisms excreting Amino Nitrogen in the form of Ammonia are called as Aminotelic. Sequence of reactions of leading to formation of urea is called as

urea cycle. This path way was first postulated by H.A. Krebs and K.Henseleit. Subsequent research establishing the details of enzymatic steps was done by S.Ratner and P.P. Cohen. Urea a nontoxic neutral compound formed in the liver of ureotelic organisms is transported through blood to kidney and excreted in urine.

Steps: Urea cycle takes place in 1. Mitochondrion and 2. Cytosol

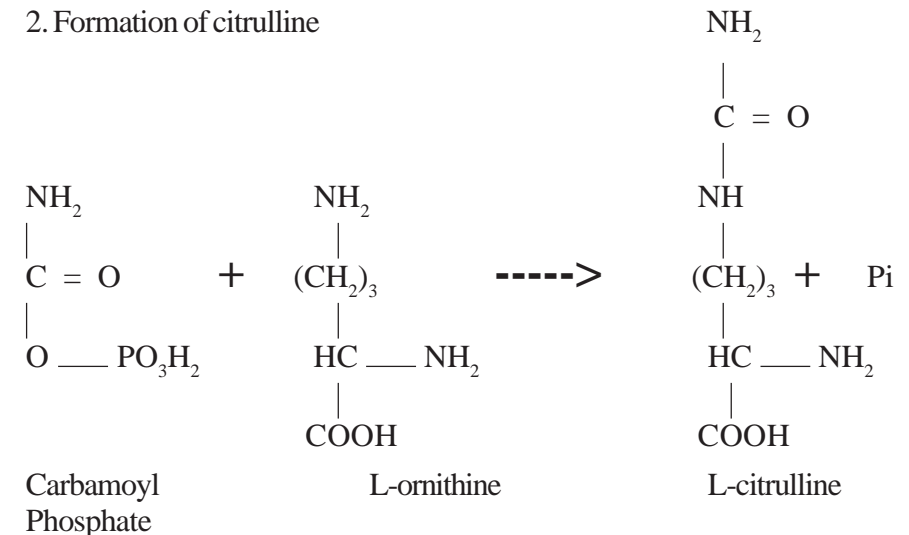
Steps taking place in mitochondrion

1. Formation of Carbamoyl Phosphate



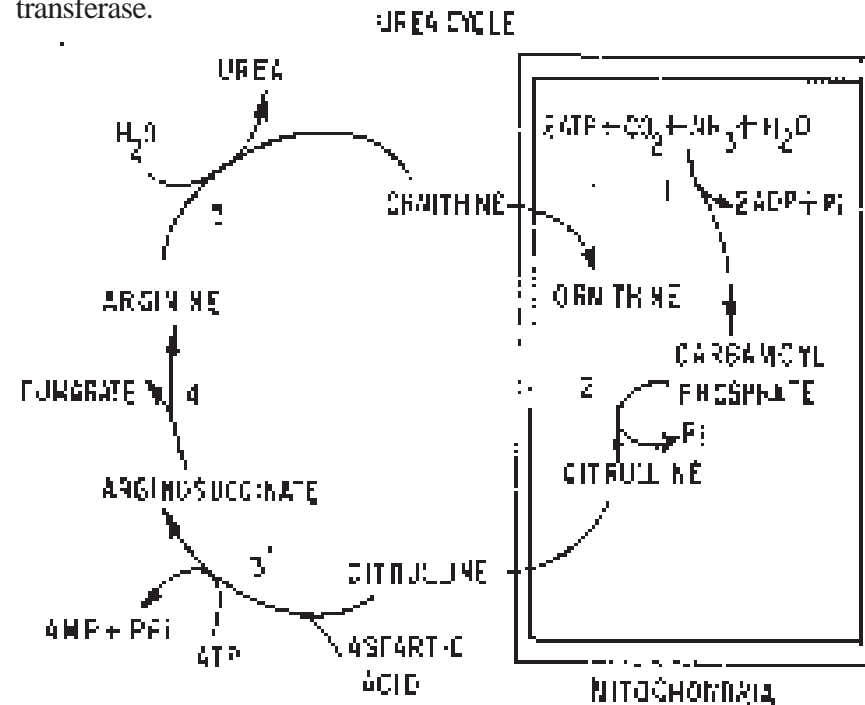
Ammonia reacts with CO_2 to form Carbamoyl phosphate. This reaction is catalyzed by carbamoyl phosphate synthetase. Two molecules of ATP are required to form each molecule of carbomoyl phosphate.

2. Formation of citrulline



Ornithine formed in the cytosol enters in to Mitochondrion through special transport system. Carbamoyl phosphate donates its carbamoyl group to

ornithine forming citrulline. This reaction is catalyzed by ornithine carbamoyl transferase.

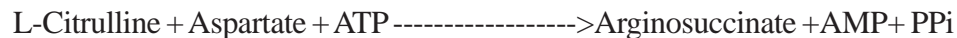


Diagrammatic representation of UREA CYCLE

Steps taking place in Cytosol :

Formation of Argino succinate:

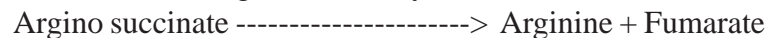
Arginosuccinate synthetase



Amino group of aspartate condenses with citrulline in the presence of ATP forming Argino succinate. This reaction is catalyzed by argino succinate synthetase.

Formation of Arginine:

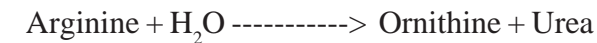
Arginosuccinate lyase



Argino succinate formed in the above step undergoes b-elimination reaction

in the body forming arginine. This reaction is catalyzed by argino succinate lyase.

Formation of urea :



Arginine undergoes hydrolysis forming urea and ornithine.

SUMMARY

Metabolism of a substance is defined as series of biochemical reactions occurring within the living organism from the time of incorporation to until its excretion. Exchange of energy between living body and its surrounding environment is in accordance with first law of thermodynamics which states that energy is neither gained nor lost when it is converted from one form to another form. Energy obtained from food sources is used in body for several purposes. Rate of production of energy in body remains more or less constant under some standard conditions known as basal metabolism. The rate of production of energy at basal conditions per hour per metre² of body surface area is called basal metabolic rate. Different metabolic processes undergone by carbohydrates in the body are - 1. Glycogenesis 2. Gluconeogenesis 3. Glycogenolysis 4. Glycolysis 5. Citric acid cycle 6. Hexosemonophosphate shunt. Glycolysis is anaerobic degradation of glucose to yield lactic acid. TCA cycle is a cyclic sequence of reactions in which pyruvate formed in glycolysis is completely oxidised to CO₂ and H₂O. Free fatty acid enters mitochondria and undergoes beta-oxidation of fatty acids. Sequence of reactions of leading to formation of urea is called as urea cycle. This path way was first postulated by H.A. Krebs and K.Henseleit.

ESSAY QUESTIONS

1. Write the introduction to metabolism .
2. Explain the significance of glycolysis and write the steps .
3. Write about significance of TCA cycle and discuss the steps .
4. Discuss the significance of b - oxidation of fatty acids and also write the steps.

5. Write the significance and steps of urea cycle.

SHORT ANSWER QUESTIONS

1. Define metabolism .
2. What is meant by anabolism and catabolism ?
3. What is the unit of energy ?
4. Write any 4 uses of energy in body .
5. What is basal metabolism ?
6. Define BMR .
7. Define a. Glycolysis b. Glycogenolysis
8. Write the other names of a. Glycolysis
b. TCA cycle
9. Name the products of first and second stages of glycolysis .
10. Write the energy balance in glycolysis and TCA cycle .
11. What is the oxidative path way of fatty acids ?
12. Define urea cycle .
13. Define a. Ureotelic organisms
b. Aminotelic organisms .
14. Name the scientists who postulated a. TCA cycle
b. Urea cycle
15. What are the reactions of urea cycle in mitochondrion ?
16. What reactions take place in cytosol leading to formation of urea ?
17. Name the end products of a. Glycolysis
b. Urea cycle
18. What are the energy contents of carbohydrates and triglycerides .
19. Name the enzymes of urea cycle in a. Arginosuccinate formation
b. Arginine formation

V - TITRIMETRIC METHODS OF QUANTITATIVE DETERMINATION, PREPARATION OF VARIOUS SOLUTIONS USED IN TITRIMETRIC ANALYSIS

Titrimetric methods offer high precision i.e. 1 part in 1000. They are advantageous over gravimetric methods in several aspects.

Advantages of titrimetric analysis :

- 1 . They offer high precision .
- 2 . They need simple apparatus.
- 3 . They can be quickly performed .
- 4 . Laborious and difficult separations can be avoided.

Definitions :

Titrimetric analysis or Volumetric analysis : It is a method of quantitative chemical analysis in which volume of solution of accurately known strength required to react quantitatively with solution of substance to be determined is measured, when these two substances react chemically.

Standard solution : Solution of accurately known strength is called Standard solution.

Titration : Titration is the process of adding standard solution to the substance being determined until the reaction is just complete.

Theoretical End point : The point at which reaction between the reactants is just complete in a titration is called theoretical or stoichiometric end point or equivalence point. Completion of titration should be detectable by some change. It can be produced by the standard solution itself (ex-Potassium permanganate) or by the addition of an auxiliary reagent called indicator.

Indicator : Indicator is an auxiliary reagent added in a titration process to notice the completion of reaction between the reactants. When a suitable indicator is not available for detection of end point, measure of potential between indicator electrode and reference electrode or change in electrical conductivity of the solution or current passing through the titration cell between indicator electrode and depolarised electrode at a suitable applied e.m.f. or change in absorbance of solution can be used for detection of end point.

End point of the titration : The point at which reaction between substance to

be determined and standard solution is practically complete and it is noticed by a clear visual change i.e. colour change or formation of turbidity by the addition of indicator is called End point of titration. . Titration involving measure of potential between indicator electrode and reference electrode for detection of end point is called Potentiometric titration. Titration involving measure of change in electrical conductivity for detection of end point of the solution is called Conductometric titration. Titration involving measure of current passing through the titration cell between indicator electrode and depolarised electrode at a suitable applied e.m.f for detection of end point the titration is called amperometric titration.. Titration involving measure of change in absorbance of solution for detection of end point is called photometric or spectrophotometric titration. Colorimetry and spectrophotometry are dealt in 1st year Biochemistry. Potentiometry is dealt as a separate chapter in second year Biochemistry.

Burette : Burette is a long and narrow graduated glass tube from which, standard solution is added to the solution of substance being determined.

Titrant : Reagent of known concentration used in titrimetric analysis reacting with the solution of substance to be determined is called Titrant.

Titrand : Substance to be determined is called Titrand.

Requirements for titrimetric analysis :

- 1 Graduated glassware like burettes, pipettes and volumetric flasks.
2. Substances of known purity for preparation of standard solutions.
3. Means of detecting end point (Chemical or instrumental).

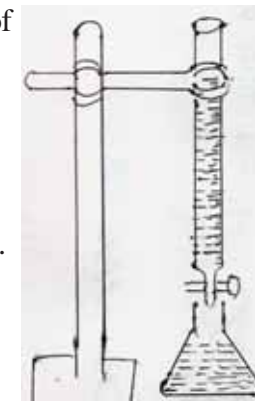


Fig.5-1 Titration

Conditions to be fulfilled by a chemical reaction in titrimetric analysis :

1. It must be a simple reaction and be expressed by a chemical equation.
2. It should be instantaneous.
3. There must be a noticeable change at the end point.
4. An indicator must be available to produce a sharp change at the end point.

Terms for expression of concentrations of solutions : Different terms for expressing the concentrations of solutions are -molarity, normality and mole.

mole is the basic unit of quantity adopted by International Union of Pure and Applied Chemistry (I.U.P.A.C.).

Mole : As defined by I.U.P.A.C., 'The mole is the amount of substance which contains as many elementary units as there are atoms in 0.012 kilogram of carbon - 12. The elementary unit must be specified and may be an atom, a molecule, an ion, a radical, an electron, or other particle or a specified group of such particles'.

Equivalent : 'The equivalent of a substance is that amount of it which, in a specified reaction, combines with, releases or replaces that amount of hydrogen which is combined with 3 grams of carbon - 12 in methane $^{12}\text{CH}_4$.

1 mole of Na_2CO_3 , 10 H_2O has a mass of 0.286004 kg.

1 mole of H_2SO_4 has a mass of 0.098078 kg.

1 equivalent of Na_2CO_3 , 10 H_2O has a mass of 0.143002 kg.

1 equivalent of H_2SO_4 has a mass of 0.049039 kg.

Normal solution : A normal solution is defined as a solution containing one equivalent of a defined species per dm^3 according to the specified reaction.

molar solution : A molar solution is defined as a solution containing 1 mole of a defined species per dm^3 .

Equivalent of an acid : equivalent of an acid is the mass of it which contains 1.0078 g of replaceable hydrogen. Equivalent of a monoprotic acid like HCl , HBr or HF is same as mole. Equivalent of a diprotic acid like H_2SO_4 is

half of a mole. Equivalent of a triprotic acid like H_3PO_4 is 1/3rd of a mole.

Equivalent of a base : equivalent of a base is the mass of it which contains 17.008 g of replaceable hydroxyl group (ionisable hydroxyl group or hydroxyl group). Equivalent of monobasic substances like NaOH , KOH is same as mole. But equivalent of a dibasic substance like $\text{Ca}(\text{OH})_2$, $\text{Mg}(\text{OH})_2$ is half of a mole.

Salts of strong bases and weak acids are alkaline in aqueous medium. A mole of Na_2CO_3 reacts with 2 moles of Hydrochloric acid and its equivalent is half a mole.

Equivalent in complex formation reactions & precipitation reactions : equivalent in complex formation reactions & precipitation reactions is mass of the substance which contains or reacts with 1 mole of univalent cation M^+ , 1/2 mole of a bivalent cation M^{2+} and 1/3rd mole of a trivalent cation M^{3+} . equivalent

of a salt in a precipitation reaction is mole divided by total valency of reacting ion.

Equivalent of an oxidising or reducing reagent : equivalent of an oxidising or reducing reagent is defined as the mass of reagent which reacts with or contains 1.008 g of available Hydrogen or 8 g of available Oxygen.

Classification of reactions in titrimetric analysis : Reactions in titrimetric analysis can be broadly classified into 2 categories. They are -

1. Reactions in which there is no change occurring in oxidation state.
2. Reactions in which there is change in oxidation state (oxidation - reduction

reactions) involving transfer of electrons.

However, for convenience sake, they can be classified into following categories. They are -

1. Neutralisation reactions (Acidimetry & alkalimetry) -
 - a. Neutralisation reaction reactions in aqueous medium
 - b. Neutralisation reaction reactions in non-aqueous medium.
2. Complexometric reactions
3. Precipitation reactions
4. Oxidation - reduction reactions

Primary standard substance : If a reagent is available in the state of high purity and is suitable for preparation of standard solution is called primary standard substance. Ex. sodium carbonate, sodium chloride, sodium oxalate, potassium chloride, potassium dichromate, potassium iodate, potassium hydrogen phthalate, benzoic acid, iodine, arsenic oxide etc.

Secondary standard substance : A reagent which is not available in the pure form, whose content of active substance is found by comparison against a primary standard and which may be used for standardisation is called secondary substance. ex. hydrochloric acid, sodium hydroxide, potassium hydroxide, barium hydroxide, potassium permanganate, sodium thiosulphate etc.

Preparation of Standard solutions : If the reagent is primary standard substance, solution of definite normality is prepared simply by weighing out an equivalent or a fraction or multiple, dissolving it in solvent (usually water) and

making up the volume by addition of more of the solvent. In practice, it is preferable to prepare the solution little more concentrated and then to dilute

it to desired normality.

If the reagent is a secondary standard substance as in the cases of most of alkali hydroxides, some of inorganic acids and deliquescent substances, solutions of approximate normality required are first prepared and they are then standardised against a primary standard.

If N_1 is required normality, V_1 is volume after dilution, N_2 is the original normality obtained and V_2 is original volume then,

$$N_1 V_1 = N_2 V_2 \quad \text{or} \quad V_1 = N_2 V_2 / N_1$$

Volume of water to be added for dilution V_d is -

$$V_d = (V_1 - V_2) \text{cc.}$$

Calculation in titrimetric analysis : Calculation in titrimetric analysis is easy with equivalent system. At the end point in a titration, number of equivalents of unknown is equal to the number of equivalents of standard solution.

$$\begin{aligned} \text{Normality} &= \frac{\text{Number of equivalents}}{\text{Number of dm}^3} \\ &= \frac{\text{Number of milliequivalents}}{\text{Number of cm}^3} \end{aligned}$$

When volumes of two reacting solutions of a titrimetric analysis are V_A and V_B in cm^3 , normalities of these solutions are N_A and N_B then.

$$V_A N_A = V_B N_B$$

V_A , V_B , and N_A being the known factors, N_B can be calculated from the above equation. i.e.,

$$N_B = \frac{V_A N_A}{V_B}$$

ex : Calculate the C.C. of 0.4N HCl required to neutralise 50 C.C. of 0.2 N - NaOH.

$$\begin{aligned} V_A &= 50 \text{ C.C.} & N_A &= 0.2 \\ V_B &= x \text{ C.C.} & N_B &= 0.4 \end{aligned}$$

Substituting the values in equation $V_A N_A = V_B N_B$,

$$50 \times 0.2 = x \times 0.4$$

$$x = \frac{50 \times 0.2}{0.4} = 25 \text{ C.C.}$$

Titration involving neutralisation reactions or Acidimetry & Alkalimetry :

These reactions involve combination of H^+ ion and OH^- ion to form salt and water.

Acidimetry : Acidimetry includes titrations of free bases or those formed from salts of weak acids by hydrolysis with a standard acid.

Alkalimetry : Alkalimetry includes titrations of free acids or those formed by hydrolysis of salts of weak bases with a standard base.

Neutralisation or acid - base indicators : Indicators used for detection of end point in acid base titrations are called neutralisation or acid base indicators. They act as indicators by virtue of possession of different colours according to Hydrogen ion concentration of the solution.

Colour change interval of indicator : Change of colour from predominantly acid to predominantly alkaline colour is not sharp and it takes place in a small interval of pH. This is called colour change interval of indicator.

Given below is list of some indicators, their pH ranges and their colours in acidic and alkaline media.

S.no. Indicator	pH range	colour in acid medium	colour in alk -ali medium
1. Methyl orange	3.1 - 4.4	red	orange
2. Methyl red	4.2 - 6.3	red	yellow
3. Phenolphthalein	8.3 - 10.0	colourless	red
4. Thymolphthalein	8.3 - 10.5	colourless	blue
5. 1 - Naphtholphthalein	7.3 - 8.7	yellow	blue
6. Quinaldine red	1.5 - 2.5	colourless	red

Preparation of indicator solutions :

Stock solutions of indicators :

General composition :

INDICATOR SUBSTANCE 0.5 - 1.0 g.

SOLVENT UPTO 1 L.

Solvent used in indicator solutions : Solvent used in indicator solutions is water when indicator substance is water soluble like sodium salt. In most other cases it is 70 - 90 % ethyl alcohol.

1. Methyl orange :

Composition with free acid : free acid of methyl orange 0.5 g.
water 1.0 L

Method of preparation : 1. Dissolve 0.5 g. of freeacid in 1 L of water.
2. Filter the cold solution if a precipitate separates.

Composition with sodium salt :

sodium salt of methyl orange 0.5 g
water 1.0 L
0.1 N. HCl 15 . 2 ml

Method of preparation : 1. Dissolve 0.5 g. of freeacid in 1 L of water.
2 .Add 15.2 ml of 0.1 M.HCL
3. Filter the cold solution if necessary.

2. Methyl red :

Composition : free acid of methyl red 1 g .
water 1 L

Method of preparation : Dissolve 1 g. of free acid of methyl red in 1 L of hot water.

or

Composition : free acid of methyl red 1 g .
ethanol 600 ml
water 400 ml

Method of preparation : 1. Dissolve 1 g. of free acid of methyl red in 600 ml of ethanol.
2. Dilute with 400 ml of water.

3. Phenol phthalein :

Composition : phenol phthalein reagent 5 g .
ethanol 500 ml
water 500 ml

Method of preparation : 1. Dissolve 500 g. of reagent in 500ml of water.
2. Add 500 ml of water with constant stirring.
3. Filter if any precipitate is observed.

or

Composition : dry phenol phthalein 1 g.
2 - ethoxy ethanol 60 ml.
distilled water upto 100 ml.

Method of preparation : 1. Dissolve 1 g. of dry indicator in 60 ml of 2 - ethoxy ethanol
2. Dilute to 100 ml with distilled water.

4. Thymolphthalein :

Composition : reagent 0.4 g.
ethanol 0.6 L.
water 0.4 L.

Method of preparation : 1. Dissolve the reagent in 600 ml of ethanol.
2. Add 400 ml of water with constant stirring.

5. 1-Naphthol phthalein :

Composition : indicator 1 g.
ethanol 0.5 L
water 0.5 L

6. Quinaldine red :

Composition : indicator 1.0 g.
80 % ethanol 100 ml

Method of preparation : Dissolve the indicator in 100 ml of 80 % ethanol.

Mixed indicators : Chief characteristic of acid base indicators is that the colour change from acid colour to alkaline colour is not sharp . It takes place in a small interval of pH called colour change interval. This interval changes widely with different indicators. It extends over 2 units of pH. However a sharp colour change can be got by use of a suitable mixture of indicators.

Some examples are given in the following table.

Indicators mixed	Ratio of mixing	Colour change	pH at Colour change
Neutral red (0.1 % solution in ethanol) Methylene blue (0.1 % solution in ethanol)	1 : 1	Violet blue - Green	7.0 pH
Phenol phthalein (0.1 % solution in ethanol) 1- Naphthol phthalein (0.1 % solution in ethanol)	3 : 1	Rose - Violet	8.9 pH
Sodium salt of thymol blue (0.1% solution in water) Sodium salt of cresol red	3 : 1	Yellow - Violet	8.3 pH

Screened indicator : A single indicator whose colour change is improved by addition of a pH sensitive dye stuff is called screened indicator.

ex : methyl orange 1.0 g.
xylene cyanol FF..... 1.4 g.
50% ethanol 500 ml.

Here colour change is from green to grey to magenta. Middle grey stage is at 3.8 pH.

Universal indicators : They are also called as multiple range indicators. Mixtures of indicators in proportions as to produce colour change over considerable range of pH are called universal or multiple range indicators. They are not use ful for quantitative determinations. They are useful for determination of approximate pH.

ex : phenolphthalein 0.1g.
methyl red0.2g.
methyl yellow0.3g.
bromothymol blue 0.4g.
thymol blue..... 0.5g,
sodium hydroxide solution until colour is yellow.

Colour changes are given below.

pH	-	colour
2	-	red
4	-	orange
6	-	yellow
8	-	green
10	-	blue

Neutralisation curves: Neutralisation curves are obtained by plotting pH as ordinates and percentage of acid neutralised as abscissae. They are useful for studying the mechanism neutralisation process.

Neutralisation of a strong acid and strong base :Neutralisation of strong acid and strong base can be studied by considering the titration of Hydrochloric acid with Sodium hydroxide solution. Both of these are completely dissociated. It can be studied by neutralising 100 ml of 1M. hydrochloric acid with same strength sodium hydroxide solution. pH of 1M HCl is zero . When 50 ml of base is added , still there is 50 ml of acid un-neutralised in total 150 ml of solution .pH rises slowly . Rise of pH is very rapid in the nearness of end point .

Neutralisation of a weak acid with a strong alkali : Neutralisation of a weak acid with a strong alkali can be studied by considering the titration of 100 ml of 0.1 M. acetic acid with same strength sodium hydroxide solution.In this titration , neither methyl orange nor methyl red can be used as indicator .pH at end point is 8.7. Indicator in pH range on slightly alkaline side such as phenolphthalein or thymolphthalein or thymol blue can be used.

Neutralisation of a weak alkali with a strong acid : Neutralisation of a weak alkali with a strong acid can be studied by considering the titration of 100 ml of 0.1 M. aqueous ammonia with same strength hydrochloric acid .End point is at pH 5.3 .Thymolphthalein or phenolphthalein cannot be used . Indicator on slightly acidic side such as methyl orange , methyl red , bromo penol blue , or bromo cresol green can be used .

Neutralisation of a weak acid with a weak alkali : Neutralisation of a weak acid with a weak alkali can be studied by considering the titration of

100 ml of 0.1 M. acetic acid with same strength aqueous ammonia. Chief feature of this titration is that change of pH during whole process of titration is gradual . A sharp end point cannot be got with any single **indicator** . A mixed indicator exhibiting sharp colour change over a limited pH range can be used . Neutral red - methylene blue mixed indicator is used in this case .On the whole , it is better to avoid to use indicator in such titrations .

Neutralisation of a polyprotic acid with a strong base : A polyprotic acid is one having more than one proton . Diprotic acids are acids containing two protons . Ex - Carbonic acid, Sulphurous acid ,Sulphuric acid etc. They will act like mixture of two monoprotic acids. Triprotic acids are acids containing three protons . Ex - Orthophosphoric acid . They will act like mixture of three monoprotic acids . There will be two stages in neutralisation of diprotic acids - primary and secondary stages. There will be three stages in neutralisation of triprotic acids - primary , secondary and tertiary stages .

Titration of anions of weak acids with strong acids : Anions of weak acids are weak bases They are Bronsted bases . Ex - carbonate ion , borate ion , acetate ion etc. Net result is weak acid is displaced by strong acid .Hence they are also called as displacement titrations .

Indicators chosen in neutralisations reactions :

a) **Indicators in titrations between strong acid and strong base :** Indicator with pH range as given below should be used for following types of solutions

SolutionpH range of indicator
0.1M solution or stronger.....	4.5 to 5.5
0.01 M solution 5.5 to 8.5
If CO ₂ is present < 5

b) **Indicators in titrations between weak acid and strong base :** Indicator with pH range as given below should be used for following types of solutions

SolutionpH range of indicator
Acid solution with $K_a > 10^{-5}$7 - 10.5
Weaker acids i.e. with $K_a > 10^{-6}$	8 -10

Average pH range in these titrations is 8 - 10.5. Thus thymol blue ,thymolphthalein or phenolphthalein will serve the purpose of detection of end point .

c) **Indicators in titrations between weak base and strong acid :** Indicator with pH range as given below should be used for following types of solutions

Solution pH range of indicator
Base with $K_b > 10^{-5}$3 - 7
Base with $K_b > 10^{-6}$ 3 - 5

Suitable indicators are methl red , methyl orange ,methyl yellow , bromo cresol green and bromo phenol blue.

Indicators in titrations between weak acid and weak base : There is no sharp raise in neutralisation curve . Simple indicator cannot be used. Titration can be avoided , if possible . Mixed indicator exhibiting colour change over limited pH range should be used . one example is neutral red - methylene blue for ammonia and acetic acid .

When it is not possible to find a suitable indicator as in case of strongly coloured solutions , electrometric methods of titrimetric analysis like conductometry , amperometry etc. should be used .

Preparation of standard solutions of acids :

Acids employed in preparation of standard solutions of acids : Standard solutions of acids are usually prepared with concentrated hydro chloric acid since chlorides are water soluble mostly .Concentrated sulphuric acid is preferable when titrating hot liquids or when determinations require boiling for some time .Sulphuric acid forms insoluble sulphates with calcium and barium hdroxides .Nitrous acid having destructive action upon most of the indicators is present invariably in nitric acid and and thus it is rarely used in preparation of standard acid solutions .

Strengths of concentrated acids : Approximate strength of commercially

available concentrated hydrochloric acid is 10.5 - 12 M . Concentrated Sulphuric acid is available in the strength of 18M approximately .

Preparation of dilute acids of approximate strength : Acid solutions of required strength can be prepared from these concentrated acids by suitable dilution .

Preparation of standard solutions of hydrochloric acid : Standard solutions of hydrochloric acid can be prepared by two methods.

- a Preparation of constant boiling point hydrochloric acid and dilution to required strength .
- b . Preparation of hydrochloric acid of desired strength by dilution and its standardisation .

a . Preparation of constant boiling point hydrochloric acid and dilution to required strength : Aqueous solutions of hydrochloric acid lose either HCl or H₂O upon boiling until they get a constant composition , which depends on the prevailing pressure . Hydrochloric acid of constant composition depending on prevailing pressure is called constant boiling point hydrochloric acid .It is neither hygroscopic nor appreciably volatile . Its concentration stands unaltered when it is stored in a well stoppered container away from direct sun light .

In method a. for preparation of standard solutions of HCl , there are two steps . They are 1 . Preparation of constant boiling point hydrochloric acid .

2 . Dilution of constant boiling point hydrochloric acid to required strength .

1 . Preparation of constant boiling point hydrochloric acid : Add 400 ml of pure concentrated acid to 250 - 400 ml of distilled water . Specific gravity of resultant acid should be 1.1. Conduct distillation using all glass distillation apparatus . Use a thermometer to read the temperature during distillation . It should remain constant . Take 500 ml of diluted acid and distil at the rate of 3-4 ml per minute . Collect distillate in a small pyrex flask . Transfer it into a measuring cylinder from time to time . When the distillate collected in the flask is 375 ml , collect further 50 ml in the small pyrex flask. Remove the receiver containing

constant boiling point hydrochloric acid and stopper it . Note the barometer reading during distillation at certain intervals . Calculate the mean value . Interpolate the concentration from the following table .

Pressure in mm	% HCl in acid	Grams of acid containing 36.47 g. of HCl
780	20.173	180.621
770	20.197	180.407
760	20.221	180.193
750	20.245	170.979
740	20.269	179.266

2 . Dilution of constant boiling point HCL to required strength : Constant boiling point HCL can be diluted to required strength for preparation of standard solution of HCl . For preparation of 1 L. of 0.1 M HCL , calculated quantity of constant boiling point HCL is taken with the help of a pipette in a flask(glass stoppered) , whose weight is determined .With the help of a dropper pipette, volume is made up .Replace the glass stopper and reweigh the flask . Add equal quantity of water to prevent loss of acid . Transfer the contents to 1 L. graduated flask with washings . Make up the final volume with distilled water . Stopper the flask and shake by inverting the flask several times .

b .Preparation of hydrochloric acid of desired strength by dilution and its standardisation :

By this method, standard acid solution of desired strength is prepared by dilution from pure concentrated hydrochloric acid and it is standardised to determine its strength .For preparation of 0.1 M. HCl ,9ml of pure concentrated HCl is measured by means of graduated cylinder or burette and poured into a 1 L.measuring cylinder containing 500ml of distilled water. Volume is made up with distilled water and thorough mixing is affected by means of shaking.

Standardisation of HCl : Some of the methods for Standardisation of HCl are

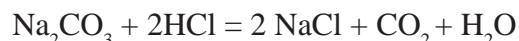
- a . Standardisation with anhydrous sodium carbonate
- b. Standardisation with sodium tetraborate

c. Standardisation by iodometric method

d. Standardisation by argentometric method

a . Standardisation with anhydrous sodium carbonate : It is an excellent method and widely employed . Titration is conducted by dissolving 0.2g of pure anhydrous sodium carbonate in 50 - 75 ml of distilled water and titrating 25 ml of it against HCl solution run from the burette .During addition of HCl to sodium carbonate solution, flask should be rotated with one hand and stop cock of burette should be handled with another hand .Methyl orange or methylorange - indigo caramine indicator may be used . During the titration , acid should be added until colour with methl orange becomes pale yellow or if methyl orange - indigo caramine is used as indicator , green colour should begin to become pale.Then , walls of the flask should be washed with little distilled water from a wash bottle and HCl to be run dropwise at this point until the end point is orange or faint pink with methyl orange and it is neutral grey with methyl orange - indigo caramine mixed indicator .Titration is repeated twice or thrice .

Calculation of normality : Normality can be calculated from equation



One method of deriving normality is in terms of primary standard substance .

Easiest method is calculating the normality using the formula -

$$V_1N_1 = V_2N_2$$

where if V_1 is volume of sodium carbonate solution ,

N_1 is normality of sodium carbonate solution ,

V_2 is volume of HCl solution and

N_2 is normality of acid solution .

$$\text{then , } N_2 = \frac{V_1N_1}{V_2}$$

$$V_2$$

if 2.778 g of sodium carbonate is dissolved in 500 ml of water , its normality is

0.1048.If 25 ml of sodium carbonate solution neutralises 25.50 ml of HCl,

$$N_2 = \frac{25.00 \times 0.1048}{25.50} = 0.1027 \text{ N}$$

$$25.50$$

Strength of HCl is determined by taking the average of values got in all titrations and they should be same .Their average value should be calculated .

Purity of sodium carbonate : A.R. quality sodium carbonate available commercially will have purity of 99.9 % . It contains a little moisture . It can be dehydrated by drying at 260 - 270⁰C for a period of half an hour and then cooled in a dessicator.

b. Standardisation with Sodium tetra borate : It is also excellent in standardisation of HCl. Moreover it has some advantages . They are- Sodium tetraborate has a large equivalent i.e 190.72g. Its purification is easy and economic.It is almost non hygroscopic. Heating sodium tetraborate is not required. End point is sharp with methylred at room temperatures.

0.4 - 0.5 g of Sodium tetra borate is accurately weighed and dissolved in 50 ml of water contained in a 250 ml conical flask . A few drops of methyl red is added to it and titrated with hydrochloric acid run from the burette. Color change at the end point is to pink from the colour of methyl red . Conduct the titration two more times . Calculate the strength of acid from weight of borate and volume of acid run from burette. Variation of values in all titrations should not be above 0.1 - 0.2 % . Calculate the strength of acid by taking arithmetic mean .

Calculation of normality : It is calculated as described in method a i.e.

Standardisation with anhydrous sodium carbonate . Equivalent of borate is 190.72.



Purity of sodium tetra borate : 15 g. of of A.R. salt is recrystallised from 50 ml of distilled water .

Care should be taken to prevent crystallisation above 55⁰C.

c. Standardisation by iodometric method

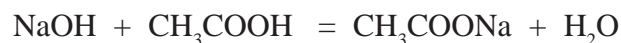
d. Standardisation by argentometric method

Refer to oxidation and reduction titrations in the same chapter .

Determination of strength of concentrated glacial acetic acid : Strength of concentrated glacial acetic acid is determined by titrating glacial acetic acid with standard solution of sodium hydroxide using phenolphthalein or thymol blue as indicator .

Weigh out a 50 ml conical flask . Introduce 2g. of acetic acid and reweigh .

Add 20ml of water and transfer to a 20ml graduated flask . Add distilled water washings of conical flask to the contents of graduated flask . Make upto mark with distilled water . Titrate 25 ml portions of acid with 0.1 M sodium hydroxide solution using phenolphthalein or thymol blue as indicator .

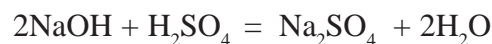


$$1\text{ml of } 0.1\text{M NaOH} = 0.006005 \text{ g. of } \text{CH}_3\text{COOH}$$

Calculate the strength of acetic acid in the given sample of concentrated acetic acid .

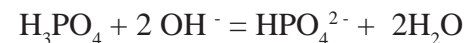
Determination of strength of concentrated Sulphuric acid : Strength of concentrated Sulphuric acid is determined by titrating sulphuric acid with standard solution of sodium hydroxide using methyl orange or methyl orange - indigo carmine as indicator .

Take about 100ml of water in a 250 ml graduated flask . Add 1.3 - 1.5 g. of sulphuric acid to flask with the help of a short stemmed funnel . Rinse the funnel with distilled water a few times and remove the funnel . Allow the flask to stand for some time to attain room temperature and make upto mark with distilled water . Shake thoroughly for proper mixing and titrate 25 ml portions with 0.1 M solution of sodium hydroxide . Use methyl orange or methyl orange - indigo carmine as indicator .



$$1 \text{ ml of } 0.1\text{M NaOH} = 0.04904 \text{ g of } \text{H}_2\text{SO}_4$$

Determination of strength of syrupy phosphoric acid : 2 g. of accurately weighed syrupy phosphoric acid is transferred into a 20 ml graduated flask and titration is continued similarly as for concentrated sulphuric acid and using phenolphthalein - 1 - naphtholphthalein as indicator .



$$1\text{ml of } 0.1\text{M NaOH} = 0.04902 \text{ g H}_3\text{PO}_4$$

Preparation of standard solutions of alkalies :

Alkalies employed in preparation of standard solutions of alkalies : Alkalies employed in preparation of standard solutions of alkalies are sodium hydroxide , potassium hydroxide , barium hydroxide etc . Aqueous solutions of ammonia above concentration of 0.5 M tend to lose ammonia . It is a weak base . It is difficult to titrate it with weak acids . Sodium and potassium hydroxides are extremely hygroscopic . Some quantities of carbonate and water are always present . Results obtained cannot be exact with some indicators in presence of carbonate and in such cases it is necessary to prepare carbonate free hydroxide solution . In all other cases , it is sufficient if sodium hydroxide A.R. containing 1 -2 % of sodium carbonate is used .

Preparation of carbonate free sodium hydroxide solution : Carbonate free sodium hydroxide solution is prepared by one of the following methods .

a. Rinsing the sticks of sodium hydroxide rapidly with water and preparation of carbonate free sodium hydroxide solution .

b. Preparation of concentrated solution of sodium hydroxide by taking equal volumes of sodium hydroxide pellets and water , allowing the solution to stand , siphoning the supernatant from insoluble carbonate and suitably diluting .

c. Electrolysis of saturated solution of sodium hydroxide with a mercury cathode and platinum anode yielding sodium hydroxide solution , completely free from carbonate .

d. Removal of carbonate completely by anion exchange method by passing through a strong anion exchange column , collecting the effluent in a special flask

and dilution of calculated quantity of it with boiled out distilled water.

Solution of sodium hydroxide prepared by any of the above methods must be standardised .

Preparation of 0.1 M . sodium hydroxide solution : Preparation of sodium hydroxide solution of 0.1 M strength involves -

a. Preparation of sodium hydroxide solution of approximate strength .

b. Standardisation of solution prepared in above step.

.a. Preparation of sodium hydroxide solution of approximate strength :

Sodium hydroxide solution of approximate strength can be prepared by any of the following methods .

Procedure A : In this procedure ,4.2 g. of sodium hydroxide A.R. is rapidly weighed on a watch glass and dissolved in quantity of distilled water enough to dissolve it in a flask and volume made to 1 L. with boiled out distilled water.

Procedure B : In this procedure , 6.5 ml of supernatant of equal volumes of sodium hydroxide A.R. and distilled water and diluting it to 1 L. with recently boiled out distilled water in a graduated flask .

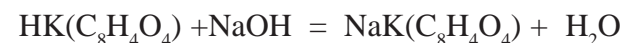
b. Standardisation of solution : In standardisation of sodium hydroxide solution prepared by procedure A, methyl orange, methyl orange-indigo caramine or bromo phenol blue can be used to indicate the end point in its titration against hydrochloric acid if the solution contains carbonate . Phenolphthalein or indicators of similar pH range are affected by carbon dioxide and thus they cannot be used to indicate the end point in titration when , sodium hydroxide solution contains carbonate . They can be employed in standardisation of carbonate free sodium hydroxide solution with hydrochloric acid ,potassium hydrogen phthalate , benzoic acid or any other organic acid .

Standardisation of sodium hydroxide solution with standard solution of hydrochloric acid : Standardisation of sodium hydroxide solution with standard solution of hydrochloric acid is done by titrating 25 ml of sodium hydroxide

solution taken in a 250 ml conical flask with standardised hydrochloric acid of 0.1 M strength using 1 -2 drops of methyl orange or 3 - 4 drops of methyl orange-indigo caramine as indicator .Titration is repeated until values agree each other within 0.05 ml. Normality is calculated by using the formula -

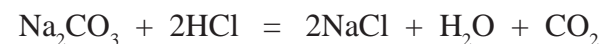
$$V_1N_1 = V_2N_2$$

Standardisation of sodium hydroxide solution with standard solution of potassium hydrogen phthalate :Standardisation of sodium hydroxide solution with standard solution of potassium hydrogen phthalate is done by titrating 0.6 - 0.7 g. of potassium hydrogen phthalate A.R. dissolved in 75 ml of boiled out distilled water in a conical flask with sodium hydroxide solution contained in a burette using phenolphthalein or thymol blue as indicator . Two more titrations are conducted and the values should agree within 0.05 ml. Normality is calculated by using the equation -



equivalent of is 204.22 g.

Determination of content of sodium carbonate : Content of sodium carbonate is determined by dissolving 3.6 g. of sodium carbonate in 250 ml of sodium carbonate and titrating 2 ml of it with standard solution of hydrochloric acid of 0.1 M strength using methyl orange or methyl orange-indigo caramine or bromo - cresol green as indicator. Two more titrations are conducted and the values should agree within 0.05 ml. Normality is calculated by using the equation -



Equivalent of sodium carbonate is 106.01.

Neutralisation reactions in non-aqueous medium : Organic compounds having poor solubility in water cannot be titrated accurately in aqueous medium due to weakly reactive nature .They are assayed using glacial acetic acid in place of water and titrated with perchloric acid , the strongest acid . Nonaqueous acidimetry and alkalimetry can be regarded as reaction between weakly protophilic substances which accept a pair of electrons and highly protophilic

substances which donate a pair of electrons .

Non aqueous titrations are simple and accurate .Difference between aqueous and nonaqueous titrations is moisture and carbondioxide should be avoided .Water being weakly basic , it would compete with weakly basic nitrogenous substances for perchloric acid (HClO_4) . Presence of water also causes loss of sharpness in end point . Moisture contact in non aqueous titrations should be less than 0.05 % .

Various substances show variations in protophilic properties in non aqueous solvents like acetonitrile , acetone and dimethyl formamide .In aqueous medium , they do not show variations due to levelling effect of water . Various acids such as perchloric acid , sulphuric acid etc . show equal strength in aqueous solutions . But their strength decreases in nonaqueous medium in the following order .



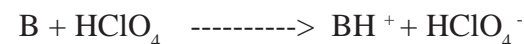
Classification of solvents : They are -

1. Aprotic solvents : These solvents have low dielectric constants.They do not have ionisable proton .They do not act as levelling agents .They are also called as neutral or inert solvents . Ex : Benzene , chloroform , acetone , etc .
2. Amphiprotic solvents : These solvents have high dielectric constant .They are partially ionised .They are also called as amphoteric solvents . Ex : Water , methanol , ethanol , isopropanol etc .Often they are used as mixtures with aprotic solvents .
3. Protogenic (acidic) solvents : These solvents have high dielectric solvents . They are ionised . They possess acidic properties .Ionic product of liberated anions and cations is greater than water .
4. Protophilic (basic) solvents : They possess high dielectric constants .They are basic in nature .Their ionic product is less than water .Ex : Dimethyl formamide , pyridine , ethylene diamine etc .

Indicators commonly used in non aqueous titrations :

Crystal violet , methyl yellow , methyl red etc .

Acidimetry in nonaqueous medium :Weakly basic substances are analysed by this method . Commonly employed titrant is N/10 perchloric acid . Solvents used are alcohol , chloroform , benzene , glacial acetic acid , acetic anhydride etc .Indicators used are crystal violet , quinaldine red or thymol blue etc . Reaction can be written as -



Preparation of N/10 perchloric acid : 8.5 ml of conc . perchloric acid is mixed with 500 ml of glacial acetic acid and 30 ml of acetic anhydride .It is cooled and volume is made upto 1000ml with glacial acetic acid.

Standardisation of N/10 perchloric acid : Standardisation of perchloric acid is done with potassium hydrogen phthalate , previously dried at 105°C for 3 hours . Solvent used is glacial acetic acid .Crystal violet is used as indicator .

1 ml of N/10 HClO_4 = 20 . 42 mg. of potassium hydrogen phthalate

Alkalimetry in non aqueous medium : Commonly employed titrant is sodium or lithium methoxide . Solvents employed are dimethyl formamide , pyridine , ethylene diamine etc .Indicators used are azoviolet , O - nitro aniline or thymol blue .

Preparation of N/10 sodium methoxide : It is prepared by adding 2.5 g. of freshly sodium metal in small portions to 150 ml of ice cold methanol contained in 1 L volumetric flask and making the volume upto 1L with benzene .

Standardisation of sodium methoxide N/10 : It is standardised by titrating with benzoic acid with thymol blue as indicator . Solvent used is Dimethyl formamide (DMF).

Titration involving complex formation reactions or complexometric titrations or Complexometry :

Complex formation reactions depend on combination of ions other than hydro-

gen or hydroxide ions to form a soluble, slightly dissociated ion or compound as in the titration of a solution of a cyanide with silver nitrate .

Ethylene diamine tetra acetic acid (E.D.T.A.) or disodium salt of E.D.T.A. is a very important reagent in complexometry .It is a metal ion indicator of great value in titrimetry . A complexation reaction with a metal ion involves replacement of one or more of the co-ordinated solvent molecules by other neutrophilic groups .

Ligands : Ligands are the groups bound to the central ion . Ligand can be either a neutral molecule or a charged ion .Successive replacement of water molecules by other ligand groups can occur until the complex ML_n is formed.

'n' is the co-ordination number of metal ion representing the number of monodentate ligands that can be bound to it .

Equation representing the reaction in aqueous solution : Equation representing the reaction in aqueous solution is -



Classification of ligands : On the basis of the number of points of attachment to the metal ion, ligands can be conveniently classified into -

a.Monodentate ligands : Ligands bound to the metal ion only at one point are called monodentate ligands . ex- halide ions or molecules H_2O or NH_3 .

b.Bidentate ligands : Ligands having two donor atoms are called bidentate ligands. When ligand molecule or ion has two atoms each having a lone pair of electrons, it is possible to form two co-ordinate bonds with the same metal ion. It is exemplified by consideration of tris(ethylene diamine) cobalt(III) complex , $[Co(en)_3]^{3+}$.

c.Multidentate ligands : Ligands containing more than two co-ordinating atoms per molecule are called multi dentate ligands . Ex : E.D.T.A.

E.D.T.A. has two donor nitrogen atoms and four donor oxygen atoms in the molecule and thus, it is hexadentate.

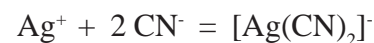
Chelation : Chelation is the process of ring formation . It is exemplified by

consideration of tris(ethylene diamine) cobalt(III) complex .In this 6-coordinated octahedral complex of cobalt(III), each of bidentate E.D.T.A. molecule is bound to metal ion through lone pair of electrons of the two nitrogen atoms. It results in the formation of three 5 membered rings, each including the metal ion.

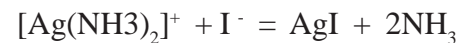
Binuclear complex : Binuclear complex is one containing two metal ions .Interaction between Zn^{2+} and Cl^- ions may result in the formation of binuclear complexes such as $[Zn_2Cl_6]^{2-}$ in addition to simple species such as $ZnCl_3^-$ and $ZnCl_4^{2-}$.

Polynuclear complex : Polynuclear complex is one containing more than two metal ions .Formation of bi and poly nuclear complexes is favoured by a high concentration of metal ion but not by presence of trace quantities of metal ion.

Simple complexometric titration : Titration of cyanide with silver nitrate solution is simple example of a complexometric titration. When these two liquids come into contact, a white precipitate is formed at first which re-dissolves on stirring due to formation of a stable complex cyanide. Its alkali salt is water soluble.



When the reaction is complete, further addition of silver nitrate solution yields insoluble silver cyanoargentate .Due to difficulty in obtaining a sharp end point, iodide ion, as potassium iodide(0.01 M) is used as indicator and aqueous ammonia (0.2M) is introduced to dissolve silver cyanide .They are added before beginning the titration.End point is indicated by the formation of silver iodide .



Silver iodide being formed during the titration will stay in the dissolved state as cyanoargentate until end point is reached due to presence of excess cyanide .

Complexone : Formation of a single complex rather than stepwise production is required for detection of end points . Amino poly carboxylic acids are found to be excellent complexing agents .The most important of these is E.D.T.A. Its

some metals . ex : Formaldehyde - acetic acid solution can be used to demask zinc and cadmium . Chloral hydrate can also be used for same purpose .

4. Kinetic masking : In this case , a metal ion does not effectively enter into complexation reaction due to kinetic inertness. It makes possible to titrate other metal ions reacting rapidly . Ex : Slow reaction of chromium (III) with EDTA makes it convenient to titrate other rapidly reacting ions .

5. Solvent extraction : Zinc is separated from copper and lead by adding excess of ammonium thiocyanate solution . Zinc thiocyanate solution extract is titrated with EDTA solution to determine the content of zinc .

Indicators used in complexation titrations : Some of the examples of metal ion indicators are murexide , solochrome black or eriochrome black T , Patton and Reeder 's indicator , solochrome dark blue or calcon , calmagite calcichrome , fast sulphon black F , catechol violet , bromopyrogallol red , xylenol orange , thymolphthalein complexone , methyl thymol blue , zincon , variamine blue etc.

Preparation of standard solutions of EDTA : Commercial disodium dihydrogen EDTA A.R. should be purified by preparing saturated solution of it at room temperature . It is done by taking 20 g. of salt per 200 ml of water and salt is precipitated by adding enough quantity of ethanol . It is filtered and to the filtrate equal volume of ethanol is added . It is again filtered and washed with acetone and ether . It is air dried at room temperature overnight and dried in hot air oven at 80°C. for 24 hours . Solutions of EDTA of 0.1M, 0.05M and 0.01M strength are used generally in experimental work . Water used for preparation of standard solutions of EDTA should be purified water i.e. water purified by distillation in all-pyrex glass apparatus , or ion exchange . Solutions should be stored in pyrex bottles , thoroughly steamed out before use . Prolonged storage can be done in borosilicate vessels , which have been boiled with 2% EDTA solution for several hours and rinsed with de-ionised water . Polyethylene bottles should be employed for storage of very dilute solutions of EDTA . Ordinary glass vessels should not be used for storage since they would yield appreciable quantities of anions and cations like calcium and magnesium.

Titration involving precipitation reactions : They depend on formation of a simple precipitate by combination of ions . There will not be change in oxidation state . Most important processes in precipitation titrations include silver nitrate as reagent. Titrimetric analysis involving silver nitrate reagent is called as argentometry . Changes in ionic concentration during titration are studied by considering the titration of 100 ml of 0.1M - sodium chloride and 0.1M silver nitrate solution .

Determination of end points in precipitation titrations : End points in precipitation titrations can be determined by several ways . The most important methods are

1. Formation of a coloured precipitate
2. Formation of a soluble coloured compound
3. Fajan's method of using adsorption indicators
4. Turbidity appearance

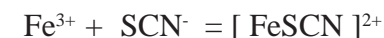
1. Formation of a coloured precipitate : It is illustrated by Mohr's method of determination of chloride and bromide . Potassium chromate is used as indicator in the titration between chloride ions and silver nitrate solution . A sparingly soluble red coloured substance silver chromate is formed at the end point .

2. Formation of a soluble coloured compound : It is illustrated by Volhard's procedure of titration of silver. It is conducted in presence of free nitric acid with standard solution of potassium or ammonium thiocyanate . Indicator used is solution of iron(III) nitrate or iron(III) ammonium sulphate .

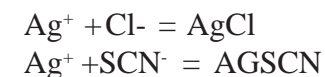
Addition of thiocyanate solution first produces precipitate of silver thiocyanate .



On completion of this reaction, a slight excess of thiocyanate forms a complex ion , which is reddish brown coloured .



This is also employed for determination of chlorides , bromides and iodides. For determination of halides , excess of silver nitrate solution is added and the excess is back titrated with standard solution of thiocyanate .



3. Fajan's method of using adsorption indicators : Adsorption indicators are adsorbed by the precipitate at the end point . During the process of adsorption , colour of the indicator changes . Substances used as indicators are either acid dyes or basic dyes . Examples of acidic adsorption indicators are - fluorescein and eosin as sodium salts . Example of basic dyes is rhodamine 6G .

Rhoamine series are applied as hydrogen salts .

4. Turbidity appearance : End point of a titration in complexometric titrations is also indicated by formation of turbidity . An example is Liebig's method for cyanides . Another example is Gay Lussac's method for determination of silver with chloride . In Gay Lussac - Stas modification , sodium chloride solution is added to the silver nitrate solution in the presence of free nitric acid and a little quantity of barium nitrate .

Preparation of 0.1 M silver nitrate solution : Preparation of 0.1 M silver nitrate solution involves two steps - a. Preparation of 0.1 M silver nitrate solution from silver nitrate

b. Standardisation of silver nitrate solution prepared in step a.

A.R. Powder some silver nitrate A.R. finely . Dry it at 120°C for 2 hours . Allow it to cool in a covered vessel in a desiccator . Weigh accurately 8.494 g. of it and dissolve in enough quantity of water . Dilute to 500 ml in a graduated flask .

Purity of silver nitrate A. R. : Silver nitrate A. R. has a purity of 99.9 % . A standard solution can be prepared by directly weighing , dissolving in enough quantity of water and diluting to the required volume .

Standardisation of silver nitrate solution : Standardisation of silver nitrate solution is necessary if commercially recrystallised silver nitrate is employed or strength of already prepared silver nitrate solution is to be checked again . Standardisation of silver nitrate solution can be done with standard solution of sodium chloride by 2 methods .

1. Standardisation of silver nitrate solution with potassium chromate solution as indicator .

2. Standardisation of silver nitrate solution with adsorption indicator .

1. Standardisation of silver nitrate solution with potassium chromate solution as indicator : Prepare indicator solution by dissolving 4.2g. of potassium chromate A.R. and 0.7 g. of potassium dichromate in 100 ml of water and add 1ml of it to the titration . Pipette 25 ml of standard solution of sodium chloride A.R. in 0.1M. strength into a conical flask and keep over a white tile . Run silver nitrate solution from burette slowly until red colour of each drop disappears . Continue the addition drop by drop until a distinct change occurs which also persists on brisk shaking . Repeat the titration with 2 - 3 twenty five ml portions of sodium chloride solution .

2. Standardisation of silver nitrate solution with adsorption indicator : Both fluorescein and dichloro fluorescein are suitable for titration . At the end point , greenish yellow colour of solution suddenly gets a pronounced reddish tint . Tartrazine is a good indicator for reverse titration .

Preparation of 0.1 M ammonium or potassium thiocyanate solution :

Preparation of 0.1 M ammonium or potassium thiocyanate solution involves two steps - a. Preparation of 0.1 M ammonium or potassium thiocyanate solution from ammonium or potassium thiocyanate A.R.

b. Standardisation of 0.1 M ammonium or potassium thiocyanate solution prepared in step a. A.R. or 10.5g. of potassium thiocyanate

a. Preparation of 0.1 M ammonium or potassium thiocyanate solution from ammonium or potassium thiocyanate A.R. : Weigh out 8.5 g. of ammonium thiocyanate A.R. or 10.5 g. of potassium thiocyanate A.R. accurately and transfer to a 1L. graduated flask and add 1 L. of water . Shake well to effect solution .

b. Standardisation of 0.1 M ammonium or potassium thiocyanate solution prepared in step a. : Standardisation of 0.1 M. ammonium or potassium thiocyanate solution prepared in step a. is done by taking 25 ml of standard solution of 0.1 M silver nitrate in a 250 ml flask , adding 5 ml of 6 M nitric acid , 1 ml of iron (III) indicator solution and running 0.1 M ammonium or potassium thiocyanate solution from burette . At the end point , a faint brown colour which does not disappear on shaking is formed . When tartrazine is used as indicator , at the end

point, supernatant liquid gets lemonish yellow colour.

4. Titrations involving oxidation - reduction reactions : All the reactions involving change in oxidation number or transfer of electrons among the reacting substances are included under these titrations. Standard solutions used are either oxidising agents or reducing agents. Principal oxidising agents are potassium permanganate, potassium dichromate, cerium sulphate, iodine, potassium bromate and potassium iodate. Some examples of frequently used reducing agents are iron compounds, tin compounds, hypo, arsenic oxide, mercury nitrate, vanadium chloride or sulphate, chromium chloride or sulphate and titanium chloride or sulphate.

Detection of end point in oxidation reduction reactions : The following are several methods for determination of end point in oxidation - reduction reactions.

1. Using internal oxidation - reduction indicators
2. Reagent itself acting as indicator
3. Using external indicators
4. Potentiometric methods

1. Using internal oxidation - reduction indicators : A redox indicator is one which exhibits one colour in oxidised state and another colour in reduced state. Some examples of redox indicators and their colours in oxidised state and reduced state are given below.

Name of indicator	color in oxidised state	colour in reduced state
Nitro ferroun	Pale blue	Red
Ferroun	Pale blue	Red
2,2'-Bipyridyl iron sulphate	Faint blue	Red
5,6-Dimethyl ferroun	Pale blue	Red
N-Phenyl anthranilic acid	Purple red	colourless
Diphenylamine sulphonic acid	Red-violet	colourless
Starch-I ₃ ⁻ , KI	Blue	colourless
Methylene blue	Blue	colourless

2. Reagent itself acting as self indicator : It is exemplified by potassium

permanganate. For the titration of colourless or slightly coloured solutions, use of an indicator is unnecessary since as little as 0.01 ml of 0.01 N potassium permanganate will give pale pink colouration to 100ml of water. However, internal indicators like ortho phenanthroline iron ion or N-phenyl anthranilic acid and starch are available to enhance the colour intensity of permanganate in very dilute solutions. Colour of iodine and cerium sulphate is also utilised in detection of end points. But in these cases, colour change is not as marked as with permanganate.

With this technique of using the reagent itself as self indicator, the drawback is that excess of oxidising reagent will always be present at the end point. Error may be reduced for highest accuracy by determining indicator blank.

3. Using external indicators : It is by removing the drops of the solution at the end point and bringing into contact with indicator on the spot plate. The best example is titration of iron(II) with standard solution of potassium dichromate using freshly prepared potassium hexacyanoferrate(III) solution. In this titration, as the equivalence point nears, drops of the solution are removed and brought into contact with indicator solution on a spot plate. At the end point, drop fails to give a blue colouration.

4. Potentiometric methods : When indicator method is inapplicable, potentiometric method is followed to determine the end point. It is by measuring e.m.f. between reference electrode and an indicator electrode at suitable intervals during titration.

Oxidation reduction titrations :

Oxidation reduction titrations are -

- a. Titrations of reducing agents by oxidising agents like potassium permanganate, potassium dichromate, cerium(IV) sulphate, iodine, potassium iodate and potassium bromate
- b. Titrations of oxidising agents by reducing agents like arsenic oxide(III) and sodium thiosulphate

Titration involving oxidation reactions with potassium permanganate as oxidising agent is called permanganometry. Titrations involving oxidation reactions with potassium dichromate as oxidising agent is called dichrometry. Titrations involving oxidation reactions with iodine as oxidising agent is called iodimetry and iodometry.

Permanganometry : Titrimetric analysis involving potassium permanganate as oxidising agent is called permanganometry. Potassium permanganate is a powerful oxidising agent. Its reducing action in dilute acids is represented by equation -



Equivalent is one fifth of mole. That is $158.03/5 = 31.606$. For providing acid medium to potassium permanganate, sulphuric acid is the most suitable acid. Hydrochloric acid is likely to cause consumption of some permanganate by formation of chlorine. Potassium permanganate also finds application in strongly alkaline solutions.

Potassium permanganate is a self indicator in the titration of colourless or slightly coloured solutions. For the titration of colourless or slightly coloured solutions, use of an indicator is unnecessary since as little as 0.01 ml of 0.01 N. potassium permanganate will give pale pink colouration to 100 ml of water. However, internal indicators like ortho phenanthroline iron ion or N-phenyl anthranilic acid and starch are available to enhance the colour intensity of permanganate in very dilute solutions.

Potassium permanganate is not a primary standard substance since it is difficult to obtain it perfectly pure and completely free from MnO_2 . Presence of MnO_2 is objectionable since it catalyses autodecomposition of potassium permanganate solution on standing.

Preparation of 0.1 N. potassium permanganate solution : 0.1 N. potassium permanganate solution is made by weighing accurately about 3.2 - 3.25 g. of potassium permanganate A.R., transferring it to a 1.5 L. beaker and adding 1 L.

water. Beaker is then covered with a clock glass, solution is then gently boiled for 15 - 30 minutes and the solution is allowed to reach the lab temperature. It is then filtered through a funnel containing a plug of glass wool. Filtrate is stored in a clean, glass stoppered bottle. The bottle is kept in dark or amber coloured bottle may be used for storing the solution.

Standardisation of 0.1 N. potassium permanganate solution : Some of the methods of standardisation of 0.1 N. potassium permanganate solution are -

1. Standardisation with arsenic(III) oxide
2. Standardisation with sodium oxalate
3. Standardisation with metallic iron
4. Standardisation with ethylenediammonium iron(II) sulphate

1. Standardisation with arsenic(III) oxide : 1.2 - 1.25 g. of arsenic oxide A.R. is dissolved in 50 ml of cool sodium hydroxide solution of 20 % w/v concentration and volume is diluted to 250 ml in a graduated flask. 25 ml of this solution is measured with the help of a burette into a 250 - 350 ml conical flask. 100 ml of water, 10 ml of pure concentrated HCl and 1 drop of potassium iodide are added to this solution. It is titrated with permanganate solution to the first permanent pink colour. Two other titrations are conducted and they should agree within 0.1 ml.

Calculation of normality :

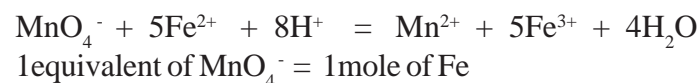


Equivalent of arsenic(III) oxide is one quarter of mole, i.e. $197.84/4 = 49.46$ g.

2. Standardisation with sodium oxalate : Weigh accurately about 1.7 g. of sodium oxalate A.R. and transfer it into a 250 ml graduated flask and dilute to mark. Take 25 ml of this solution, add 150 ml of M-sulphuric acid. Titrate with permanganate solution until first pink colour appears. Allow to stand so that the solution becomes colourless. Warm it to 50 - 60°C and continue the titration till a permanent faint pink colour.

Calculation of normality : It is 67.00g.

3. Standardisation with metallic iron : Arrange a conical flask to a retort stand in slant position . Arrange a bent delivery tube through the one holed rubber stopper fitted to the conical flask into a beaker kept on a wooden slab near the retort stand . Beaker should contain saturated solution of sodium bicarbonate or 20% potassium bicarbonate so that the lower end of bent tube dips into this solution . Take 100ml of 3N sulphuric acid prepared from 92 ml of water and 8 ml of concentrated sulphuric acid in the flask . Add 0.5 g. of sodium hydroxide in two portions . Carbon dioxide produced will drive away the air in the flask . Weigh out 0.15 g. of iron wire A.R. and place it quickly in the flask . Replace the stopper and bent tube very quickly , warm gently to cause dissolution of wire . Cool the flask rapidly under stream of cold water . Titrate with permanganate run from a burette until faint pink colour is permanent . Addition of 5 ml of syrupy phosphoric acid will facilitate detection of end point . Repeat the titration with two other samples of iron wire .



4. Standardisation with ethylenediammonium iron(II) sulphate : Weigh out accurately 1.3 - 1.5 g. of ethylenediammonium iron(II) sulphate and transfer into 350 ml conical flask . Add 60 ml of 1M. sulphuric acid and dissolve the solid in the acid . Titrate with permanganate solution run from a burette until a permanent pale pink tinge .

Dichrometry : Titrimetric analysis involving potassium dichromate as oxidising agent is called dichrometry . Potassium dichromate is not as powerful oxidising agent as permanganate . However , it is advantageous over permanganate due to several reasons . It is an excellent primary standard substance since - It is obtainable in pure state .

- It is stable upto its fusion point .
- Standard solutions of known strength can be prepared by weighing pure and dry salt and dissolving in suitable quantity of water.
- Aqueous solutions are stable if protected from evaporation .

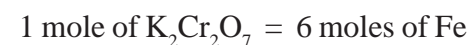
- Dichromate solutions are less easily reduced by organic matter than permanganate .
- They are stable towards light .

In acid solution , its reduction may be represented as -



Preparation of 0.1 N potassium dichromate solution : Powder about 6 g. of potassium dichromate A.R. finely . Heat for 30 - 60 minutes . in an oven at 140 - 150°C . Weigh out 4.9 g. of powder from this salt and dissolve in enough quantity of water and dilute to 1 L. in a graduated flask .

Standardisation of potassium dichromate solution : Potassium dichromate solution can be titrated with iron solution . It can be done by first dissolving iron wire A.R. in acid solution as in the titration of potassium permanganate with iron wire A.R. and titrated with dichromate solution .



Iodimetry and iodometry : Titrimetric analysis involving iodine as oxidising agent is called iodimetry and iodometry . Iodimetry refers to direct method in which , standard solution of iodine is used as oxidising agent . Iodometry refers to indirect method in which iodine liberated in chemical reaction is used as oxidising agent .

Detection of end point in titrations involving iodine : End point in titrations involving iodine can be detected by several methods .

1. Iodine itself acting as self indicator
2. Use of starches
3. Use of sodium starch glycollate
4. Use of carbon tetra chloride

1. Iodine itself acting as self indicator : In titrations with colour less solutions , iodine itself can serve as indicator owing to its colour . 1 drop of 0.1 N-Iodine solution can impart pale yellow colour to 100 ml of water

2. Use of starches : Detection of end point with iodine can be made more sensi-

tive by use of starch . Starch reacts with iodine in presence of potassium iodide to form intensely blue coloured complex .Starches can be separated into amylose and amylopectin . Amylose is abundant in potato starch and produces blue colour with iodine . Amylopectin produces red - purple product .Its merit is that it is less expensive .

- Demerits of starch :
- It is insoluble in water .
 - Its aqueous solutions are unstable .
 - It produces water insoluble complex with iodine and thus it should be added in titration just prior to end point .
 - There is some times a drift end point .

Composition and preparation of starch solution :

Composition :

Starch	----	1g.
water	----	100 ml

Preparation :

Make a paste of starch with 1g . of starch and a little quantity of water in a mortar with pestle . Add the paste to 100 ml of boiling water , boil for 1 minute and cool to room temperature . Dissolve 2 - 3 g. of potassium iodide in this solution and store in a stoppered bottle .

Solution of starch in urea may also be employed . It is prepared by refluxing procedure and quantities of the components are - Starch --- 1g .

Urea --- 19 g.

Xylene is also employed in the procedure of preparing starch in urea solution .

3. Use of sodium starch glycollate : It is a white hygroscopic substance and is readily soluble in hot water .Its opalescent solution is stable for months .Indicator may be added at any time during titration unlike starch since it does not form insoluble complex with iodine . It gives a sharp end point and it is reproducible .

Composition and preparation of sodium starch glycollate stock solution :

Composition :	Sodium starch glycollate	-----	5 g.
	Ethanol	-----	2 ml
	Water	-----	100 ml

Preparation : Sodium starch glycollate stock solution is prepared by mixing 5 g . of sodium starch glycollate with 1 - 2 ml of ethanol and boiling with 100ml of water for a few minutes . A faintly opalescent solution results on vigorous stirring and boiling with water .

Sodium starch glycollate working solution : It is prepared by diluting stock solution 5 times and 1 ml of it is added to 100 ml of solution in titration .

4. Use of carbon tetra chloride : Carbon tetrachloride is used as indicator owing to its ability of solubilising iodine . Iodine is 85 times more soluble in carbon tetra chloride than in water .

Preparation of 0.1 N. - Sodium thiosulphate solution : Weigh out 25 g. of sodium thiosulphate A.R. , dissolve it in sufficient quantity of boiled out distilled water and dilute to 1 litre with boiled out water in a graduated flask .

Standardisation of 0.1 N. - Sodium thiosulphate solution : Standardisation of 0.1 N. - Sodium thiosulphate solution can be done with

- 1 . Potassium iodate
- 2 .Potassium dichromate
- 3. Standard solution of iodine
- 4. Cerium sulphate

Standardisation of 0.1 N. - Sodium thiosulphate solution with Potassium iodate : Weigh out 0.14 - 0.15 g. of pure dry potassium iodate . Dissolve in 25 ml of cold, boiled out water . Add 2g. of iodate free potassium iodide and 5 ml of 1M -sulphuric acid . Titrate the iodine liberated in reaction with sodium thiosulphate solution . On the solution becoming pale yellow , dilute to 200 ml with distilled water . Add 2 ml of starch solution , titrate until colour turns from blue to colourless . Repeat the titration two more times .

2. Standardisation of 0.1 N. - Sodium thiosulphate solution with potassium dichromate solution : Take 100 ml of cooled, boiled out distilled water in a glass stoppered conical flask .Dissolve 3 g. of iodate free potassium iodide and 2 g. of pure sodium bicarbonate by shaking in the water taken in flask .Add 6ml of concentrated hydrochloric acid with shaking . Run 25 ml of 0.1 N. potassium dichromate solution . Wash the sides of the flask with boiled out water from wash bottle . Stopper the flask and keep in dark for 5 minutes . Rinse the stopper with cooled , boiled out distilled water. Dilute with 300 ml of cooled , boiled distilled water . Titrate the liberated iodine with hypo solution run from burette . Add 2 ml of starch solution when the solution has acquired yellowish green colour . Colour on addition of starch solution will become blue . Continue the addition until 1 drop changes from greenish blue to light green . Carry out blank titration with distilled instead of potassium dichromate .

3. Standardisation of 0.1 N. - Sodium thiosulphate solution with standard solution of iodine : Measure 25 ml of standard solution of iodine into a 250 ml flask and add 150 ml of distilled water . Titrate with hypo solution and add 2 ml of starch solution when colour of liquid becomes pale yellow in colour .

4. Standardisation of 0.1 N. - Sodium thiosulphate solution with standard solution of cerium sulphate : Take 25 ml of 0.1 N hypo solution in a flask . Add 0.3 - 0.4 g. of pure potassium iodide , 2 ml of 0.2 % starch solution and dilute to 250 ml with distilled water . Titrate with cerium sulphate solution to a first permanent blue colour .

Preparation of 0.1 N. - Iodine solution : Iodine is slightly soluble in water . 0.335 g. of iodine dissolves in 1 L . of water at 25 C . In addition to its poor solubility in water , its concentration in aqueous solutions decrease with time due to appreciable vapour pressure of iodine .Both these problems are overcome by dissolving iodine in aqueous solution of iodide of potassium .Increased solubility of iodine in aqueous solution of iodide of potassium is due to formation of tri-iodide ion with iodide of potassium .



Vapour pressure of the resulting solution is considerably reduced and thus loss by volatilisation is also reduced .

Procedure : Weigh accurately 20 g. of iodate free potassium iodide and dissolve in 30 - 40 ml of water in a glass stoppered 1L . graduated flask . Weigh out accurately 12.7 g . of resublimed iodine or iodine A.R. on a watch glass on a rough balance . Transfer it into flask of iodide solution . Stopper the flask and shake to dissolve iodine . Allow the solution to acquire room temperature and dilute to mark with distilled water . Preserve the solution in small glass stoppered bottles in cool and dark place .

Standardisation of 0.1 N. Iodine solution : Standardisation of 0.1 N.- Iodine solution can be done with - 1. Arsenic oxide

- 2. Standard solution of hypo

Standardisation of 0.1 N.- Iodine solution with Arsenic oxide :

Weigh out accurately 2.5 g. of finely powdered arsenic oxide A.R. and transfer to a 400 ml beaker . Dissolve it in aqueous solution of 2g. of sodium hydroxide dissolved in 20 ml of water . Dilute to 200 ml and neutralise with 1 M hydrochloric acid using a litmus paper . When the solution under titration is still faintly acidic , remove the litmus paper by means of glass rod , rinse both litmus paper and glass rod . Transfer the contents into a 500 ml graduated flask . Dissolve 2 g. of pure sodium bicarbonate in this solution and dilute to mark by shaking . Measure 25 ml of arsenite solution by means of a burette into a 250 ml conical flask .Add 25 - 50 ml of water , 5 g. of sodium bicarbonate and 2 ml of starch solution . Swirl the solution to dissolve sodium bicarbonate and titrate with iodine solution from a burette to the first blue colour .

Standardisation of 0.1 N.- Iodine solution with standard solution of hypo :

Transfer 25 ml of iodine solution to a 250 ml conical flask . Dilute to 100 ml . Titrate with standard solution of sodium thiosulphate until pale yellow . Add 2 ml of starch solution and complete the titration with hypo solution until the contents of the flask become colourless .

SUMMARY

Titrimetric analysis is a method of quantitative chemical analysis in which the volume of solution of accurately known strength required to react quantitatively with a solution of substance to be determined is measured, when these two substances react chemically. Reactions in titrimetric analysis can be broadly classified into-

1. Neutralisation reactions (Acidimetry & alkalimetry)

2. Complexometric reactions
3. Precipitation reactions
4. Oxidation - reduction reactions.

Acidimetry includes titrations of free bases or those formed from salts of weak acids by hydrolysis with a standard acid. Alkalimetry includes titrations of free acids or those formed by hydrolysis of salts of weak bases with a standard base. Indicators used for detection of end point in acid base titrations are called neutralisation or acid base indicators. Standard solutions of acids are usually prepared with concentrated hydrochloric acid. Alkalies employed in preparation of standard solutions of alkalies are sodium hydroxide, potassium hydroxide, barium hydroxide etc. Complex formation reactions depend on combination of ions other than hydrogen or hydroxide ions to form a soluble, slightly dissociated ion or compound as in the titration of a solution of a cyanide with silver nitrate. Selectivity in complexometry is enhanced by some procedures. They are -

1. Control of pH of the solution

2. Masking
3. Demasking
4. Kinetic masking
5. Solvent extraction.

Some of the examples of metal ion indicators are murexide, solochrome black or eriochrome black T, Patton and Reeder's indicator, solochrome dark blue or calcon etc. Different types of titrations of E.D.T.A. are

1. Direct titrations
2. Back titrations
3. Replacement or substitution titrations
4. Alkalimetric titrations
5. Miscellaneous methods.

Titrations involving precipitation reactions depend on formation of a simple precipitate by combination of ions. End points in precipitation titrations can be determined by several ways. The most important methods are

1. Formation of a coloured precipitate
2. Formation of a soluble coloured compound
3. Fajan's method of using adsorption indicators
4. Turbidity appearance.

All the reactions involving change in oxidation number or transfer of electrons among the reacting substances are included under these titrations

Following are several methods for determination of end point in oxidation - reduction reactions. They are

1. Using internal oxidation - reduction indicators
2. Reagent itself acting as indicator
3. Using external indicators
4. Potentiometric methods.

Titrations involving oxidation reactions with potassium permanganate as oxidising agent is called permanganometry. Titrations involving oxidation reactions with potassium dichromate as oxidising agent is called dichrometry. Titrations involving oxidation reactions with iodine as oxidising agent is called iodimetry and iodometry.

ESSAY QUESTIONS

1. Write about indicators in acidimetry and alkalimetry.
2. Write the procedures of preparation of standard solution of hydrochloric acid.
3. Explain the method of standardisation of hydrochloric acid with anhydrous sodium carbonate.
4. Describe the method of standardisation of hydrochloric acid with sodium tetraborate.
5. How do you prepare carbonate free sodium hydroxide solution?
6. Write about nonaqueous acidimetry and alkalimetry.
7. Classify ligands and explain a simple complexometric titration.
8. Write about selectivity in complexometry.
9. How do you prepare 0.1 M silver nitrate solution from silver nitrate and also describe standardisation of silver nitrate solution.
10. Explain determination of end points in precipitation titrations.
11. Write about determination of end point in oxidation - reduction reactions.
12. Explain permanganometry.
13. Write about dichrometry.
14. Define iodometry and explain it.

SHORT ANSWER QUESTIONS

1. Define titrimetric analysis .
2. Write the advantages of titrimetric analysis .
3. What is a standard solution ?
4. Write the definition of indicator .
5. Define the terms a. Titrant
b. Titrand .
6. Mention the terms to express the concentrations of solutions .
7. Name the classes of reactions in titrimetric analysis .
8. What is a primary standard ? Give two examples .
9. Define acidimetry and alkalimetry .
10. What acids are employed in preparation of standard solutions of acids ?
11. Mention the strengths of commercial hydrochloric acid and sulphuric acid.
12. What is constant boiling point hydrochloric acid ?
13. Name the alkalis employed in preparation of standard alkalis .
14. Mention the methods of preparing carbonate free sodium hydroxide .
15. What is nonaqueous acidimetry and alkalimetry ?
16. Explain levelling .
17. Define aprotic solvent and give examples .
18. Name the strongest acid in nonaqueous medium .
19. Write some examples of indicators in non aqueous medium .
20. What is complexometry ? Define ligand .
21. Write the definition of chelation .
22. Mention the types of EDTA titrations .
23. What is back titration ?
24. What do you mean by masking ?
25. Mention any 4 indicators in complexometry .
26. What is Mohr's method ?
27. Write Fajan's method of using adsorption indicator .
28. Write about purity of silver nitrate A.R.
29. Mention the methods of detection of end point in redox titrations .
30. Write the names of any 4 indicators in redox titrations .
31. What is meant by self indicator ?
32. Define permanganometry .
33. Mention the methods of standardisation of 0.1 N potassium permanganate solution .
34. Write the definition of dichrometry .
35. What is meant by Iodimetry and Iodometry ?
36. What are the demerits of starch in titrations involving iodine ?
37. Write the composition of starch solution .
38. Write about preparation of 0.1 N sodium thiosulphate solution .
39. Write about solubility of iodine in water and enhancement of solubility of solubility..

VI. LIVER FUNCTION TESTS : BASIC CONCEPTS

1) BASIC CONCEPTS INCLUDING NORMAL AND ABNORMAL BILIRUBIN METABOLISM

:Liver is the largest organ in the body. It is the main metabolic organ of the body. It is located at the upper right side of abdominal cavity. It is both secretory and excretory in its functions.

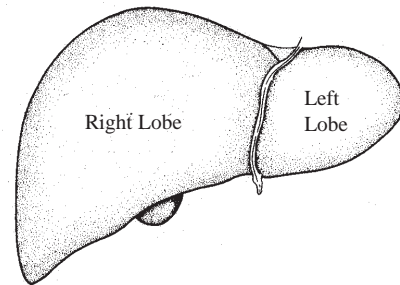


Fig. 6-1 Liver

Liver is divided into two lobes 1) Right lobe 2) Left lobe. Right lobe is larger than left lobe. Each lobe is divided into lobules. Lobules are called as hepatic lobules and they are hexagonal in shape. Lobules consist of liver cells which are also called as hepatic cells. Each lobule (1mm in diameter) has a central intralobular vein. Intralobular vein is tributary of hepatic vein. Portal canals are present around the edges of lobules each containing an interlobular vein, a branch of hepatic artery and a small bile duct. These three structures are combinedly called as portal triad.

Functions of Liver :

Functions of liver can be classified into -

- 1) Metabolic functions
- 2) Storage functions
- 3) Secretory functions

1) *Metabolic Functions* :Metabolic functions of liver are-

- a) Carbohydrate metabolism - Glycogenesis, glycogenolysis and

neoglycogenesis , conversion of all monosacharides into glucose

b) Protein metabolism - Deamination of amino acids to form urea.

c) Fat metabolism - Conversion of unsaturated fats into saturated fats, synthesis of fat from carbohydrates and proteins.

d) Detoxification of drugs and poisons

e) Conversion of excessive carbohydrates into fat

f) Synthesis of prothrombin, fibrinogen, heparin, antibodies and antitoxins

g)Hormone metabolism

h)Alcohol metabolism

i) Heat regulation

j) Bilirubin metabolism

2) *Storage functions*: Storage functions of liver are -

a) Storage of Vitamin A , B , D and K.

b) Storage of Anti anaemic factor.

c) Storage of Iron from diet and worn out RBC Vitamin B₁₂ necessary for erythropoiesis.

d) Storage of glucose in the form of glycogen.

3) *Secretory Functions*: Liver has secretory function. It secretes Bile, which is used for-

a) Emulsification and Saponification of fats.

b) Peristaltic moment in the intestine.

c) Excretion of pigments and toxic substances.

d) Deodorization of faeces.

Synthetic functions : a. . Synthesis of natural anticoagulant called as heparin

synthesis of plasma proteins - Albumin, globulin, fibrinogen and prothrombin, synthesis of vitamin A from carotene, manufacture of antibodies and antitoxins, formation of urea and uric acid from worn-out tissue cells.

Normal Bilirubin metabolism:

- 1) Red blood cells are subjected to haemolysis at the end of their life span. Cell wall of R.B.C. is destroyed and haemoglobin is released. This process of haemolysis takes place in reticuloendothelial system.
- 2) Haemoglobin released from R.B.C. in haemolysis is broken down to haem and globin. Iron is released from haem, which is stored or again used in the synthesis of haemoglobin.
- 3) Green coloured Biliverdin is formed from protoporphyrin (non-iron residue of haemoglobin). This biliverdin on reduction forms bilirubin. This bilirubin is water insoluble and is called as indirect bilirubin. It circulates in blood, bound to plasma protein albumin.
- 4) In liver, protein bound bilirubin is liberated and subjected to conjugation with glucuronic acid to form glucuronide. Conjugated form is water soluble and is called as direct bilirubin or conjugated bilirubin. Conjugated form of bilirubin is excreted into intestine. In large intestine, it is subjected to bacterial action and reduced to urobilinogen.
- 5) Most of the urobilinogen is excreted in faeces. Partly it is absorbed into portal circulation and some part of it is excreted through urine and some part is re-excreted into bile. Urobilinogen excreted in faeces is oxidised in the presence of air to pinkish brown coloured urobilin.

Normal value of total bilirubin in serum is less than 1 mg/dL.

Abnormal Bilirubin Metabolism:

Abnormal bilirubin metabolism is manifested as -

Cholestasis, excessive production of bilirubin, abnormal uptake of

bilirubin by liver cells and defective conjugation. Accumulation of unconjugated bilirubin is caused by excessive production of bilirubin, abnormal uptake of bilirubin by liver cells and defects in conjugation. This condition is called as retention jaundice. Cholestasis is caused by blockage of passage of bile into duodenum or intracellular obstruction of bile circulation and it results in its passing back into circulation. It is called as regurgitation jaundice. Disturbed bile pigment metabolism causes jaundice. Prehepatic, Hepatic and post hepatic conditions cause jaundice. In jaundice, there is yellow pigmentation of skin, conjunctiva and mucous membranes by bilirubin.

Pre-Hepatic Conditions:

Haemolytic jaundice is example of jaundice caused by prehepatic conditions. Haemolytic jaundice is due to excessive destruction of RBC resulting in excessive formation of bilirubin. This condition is also characterised by anaemia, splenomegaly and chronic ulceration over the internal or external malleoli.

Hepatic Conditions:

Hepatic conditions causing jaundice are -

- a) Impairment of uptake of bilirubin by liver cells.
ex: Gilbert's syndrome, Crigler-najjar syndrome
- b) Infections: Viral, bacterial and protozoal.
- c) Effect of hepatotoxic drugs ex: tetracyclines, paracetamol, alcohol etc.
- d) Impairment in the transport of bilirubin. Ex:- Dubin – Johnson syndrome, Rotor syndrome etc.
- e) Intra hepatic cholestasis. ex: Primary biliary cirrhosis, bile duct carcinoma etc.

Different viruses causing viral hepatitis are Hepatitis A, Hepatitis B, Non A, Non B viruses etc.

Post Hepatic Conditions:

They are due to obstruction of bile into duodenum. It is also called as obstructive jaundice. Clinical features of post hepatic jaundice are Icterus, Pruritus, Hepatomegaly and Purpura etc.

2) CLASSIFICATION

Tests which help in assessment of liver functioning are called liver function tests.

. They are classified into-

- 1) Group 1 tests
- 2) Group 2 tests
- 3) Group 3 tests
- 4) Other tests

1) *Group 1 tests* : Group 1 tests are a) Tests conducted with serum specimen
b) Tests conducted with urine specimen

Tests conducted with serum specimen are total bilirubin, direct bilirubin and indirect bilirubin.

Tests conducted with urine specimen are Fouchet's test, Hay's test, Urobilinogen test etc.

2) *Group 2 tests*: Group 2 tests are-

Determination of SGPT

Determination of SGOT

Determination of Alkaline Phosphatase etc.

3) *Group -3 tests* : Group 3 tests are-

Determination of Serum total proteins

Determination of Albumin

Determination of Globulin and

Determination of A/G ratio

Thymol turbidity test

4) *Other tests* : They are -

Bromosulphthalein test

Determination of fibrinogen

Flocculation tests etc.

3) SERUM BILIRUBIN DETERMINATION

There are different methods of determination of bilirubin. They are -

- 1) Malloy & Evelyn method
- 2) DMSO method
- 3) Jendrasic & Grof method.

Determination of total serum bilirubin by Malloy and Evelyn method:

Principle: This method is based on Vandenbergh reaction.

Bilirubin present in serum reacts with diazo reagent and produces purple coloured azobilirubin. Total serum bilirubin is soluble in methyl alcohol. Hence it is used as solvent for total serum bilirubin in the determination of total serum bilirubin.

Requirements:

- 1) Test tubes
- 2) Serological pipettes.
- 3) Photo electric colorimeter

Reagents:

1) Diazo reagent:

Composition: Diazo reagent A-5 parts.

Diazo reagent B-0.15 parts.

Method of preparation :

Mix 5 parts of diazo reagent A and 0.15 parts of diazo reagent B.

Stability : This reagent is stable for one day only. Hence it should be prepared freshly.

2) Diazo reagent 'A':

Composition:

Sulphanilic acid- 0.1g.

1.5% v/v hydrochloric acid-100 ml.

Method of preparation:

Dissolve 0.1 g of Sulphanilic acid in 100 ml of 1.5% v/v Hydrochloric acid.

Stability: This reagent is stable at room temperature for one year.

3) Diazo reagent –'B':

Composition:

Sodium Nitrite-0.5g.

Distilled water-100ml.

Method of preparation:

Dissolve 0.5 g of sodium nitrite in 75 ml of distilled water and dilute to 100ml with distilled water.

Stability: This reagent is stable at 2-8°C and should be stored in amber coloured bottle.

4) Diazo blank reagent:

Composition:

Conc. Hydro Chloric acid-1.5 ml.

Distilled water- upto 100 ml.

Method of preparation:

Dilute 1.5 ml of conc. H Cl with approximately 75 ml of distilled water taken in a 100 ml volumetric flask and make the volume upto 100 ml with distilled water.

Stability: This reagent is stable at room temperature ($25 \pm 5^\circ\text{C}$) for one year.

5) Methyl alcohol

6) Artificial bilirubin stock standard:

Composition:

Methyl red-0.2g.

Glacial acetic acid-100 ml.

Method of preparation:-

Dissolve 0.2g of methyl red in approximately 75 ml of gl. acetic acid and dilute to 100 ml with glacial acetic acid.

Stability: This reagent is stable at room temperature for one year.

7) Artificial bilirubin working standard:

Composition:

Artificial bilirubin stock standard –0.1 ml.

Glacial acetic acid-0.5 ml.

Sodium acetate-1.44 g.

Distilled water upto-100 ml.

Method of preparation:

Mix 0.1 ml of artificial bilirubin stock standard, 0.5 ml of gl. acetic acid and 1.44 g of sodium acetate with about 75 ml of distilled water and dilute to 100 ml with distilled water.

Specimen: Serum or Heparinised plasma.

Wave length: 540 nm (green filter).

Procedure:

1) Take 3 test tubes and label them as total test, total blank and std.

2) Pipette the reagents into the tubes as follows.

Total test

Total blank

std.

Distilled water	1.8 ml	1.8 ml	-
Serum	0.2 ml	0.2 ml	-
Methanol 2.5 ml	2.5 ml	-	-
Diazoreagent	0.5 ml	-	-
Diazo blank	-	0.5 ml	-
Undiluted artificial bilirubin std - -	-	5ml.	-

- 4) Allow the tubes to stand in dark for 30 minutes.
- 5) Read the O.D.s at 540 nm wave length (green filter).
- 6) Total Bilirubin = $\frac{\text{O.D. of total test} - \text{O.D. of total blank.}}{\text{O.D of standard}} \times 10 \text{ mg\%}.$

Normal range:

- 1) Total Bilirubin upto 1 mg /dl.
- 2) Direct Bilirubin upto 0.5 mg/dl.
- 3) Indirect Bilirubin upto 0.5 mg/dl

Precautions:

- 1) There should not be haemolysis in the specimen.
- 2) Specimen should not be exposed to light.

Determination of total serum bilirubin by DMSO method:

Requirements:

- 1) Test tubes
- 2) Serological pipettes.
- 3) Photo electric colorimeter.

Reagents:

- 1) Diazo 'A'-DMSO Reagent:

Composition:

- Sulphanilic acid-0.5 g
- Hydrochloric acid-1.2 ml
- Dimethyl sulfoxide(DMSO)-51.2 ml
- Distilled water-100 ml.

Method of preparation:

Mix 0.5 g of sulphanilic acid . 1.2 ml of Hydrochloric acid and 51.2 ml. of DMSO with 100 ml of distilled water.

Stability : This reagent is stable at room temperature for one year

- 2) Diazo reagent-B:

Composition:

- Sodium nitrite-0.5 g.
- Distilled water upto-100 ml.

Method of preparation:

Dissolve 0.5 g of sodium nitrite in 75 ml of distilled water and dilute to 100 ml with distilled water.

Stability : This reagent is stable at 2-8°C for 6 months.

- 4) Artificial Bilirubin stock standard :

Composition and method of preparation same as in Malloy & Evelyn method.

Stability : This reagent is stable at room temperature for one year.

- 5) Artificial bilirubin working standard:

Composition and method of preparation same as in Malloy & Evelyn method.

Specimen: Serum

Wave length: 555 nm (green filter)

Procedure:

- 1) Take three test tubes and label them as total test, total blank and standard.
- 2) Pipette the reagents into these tubes as follows.

	Total test	Total blank	Std.,
Serum	0.1 ml.	0.1 ml.	-
Diazo A-DMSO	2.8 ml.	2.8 ml	-
Diazo reagent 'B'	01. ml.	-	-
Undiluted Bilirubin Std.,	-	-	3 ml.,

- 3) Mix thoroughly.
- 4) Read the optical densities.
- 5) Calculate the conc. of serum total bilirubin with the formula.

Serum total bilirubin =

$$\frac{\text{O.D. of total test} - \text{O.D. of total blank.}}{\text{O.D of standard}} \times 10 \text{ mg\%}$$

Determination of Total Serumbilirubin by Jendrassic & Grof Method on Auto Analyser:

Principle:

- 1) Nitrous acid is produced by reaction between sodium nitrite and hydrochloric acid.
- 2) Sulphanilic acid is subjected to diazotisation on treatment with nitrous acid.
- 3) In the presence of caeffine, bilirubin reacts with diazotised sulphanilic acid to form azobilirubin.

Reagents:

- 1) Sulphanilic acid reagent

Composition:

Sulphanilic acid-500 mg.
1.5% v/v Hydrochloric acid-upto 100 ml

Stability: It is stable at room temperature.

Method of preparation:

Dissolve 500 mg of Sulphanilic acid in 75 ml. of 1.5% v/v Hydrochloric acid and dilute to 100 ml with 1.5% v/v Hydrochloric acid.

- 2) Sodium nitrite reagent :

Composition:

Sodium nitrite-170 mg.
Distilled water-up to 100 ml.

Method of preparation:

Dissolve 170 mg of Sodium nitrite in 75 ml of distilled water and dilute to 100 ml with distilled water.

Stability : This reagent is stable at 2-8°C in amber coloured bottle

- 3) Caeffine reagent:

Composition:

Caeffine -5.048g
Sodium benzoate -7.49 g
Distilled water upto 100 ml

Method of preparation:

Mix 5.048 g of caeffine and 7.49 g of sodium benzoate with 75 ml of hot distilled water. Dilute to 100ml volume with distilled water. Volume of cooled solution should be 100ml.

Stability : This reagent is stable at room temperature.

4) Tartarate Reagent:

Composition:

- Sodium potassium tartrate -26.247g
- Sodium hydroxide -7.6g
- Distilled water-up to 100ml

Method of preparation:

Dissolve 26.247g of Sodium Potassium Tartarate and 7.6g of sodium hydroxide in 75 ml of distilled water and make the volume to 100 ml. with distilled water.

Stability : This reagent is stable at room temperature.

Specimen : Serum or heparinised plasma.

System parameters:

- 1) Type of reaction: end point
- 2) Wave length : 578nm
- 3) Flow cell temperature :25°C
- 4) Incubation:15 min at R.T.
- 5) Specimen volume:0.1ml
- 6) Reagent volume:1.2ml
- 7) Factor :10.8
- 8) Zero setting: With sample blank

Procedure :

- 1) Pipette reagents into the labelled tubes as follows.

S.No	Reagent	Total test	Total blank
1	Sulphanilic acid reagent	0.1ml	0.1ml

2	Sodium nitrite reagent	0.025ml	-
3	Caefine reagent	0.5ml	0.5ml
4	Specimen	0.1ml	0.1ml
Mix and keep at R.T. for 10 minutes			
5	Tartarate reagent	0.5ml	0.525ml

- 2) Mix thoroughly.
- 3) Keep at room temperature for 5 minutes.
- 4) Read the optical densities.

4) VANDENBERGH TEST

Vandenbergh reaction is made use of in differential diagnosis of jaundice.

Principle: Same as in Malloy & Evelyn method.

Requirements:

- | | |
|--------------------|----------------|
| 1) Diazo reagent A | 4) 95% Alcohol |
| 2) Diazo reagent B | 5) Centrifuge |
| 3) Diazo reagent | 6) Test tubes |

Compositions and methods of preparation for above three reagents:

Same as in Malloy & Evelyn method.

Procedure : Separate serum from clotted blood . Take one ml of serum in a test tube and add equal amount of Diazo reagent for direct reaction and Biphasic reaction. For indirect reaction, treat one ml of serum with 2 ml of 95% alcohol, shake and centrifuge. Add 0.25 ml of Diazo reagent to one ml of supernatant.

Interpretation:

- 1) In obstructive jaundice, Vandenbergh test is direct and prompt.
- 2) In Haemolytic jaundice, Vanderbergh test is indirect

- 3) In toxic or haemolytic jaundice, Vanderbergh test is delayed, direct or biphasic.

Types of reactions in Vandenberg's test:

- 1) Direct reactions : They are two types
- a) Immediate or prompt
 - b) Delayed

In immediate or prompt type of direct reaction, bluish-violet color immediately appears within 10-30 secs. In delayed type direct reaction, red colour appearing turns violet gradually with in a period of 5-30 minutes.

- 2) Biphasic reaction: In Biphasic reaction, reddish colour appears and it turns violet after much longer time.
- 3) In indirect reaction, reddish-violet colour appears immediately.

5) TOTAL PROTEINS AND A/G RATIO

Determination of total serum proteins and A/G ratio belong to group-3 tests for hepatic functioning.

Clinical significance : In cirrhosis of liver and other liver diseases , values of total proteins are decreased . In advanced liver disease , globulins increase .

Clinical conditions other than hepatic conditions which require determination of total serum proteins and A/G ratio are -

- a) Nephrotic syndrome b) Malnutrition
- c) Multiple myeloma. d) Chronic infections
- e) Dehydration

In nephrotic syndrome and malnutrition , values of the total proteins are decreased. In multiple myeloma, chronic infections and dehydration, values of total proteins are found increased. Globulins also increase in multiple myeloma and chronic infections like tuberculosis , rheumatoid arthritis , sub

acute bacterial endocarditis and lupus erythomatosus disseminatus etc.

Methods of determining total proteins:

- 1) Biuret method
- 2) Reinhold method
- 3) Philips etal method

Biuret method of determination of total serum proteins :

Principle : Proteins on reaction with cupric ions in presence of alkaline medium form a violet coloured complex. Colour intensity is directly proportional to the concentration of total proteins in the specimen.

Requirements:

- 1) Test tubes.
- 2) Serological pipettes.
- 3) Photo electric colorimeter.

Reagents:

- 1) Stock Biuret reagent:

Composition:

Rochelle salt	-	45g
Copper sulphate	-	15g
Potassium iodide	-	5g

0.2 N sodium hydroxide up to one litre

Method of preparation:

- 1) Dissolve Rochelle salt in about 400ml of 0.2 N sodium hydroxide solution.
- 2) Dissolve 15g of copper sulphate in the solution formed in step-1 with constant stirring.
- 3) Dissolve 5g of potassium iodide in the solution formed in step-2.

4) Make the final volume to one litre with 0.2 N sodium hydroxide solution.

2) Working Biuret Reagent :

Composition :

Reagent 1	- 200 ml
0.2 N Sodium hydroxide	- up to one litre.

Method of preparation:

Dilute 200ml of stock biuret reagent to one litre with 0.2N sodium hydroxide solution.

Stability : It is stable at R.T. for one year.

3) Protein standard solution:

Composition:

Bovine albumin	-	6g
Sodium azide	-	0.1g
Normal saline	-	up to 100ml

Method of preparation:

Dissolve 6g of Bovine albumine and 0.1g of sodium azide in 75ml of Normal saline and dilute to 100ml with normal saline.

Stability : This reagent is stable at 2-8°C for one year.

Sample blank reagent : Contains 9g of Rochelle salt, 5g of KI in one litre solution with 0.2 N NaOH as solvent. It is prepared by dissolving 9g. of rochelle salt and 5g. of KI in enough quantity of 0.2N NaOH solution and making upto 1L with

0.2N NaOH solution .

Stability : One year at R.T.

Specimen : Serum.

Wave length : 530 nm (green filter)

Procedure:

1) Take three test tubes labelled as test (T), standard (S) and Blank (B)

2) Pipette reagents in to the tubes as follows

	T	S	B
Working biuret reagent	5ml	5ml	5ml
Serum	0.05ml	-	-
Protein standard	-	0.05ml	-
Distilled water	-	-	0.05ml

3) Mix thoroughly.

4) Keep at room temperature for 10 minutes exactly.

5) Measure the optical densities of test and standard against blank.

6) Calculate using the formula.

$$\text{Total serum proteins} = \frac{\text{O.D of Test}}{\text{O.D of Standard}} \times 6 \text{ gm \%}$$

7) If serum is Icteric or lipaemic, proceed as follows -

a) Mix sample blank – 5ml, Specimen – 0.05ml and keep at R.T. for 10 minutes

b) Read O.D against blank.

c) Subtract this O.D from O.D of test got in step 5.

d) Calculate total serum proteins with the following formula

$$\text{Serum total proteins} = \frac{\text{O.D. of test in step 7}}{\text{O.D of standard}} \times 6 \text{ gm \%}$$

Normal range: 6-8 g%

Determination of A/G ratio :

Determination of A/G ratio requires determination of albumin and globulin and dividing serum albumin by serum globulin. By determining serum albumin and on subtracting this value from total serum proteins, value of serum globulin can be got.

Determination of serum albumin :

Name of the method :- Bromo cresol green method.

Principle: Serum albumin on binding with bromocresol green forms green coloured complex at pH-4.1. Color intensity of this complex is directly proportional to the concentration of serum albumin.

Requirement:

- 1) Test tubes
- 2) Serological pipettes
- 3) Photo electric colorimeter

Reagents:

1)Albumin reagent:

Composition:

Succinic acid – 8.85g,

Bromocresol green –108mg,

Sodium azide - 100mg,

Brij 35 – 3.54ml.

1 N Sodium hydroxide _ quantity sufficient

Distilled water _ up to one litre.

Method of preparation:

- 1) Dissolve 8.85g of succinic acid, 108mg of bromocresol green, 100mg of

sodium azide and 4ml of brij 35 in 750ml of distilled water.

- 2) Make the final volume to one litre with distilled water.

- 3) Adjust pH to 4.1 with 1 N NaOH solution.

Stability : This reagent is stable at R.T. for one year.

2) Albumin standard solution(4g%):

Bovine albumin- 4g,

Sodium azide - 0.1g,

Normal saline up to 100ml.

Method of preparation:

Dissolve 4g of bovine albumin and 0.1g of sodium azide in 75ml of Normal saline. Make the final volume to 100ml with normal saline .

Stability : This reagent is stable at 2-8°C for one year.

3) Sample blank reagent :

Composition:

Succinic acid – 0.885g, Sodium azide –10mg, Brij 35 – 0.4ml, pH-4.1.

Stability : This reagent is stable at R.T. for one year.

Specimen: Serum.

Wave length:- 640nm(Red filter).

Procedure :

- 1) Take three test tubes and label them as Test(T), Standard(S), and Blank(B).

- 2) Pipette reagents into the tubes as follows

S.No	reagent	Test	Std.	Blank
1	Albumin reagent	5ml	5ml	5ml
2	Albumin standard	-	0.05ml	-

3	Serum	0.05ml	-	-
4	Distilled water	-	-	0.05ml

- 3) Mix thoroughly.
- 4) Keep at room temperature for 10 min exactly.
- 5) Read O.D. values against blank.
- 6) Calculate using the formula

$$\text{Serum albumin} = \frac{\text{O.D. of test}}{\text{O.D. of Standard.}} \times 4 \text{ gm \%}$$

- 7) If serum is lipaemic or icteric, proceed as follows-
 - a) Mix 5ml of sample blank and 0.05ml specimen
 - b) Keep at R.T for 10 min.
 - c) Read the O.D. against distilled water
 - d) Subtract O.D. got in step 'c' from O.D. of test got in step '5'.
This is corrected O.D value of test.
 - e) Calculate with the formula

$$\text{Serum albumin} = \frac{\text{Corrected O.D. of test}}{\text{O.D. of standard}} \times 4 \text{ gm \%}$$

Serum globulin= Total serum proteins – serum albumin

A/G ratio = serum albumin/ serum globulin

Normal range:- 1.2:1 to 2:1

6) ENZYME ESTIMATIONS AS LFT

Determination of some enzymes helps in assessing liver functioning. Among them are serum transaminases, serum alkaline phosphatase etc. Serum

transaminases are - 1) Serum glutamate oxalo acetate transaminase(SGOT) & 2) Serum glutamate pyruvate transaminase(SGPT).

Clinical significance : SGOT is enzyme of mitochondria and SGPT is enzyme of cytosol. In prehepatic conditions, where there is no damage to liver cells, SGOT and SGPT levels are normal. In hepatic conditions these enzymes are elevated as these enzymes are released into serum from liver cells. Peak values are observed with in 12-14 hours of onset of jaundice. Usually their values will be elevated 10-20 folds the upper limit of normal ranges. During recovery phase normal levels are reached in 2-5 weeks. In chronic phase, moderate elevation may be found for a long duration. In post hepatic conditions, elevation will be 3-4 fold the upper limit of normal ranges. In hepato cellular damage, elevation of SGPT is more than the elevation of SGOT levels. In infective hepatitis, their values elevate several hundred fold. In obstructive jaundice, there is only moderate elevation. In toxic hepatitis observations are similar as in viral hepatitis. In cirrhosis, elevation of transaminases is moderate. In cirrhosis SGOT elevation is greater than SGPT.

Serum alkaline phosphatase enzyme is normally present in liver and excreted in the bile. Thus, its elevation is a manifestation of retention which occurs in obstructive jaundice. Alkaline phosphatase also gets elevated in acute and chronic hepatocellular disease. But this elevation will not be as high as in obstructive jaundice. In obstructive jaundice, the values are normal in early stage and also with relief of obstruction. Values should be normal in haemolytic jaundice. In some cases of metastatic carcinoma of liver, values may rise in the absence of jaundice also.

Determination of SGOT:

Refer to chapter X I (Clinical enzymology)

Determination of SGPT:

Refer to chapter X I (Clinical enzymology)

Determination of serum alkaline phosphatase:

Refer to chapter XI (Clinical enzymology)

Summary

Liver is the largest organ in the body which has secretory as well as excretory functions. It is the main site of metabolism for various substances. Abnormal conditions related to liver functioning can be divided into prehepatic conditions, Hepatic conditions and post hepatic conditions. Liver function tests can be classified into group 1 tests, group 2 tests, group 3 tests and other tests.

For determination of total serum bilirubin, Malloy & Evelyn method, DMSO method and Jendrassic & Grof method are available. Van den Bergh's test is useful in differential diagnosis of jaundice. For determination of total serum proteins, Biuret's method, Reinhold's method and Philips et al method are available. A-G ratio is ratio of concentration of Albumin to the concentration of globulin. Serum albumin is determined by Bromocresol-green method. Serum globulin is determined by subtracting serum albumin value from total serum proteins value. A-G ratio is got by dividing serum albumin value with serum globulin value.

Determination of Enzymes such as SGOT, SGPT and alkaline phosphatase also help in assessment of liver functioning owing to their release into blood stream due to destruction of hepatic cells causing elevation of their values in blood above normal.

ESSAY QUESTIONS

- 1) Write about the anatomical, physiological and pathological aspects of liver.
- 2) Write about normal and abnormal metabolisms of bilirubin.
- 3) Classify liver functioning tests.
- 4) Mention different methods of serum bilirubin determination and describe Malloy & Evelyn method.

- 5) Write about determination of total serum bilirubin by DMSO method.
- 6) Describe determination of serum bilirubin by Jendrassic & Grof method.
- 7) What are the different methods of determination of serum proteins? Describe Biuret method. What is A-G ratio?
- 8) Explain the determination of serum albumin by Bromocresol green method.
- 9) Give the clinical significance for determination of serum transaminases in liver functioning.
- 10) Write about determination of SGOT and its significance in LFT.
- 11) Explain about determination of SGPT and its significance in LFT.
- 12) Describe the determination of serum alkaline phosphatase and its significance in LFT.

SHORT ANSWER QUESTIONS

- 1) What is portal triad?
- 2) Mention the functions of liver.
- 3) Exemplify prehepatic condition.
- 4) Give few examples of hepatic conditions.
- 5) What are post hepatic conditions?
- 6) Name group-1 liver function tests.
- 7) Mention group-2 liver function tests.
- 8) List out group-3 liver function tests.
- 9) What are different methods of determination of total serum bilirubin?
- 10) Write the composition of Diazo reagent.
- 11) Write the stability aspects of Diazo reagent 'A' and Diazo reagent-'B'.
- 12) Mention the normal ranges of-
 - a) total bilirubin
 - b) direct bilirubin
 - c) indirect bilirubin

- 13) Write the principle of Total serum bilirubin.
- 14) Give the principle of Vandenberg test.
- 15) What are different types of reactions in Vandenberg test?
- 16) Write the formulae for a) Serum globulin
b) A/G ratio.
- 17) Give the principle of total serum proteins determination by Biuret's method.
- 18) Write the composition of protein standard solution.
- 19) Name the LFT enzymes .
- 20) What type of hepatic conditions are indicated by elevation of serum alkaline phosphatase ?

VII. RENAL FUNCTION TESTS

1) BASIC CONCEPTS AND CLASSIFICATION

Anatomy :Urinary system consists of -

KIDNEYS-2

URETERS -2

URINARY BLADDER

URINOGENITAL TRACT/URETHRA

Kidneys are the main organs of urinary system. They are 2 bean shaped organs lying on the posterior wall of upper abdomen, one on each side of vertebral column. They lie at the level of twelfth thoracic to third lumbar vertebrae.

Structure of Kidney :

Longitudinal section of Kidney : Kidney is surrounded by a fibrous capsule. It can be stripped off easily. Portion inside this fibrous capsule can be divided into -

1) Cortex 2) Medulla

Cortex is the outer reddish brown coloured portion. Medulla is the inner lighter area.

Medulla is subdivided into 10 to 15 conical

areas called renal pyramids. Pyramids have their broad base towards cortex and apex projecting into lumen of minor calyx. Columns of Bertin are the projections of cortex. They form the boundaries of the pyramids.

Microscopic structure of kidney :

Kidneys consist of number of minute units called nephrons. They are basic structural and functional units of kidney. They are about one million nephrons in each kidney. Nephrons drain into pelvis of ureter and then into urinary bladder.

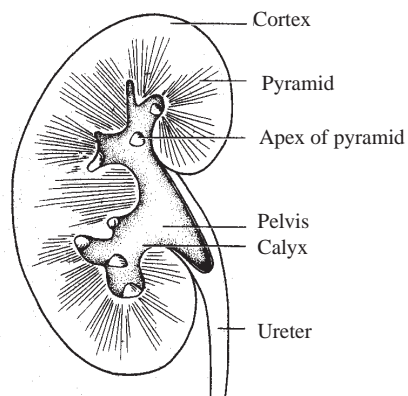


Fig.7-1 L.S of kidney

Nephrons : Uriniferous tubules consist of two portions 1) Nephron 2) Collecting tubule. Nephron is the secretory portion of uriniferous tubule.

Parts of Nephron : Nephron consists of the following parts in succession.

1) Malphigian body

a) Glomerulus

b) Bowman's capsule

2) Renal tubule :

a) Proximal convoluted tubule

b) Loop of Henle

c) Distal convoluted tubule

Proximal and distal parts of the convoluted tubule lie in cortex where as loop of Henle extends from cortex to medulla.

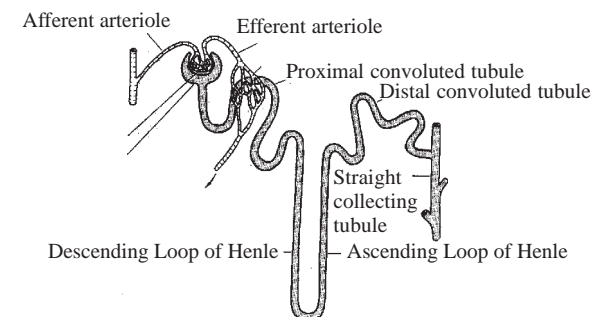


Fig.7-2 Nephron

Malphigian body : It is also called as renal capsule. It lies in cortex of kidney. Malphigian capsule consists of two parts a) Glomerulus and b) Bowman's Capsule

Glomerulus is tuft of about 6-8 renal capillaries invaginating into the end of tubule. Glomerulus has two poles 1) Vascular pole - where bloods vessels are attached 2) Tubular pole - Where renal tubule begins. Afferent arteriole brings blood to glomerular tuft. It is short and wide. This capillary tuft reunites and forms efferent arteriole. It is long and narrow. This arrangement builds up a pressure gradient of 70 mmHg. and facilitates filtration. Bowman's capsule is the dilated end of nephron. It is invaginated by glomerular tuft. It is made of two layers called parietal and visceral layers. It gradually continues with tubule.

Renal tubule : Renal tubule begins at the tubular pole of glomerulus. Short constricted part of tubule just below the glomerulus is - neck.

Parts of the renal tubule after neck are -

- Proximal convoluted tubule,
- Loop of Henle and
- Distal convoluted tubule

Proximal convoluted tubule : It is also called as pars convoluta. This portion of nephron lies in cortex of kidney.

Loop of Henle : It is also called as pars recta. Pars recta is U-shaped loop. Pars recta is anatomically divided into-

- 1) Descending limb of loop of Henle.
- 2) Thin walled ascending limb of loop of Henle
- 3) Thick walled ascending limb of loop of Henle.

Distal convoluted tubule : It is lined by cubical epithelium.

Collecting tubule : It is non-secretory portion of uriniferous tubule. It is collecting system. It is lined by pale cuboidal cells. Several collecting tubules from nephrons join to form duct of Bellini. It opens at apex of renal pyramid. Nephrons ultimately drain into pelvis of ureter. From here urine collects into urinary bladder. Urine is passed out into exterior through urethra.

Physiology :

Functions of kidney :

- 1) Excretion of waste products of cellular metabolism
- 2) Maintenance of pH and acid-base equilibrium.
- 3) Maintenance of water balance
- 4) Elimination of drugs and toxic substances from body.
- 5) Maintenance of optimum concentration of certain blood constituents by means of selective reabsorption.
- 6) Regulation of blood pressure by its endocrine function

7) Manufacture of new substances like ammonia, hippuric acid, inorganic phosphates etc.

8) Maintenance of osmotic pressure in blood and tissues.

9) Regulation of erythropoiesis

Renal functioning is dependent on 1) Glomerular functioning 2) Tubular functioning.

Glomerular functioning : Glomerulus acts as ultra filter to separate cells, proteins and fats from blood. Thus, glomerular filtrate has the composition of plasma except proteins and fats.

Glomerular filtration is brought about due to pressure gradient in the glomerular tuft. This pressure gradient in glomerular tuft is caused by the special anatomical set up i.e. afferent arteriole is wider than efferent arteriole.

Tubular functioning : Functions of renal tubules are -

- 1) Selective reabsorption
- 2) Tubular secretion
- 3) Formation of new substances.

1) Selective reabsorption :

Reabsorption of water and other substances by renal tubules is differential and selective. Substances reabsorbed by renal tubules are water, proteins, glucose, sodium, chloride, potassium, bicarbonate, phosphate etc.

2) Tubular secretion :

Renal tubular cells secrete some substances. Tubular secretion is an active process. Ethereal sulphates, steroids, glucuronides, 5 hydroxy indole acetic acid etc. are secreted by tubules. Certain substances like phenol red, diodrast, penicillin, para amino hippuric acid are actively transferred from blood stream into tubules. Tubules also secrete potassium, creatinine and hydrogen ion. Potassium and hydrogen ion are secreted in tubules when sodium ion is reabsorbed.

3) Formation of new substances :

Some new substances are formed by renal tubules. They are -ammonia, hippuric acid and inorganic phosphates.

Micturition : Micturition is the process of filling up of bladder by urine and emptying of same from it from time to time.

Clinical significance of RFT : Renal functioning is affected by different conditions. They are 1) Pre renal conditions 2) Renal conditions 3) Post renal conditions. Prerenal conditions affecting renal functioning are diseases of heart and circulatory system. Ex:- In cardiac failure, blood flow to the kidneys is diminished causing diminished renal functioning, though kidneys are normal. Renal conditions are due to glomerular as well as tubular damage. Glomerular damage causes defective glomerular filtration and tubular damage causes defective tubular functions that is reabsorption. Postrenal conditions such as formation of calculi in pelvis of kidney, ureter or bladder cause back pressure. This reduces filtration rate and urinary excretion. Patients with inherited deficit enzymatic systems also cannot carry one or more normal metabolic processes. Renal glycosuria, aminoaciduria are examples of such cases.

Renal function tests : They are the laboratory tests done to assess renal functioning. However, renal function tests can detect renal damage in its early stage very rarely. Two thirds of the renal tissue must be damaged to show any abnormal situation. One healthy kidney is enough to give a normal response in renal functioning tests.

Renal function tests can be classified into

- 1) Tests performed by determining blood constituents.
- 2) Tests related to urine analysis.
- 3) Elimination tests.
- 4) Clearance tests.

1) Tests performed by determining blood constituents: Variation in renal functioning causes variations in the concentrations of certain blood constituents. Some of the determinations of blood constituents are :

- a) Determination of Blood urea Nitrogen (BUN).
- b) Determination of Non protein Nitrogen (NPN).
- c) Determination of Serum creatinine.
- d) Determination of Serum uric acid.
- e) Determination of Serum cholesterol.
- f) Determination of Albumin/Globulin ratio.

2) Tests related to urine analysis:

- a) Determination of urine bio-chemical parameters such as urea, proteins etc.
- b) Determination of Volume, Reaction, Ammonia coefficient etc.
- c) Determination of Specific gravity (Concentration-dilution tests).
- d) Qualitative/Semi-Quantitative and Microscopic procedures for abnormal substances such as Proteins, Casts, Blood cells.

3) Elimination tests :

They depend on elimination of some substance, administered by oral or parenteral route. They are-

- a) Water elimination test
- b) Urea elimination test
- c) Indigo carmine test
- d) Iodoxyl test

4) Clearance tests: Different clearance tests are :

- a) Creatinine clearance test.
- b) Urea clearance test.
- c) Inulin clearance test.

2) CLEARANCE TESTS

Tests to measure capacity of kidneys to clear waste products from blood into urine are called clearance tests . They are relatively more sensitive and useful than retention tests. They measure amount of substance excreted in urine as compared to conc. of the substance in plasma. Clearance value of a suitable

substance gives glomerular filtration rate.

Definition : Clearance of a substance is defined as number of ml of plasma which contains the amount of substance excreted in urine in one minute. Test used for measuring clearance of a substance is called clearance test.

a) Creatinine clearance test:

Definition : Test conducted to determine the clearance of creatinine is called Creatinine clearance test . Clearance of creatinine can be defined as number of ml of plasma which contains the amount of creatinine excreted in urine in one minute.

Specimens:

- 1) Blood
- 2) 24 hours urine specimen preserved by thymol crystals.

Procedure:

- 1) Separate serum/plasma from blood.
- 2) Determine serum creatinine conc.
- 3) Measure the volume of 24 hours urine and determine urine creatinine conc.
- 4) Calculate creatinine clearance from the formula.

$$C_c = \frac{U_c V}{P_c} \times \frac{1.73}{\text{Estimated surface area}}$$

Where C_c = Creatinine clearance

U_c = Conc. of creatinine in urine in mg/ml.

V = Volume of urine per minute.

P_c = Conc. of Creatinine in plasma/serum in mg/ml.

Normal values:

110-150 ml./min (males)

105-132 ml/min (females)

Correction on basis of age : For age between 50 - 75 subtract 5 ml for each 5

years . For age above 75 , subtract 8ml for every 5 years .

Clinical significance : Clinical conditions like shock , hypovolemia , nephrotoxic chemicals , acute glomerulonephritis , malignant hypertension , eclampsia etc cause acute reduction of creatinine clearance . Glomerulonephritis , pyelonephritis , hypertensive nephrosclerosis , polycystic kidneys etc . cause chronic reduction of creatinine clearance .

b) Urea clearance test (Von slyke method) :

Definition : Test conducted to determine the clearance of urea is called urea clearance test . Clearance of urea can be defined as number of ml of plasma which contains the amount of urea excreted in urine in one minute.

Procedure :

- 1) Collect two one hourly samples of urine.
- 2) Collect blood at the end of first hour and its urea content.
- 3) Measure the volumes of urine sample and their urea concentrations.
- 4) Calculate urea clearance maximum and urea clearance standard.

$$C_m = \frac{U_u \times V}{P_u}$$

Where C_m = Maximum clearance of urea.

U_u = Urine urea concentration in mg/ml.

V = Volume of urine in ml per minute.

P_u = Concentration of urea in plasma in mg/ml.

Normal value : 60-100ml/min.

$$C_s = \frac{U_u \times V}{P_u}$$

Where C_s = Standard clearance of urea.

Note:- Normal value 40-65 ml/min.

- 1) If output of urine per minute is less than 2 ml , clearance is termed standard clearance.

- 2) If output of urine per minute is more than 2 ml , clearance is termed maximum clearance.

c) Inulin clearance test :

Definition : Test conducted to determine the clearance of inulin is called inulin clearance test . Clearance of inulin can be defined as number of ml of plasma which contains the amount of inulin excreted in urine in one minute.

Procedure:

- 1) Dissolve 10g of inulin in 100ml of normal saline.
- 2) Administer it intravenously at the rate of 10ml per minute.
- 3) Calculate using the formula.

$$C_{in} = \frac{U_{in} \times V}{P_{in}} \text{ ml/min}$$

Where C_{in} = inulin clearance.

U_{in} = Conc. of inulin in urine in mg/ml.

V = Volume of urine in ml per minute.

P_{in} = Conc. of inulin in plasma in mg per ml.

- 4) Correct to the body surface area.

Normal values:

110-150 ml/min (males)

105-132 ml/min (females)

3) CONCENTRATION AND DILUTION TESTS

CONCENTRATION TEST

Concentrating test is specific gravity test to test the concentrating power of kidneys . It is carried out in the following way . This test may detect the renal defect in cases of normal blood urea .

1. On a selected day , at 7 p.m., patient is given a good protein meal . Fluid shall not exceed 200 ml . Any fluids will not be given afterwards .
2. Patient shall empty the bladder on going to rest and this sample is discarded .

3. On the next day , morning 3 urine samples are collected at one hour intervals from 9A.M. to 11 P.M.

4. Specific gravities of the three samples are determined .

Interpretation of results : 1. Specific gravity of atleast one sample should be more than 1.022.

2. Specific gravity of more than 1.022 in maximum samples indicates impaired renal function .

DILUTION TEST

Dilution test is specific gravity test to determine the diluting power of kidneys . This test is carried out in the following way .

1. Patient should not drink water after mid night .
2. Patient will empty the bladder at 7 A.M.
3. Within next half an hour, 1.2 litres of water should be drunk by the patient .
4. 4 samples of urine are collected from 8 A.M. to 11 A.M. at an interval of 1 hour .
5. Volume and specific gravities of the 4 samples are determined .

Interpretation of results : 1. Specific gravity of atleast one sample should be 1.003 or less .Such a low values of specific gravity are not reached in impaired renal function .

2. All the water drunk will be excreted in four hours .
3. In impaired renal function , volumes will be less .
4. In advanced cases of renal impairment , volumes may be less than 100 ml and specific gravity 1.10.

4) URINE EXAMINATION IN ASSESSING KIDNEY FUNCTION VOLUME

Volume excreted in 24 hours in healthy adults on average is 1.2 - 1.5 L. Volume

of urine passed in night should not be more than 400 ml. 24 hours urine may be 0.6 - 2 L. also .Abnormal clinical condition of excreting more than 2 L. of urine in 24 hours is called polyuria . Oliguria is an abnormal clinical condition of excreting less than 500 ml of urine in 24 hours . Anuria is an abnormal clinical condition of completely suppressed excretion of urine . Nocturia is an abnormal clinical condition of excreting more than 500 ml of urine at night with specific gravity of less than 1.018.

Measurement of volume of urine is done using measuring cylinder . Collection of 24 hours urine is done with the help of preservative .

Interpretation with renal functioning :

1. Polyuria occurs in chronic renal failure .
2. Oliguria occurs in
 - a. Renal ischaemia
 - b. Acute renal tubular necrosis
 - c. Acute glomerulonephritis
 - d. Obstruction to urine outflow
3. Nocturia is characteristic feature of chronic glomerulonephritis .

SPECIFIC GRAVITY

Specific gravity is weight per ml. The presence of various solutes in the urine changes the specific gravity of urine. Specific gravity depends up on concentration of various solutes in the urine. Pure water has specific gravity of 1. Since urine is aqueous solution of inorganic and organic substances, its normal specific gravity range over 24 hours period is 1.015 to 1.030. Normal range for a random specimen is 1.003 to 1.035.

Specific gravity of urine can be measured with

- | | |
|----------------------------|---------------------------|
| 1. Urinometer | 2. Refractometer |
| 3. Dip Sticks | 4. Osmometer |
| 5. Specific gravity bottle | 6. Specific gravity beads |

1. Measurement of specific gravity of urine with urinometer :

- Requirements :
1. Urinometer
 2. Beaker
 3. Distilled water
 4. Filter paper

Specimen : Urine



Fig. 7-3 Urinometer

Procedure : Take the urine sample in to a beaker nearly to full. By means of filter paper remove the froth or bubbles. Float the urinometer so that it does not touch the bottom or sides of the beaker. Take the reading of the lower meniscus. If the urine amount is less, dilute the urine to raise the volume up to 70-80 ml. Take the reading and multiply the last 2 figures after decimal with dilution factor.

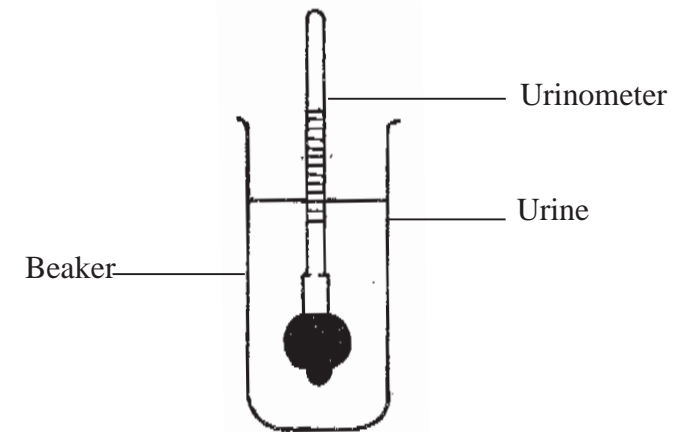


Fig.7-4 Determination of specific gravity of urine with urinometer

Correction of specific gravity value for variations from the calibrated temperature : For every 3°C raise of temperature from the calibrated temperature, 0.001 should be added. For every 3°C decrease of temperature from the calibrated temperature, 0.001 should be subtracted from the noted specific gravity reading.

Correction for Albumin : For each gram of Albumin for 100 ml., 0.003 should be added.

- 2) Measurement of specific gravity of urine with refractometer : Small quantity of urine is enough for measuring specific gravity by refractometry. It measures the concentration of dissolved substances. Goldberg refractometer gives direct reading of specific gravity.
- 3) Measurement of specific gravity of urine with Dip sticks : This method requires only one drop of urine. Specific gravity dip sticks are available from Miles India.
- 4) Measurement of specific gravity of urine with Osmometer : It is called as osmometry. It gives the most accurate values.
- 5) Measurement of specific gravity of urine with specific gravity bottle : Weigh the empty bottle. Weigh the bottle with urine. Subtract the former from the later and divide with volume of urine. It gives specific gravity of urine tested for.
- 6) Measurement of specific gravity of urine with specific gravity beads: Dip the specific gravity beads one after other. Take the reading of the bead which neither sinks nor floats.

Clinical Significance:

Hypersthenuria : Hypersthenuria is a clinical condition in which urine of unusually high specific gravity is excreted.

Isothenuria : Isothenuria is condition of excretion of urine of fixed specific gravity - 1.010. It indicates poor tubular reabsorption.

Hyposthenuria : Hyposthenuria is a clinical condition in which urine of specific gravity less than 1.007 is excreted.

Interpretation with renal functioning :

1. Hypersthenuria is present in
 - a. Acute nephritis
 - b. Albuminuria
 - c. All cases of albuminuria
2. Hyposthenuria is seen in
 - a. Chronic nephritis (when concentrating power of

renal tubules is low)

b. All causes of polyuria (not diabetes mellitus)

3. Isothenuria is seen in

- a. Chronic nephritis

b. Arteriosclerotic kidney

PROTEINS

There are three main tests for proteins in urine. They are -

- a) Boiling test for albumin
- b) Sulphosalicylic acid test for albumin
- c) Nitric acid test for albumin (Heller's ring test)
- d) Albustix method
- e) Esbach's Albumino meter method
- f) Turbidimetric procedure

a) Boiling test for albumin (coagulation test or heat and acetic acid test):

Principle : Proteins are subjected to coagulation by the action of heat. Coagulation causes changes in solubility of the protein, thus precipitating it. Acid also has coagulating effect on proteins.

Requirements :

1. Test tube
2. Funnel and Filter paper
3. Litmus paper
4. 3% acetic acid
5. Spirit lamp or Bunsen burner

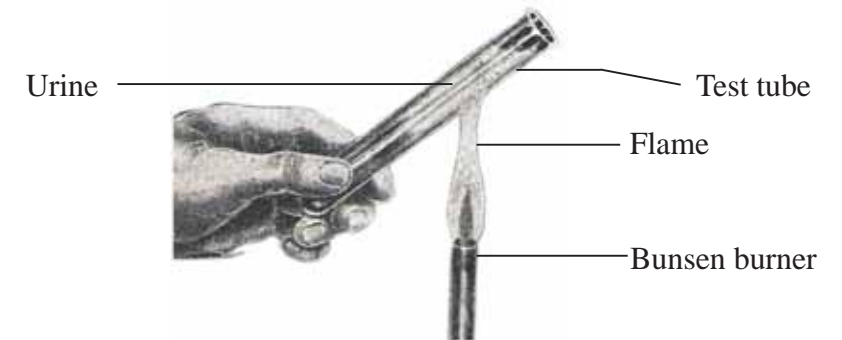


Fig. 7-5 Boiling test for albumin

Procedure: 1) If the collected urine sample looks turbid, filter it. 2) If it is alkaline, make it slightly acidic to litmus by adding a few drops of 3% acetic

acid. 3) Fill the urine in to a clean dry test tube up to 1 inch from top portion of the test tube. 4) Boil the urine present in the top one third portion of test tube over a spirit lamp or Bunsen flame. 5) If any cloudiness appears, add a few drops of 3% acetic acid.

Result : 1) If the cloudiness appears while the tube is being heated but disappears when it reaches the boiling point, it is Bence Jones protein. 2) If the cloudiness disappears on addition of 3% acetic acid, it indicates the presence of phosphates in urine. 3) If the cloudiness still remains after adding acetic acid, then it is due to the presence of albumin in urine sample. 4) If urine remains clear no albumin is present.

Varying degrees of turbidity are reported as follows

S.No.	Observation	Degree of turbidity	Inference
1)	Clarity	-	Proteins are absent
2)	Faint haziness	+	
3)	Cloudiness	++	Present in varying
4)	Dense Cloudiness	+++	degrees
5)	Formation of flocculent precipitate	++++	

b) Sulphosalicylic acid test for albumin :

Principle : Proteins are subjected to coagulation by the action of sulfosalicylic acid.

Procedure : Take a clean test tube and to it, add 5 ml. of clear urine and then to it, add 10 drops of 20% sulphosalicylic acid.

Report : If protein is present there will be a white precipitate increasing with the increasing quantity of protein.

c) Nitric acid test for albumin (Heller's ring test) :

Principle : Proteins are subjected to coagulation by the action of Nitric acid.

Procedure : Take a clean test tube and to it, add 2 to 3 ml. of nitric acid and then gently float equal volume of clear urine.

Report : Presence of white ring at the junction indicates the presence of albumin.

d) Albustix method : It is also known as paper strip method.

Principle : Paper strips are impregnated with Bromophenol blue and salicylate buffer. Presence of proteins causes change of colour of indicator from light yellow to blue.

Procedure : Dip the paper strip in urine and observe for colour change.

Result : 1. No change in colour indicates -ve result.

2. Change of colour from light yellow to blue indicates +ve result i.e., presence of proteins.

The above four methods of protein testing are qualitative and semi quantitative in nature. The following methods are quantitative.

MICROSCOPIC EXAMINATION : Identification or detection of normal or abnormal components like cells, casts, crystals, mucus threads, parasites and bacteria using microscope is known as microscopic examination.

First collect the urine sample (clear, fresh morning specimen). Obtain urinary sediment by centrifuging urine at 3000 rpm for 5 minutes. Withdraw the clear supernatant fluid, place a drop of the sediment on a glass slide and cover it with a coverslip. Examine first under low power objective, then observe under high power objective of a microscope. Vary the intensity for screening casts. If protein is present, look for casts R.B.C.'s, pus cells and epithelial cells.

Note: Overnight 12 hrs. sample with restricted fluid intake- preferably a fresh concentrated specimen should be examined for cells, casts and crystals. If urine is diluted or contaminated, cells and casts dissolve very quickly.

After examining under microscope, the following components are observed in the sediment.

A) Cells, B) Casts C) Crystals D) Miscellaneous

A) CELLS :

i) RBC's : Under high power objective of a microscope, RBCs appear as pale discs. More than one RBC per high power field is abnormal. The red blood cells swell up and broken down in diluted urine. The red cells may show crenated margins.

Clinical Significance: Increase in number of red blood cells is found in

a) Pyelonephritis b) Renal stones c) Cystitis d) Polycythemia vera

Decrease in number of red blood cells is found in Anemias.

ii) WBC : Normal values 0-5 per high power field. More than this is abnormal.

Clinical Significance : Large number of WBCs indicate bacterial infection of urinary tract.

Increased white blood cells are found in Leucytosis, Leukaemia.

Decreased white blood cells are found in Leucopenia.

iii) Epithelial cells : These cells have a single rounded nucleus. Squamous epithelial cells present in urine in moderate numbers have no pathological importance.

Clinical Significance : Presence of all other epithelial cells indicates pathological condition.

iv) Renal tubular epithelial cells: Unstained cells have almost the same size as that of a neutrophil but contain a large round nucleus. Oval fat bodies are the cells containing fat globules. The nucleus, is not visible.

v) Bladder epithelial cells : Unstained cells are larger than renal tubular cells having a round nucleus and vary in size depending on depth of origin.

vi) Squamous epithelial cells: These are unstained large flattened cells with abundant cytoplasm and a small round nucleus. The cell may be folded or rolled.

B) CASTS :

Urinary casts are formed in the lumen of the tubules of the nephrons and are believed to originate from Tamm-Horsfall proteins secreted by renal tubules.

Casts dissolved in alkaline urine. Casts have parallel edges, round or broken. They may be curved or straight, short or long. The finding of casts is very important. The presence of casts in urine usually indicates some form of kidney disorder.

i) Hyaline casts : More than one per low power field is considered to be abnormal. They are colourless, semi transparent, homogeneous with rounded ends & cylindrical in shape. Hyaline casts cannot be seen, if illumination is not proper.

Note: A few hyaline casts may be present in normal urine.

ii) Granular casts : These are hyaline casts containing fine granules or coarse granules and are called as fine granular or coarse granular casts. Degeneration of cellular casts produce these casts.

Clinical Significance : The presence of granular casts in urine indicates renal disease.

iii) Waxy casts : These are greyish yellow or colourless and are homogeneous in appearance. The waxy casts result from the degeneration of granular casts.

Clinical Significance : The presence of waxy casts in urine indicates advanced chronic nephritis and amyloid disease.

iv) Epithelial casts: Epithelial casts are present in urine occasionally. These epithelial casts have the same significance as granular casts.

Clinical Significance : The presence of epithelial casts in urine indicates tubular degeneration and necrosis, severe chronic renal disease.

v) Cyliindroids : These will closely resemble hyaline casts, but appear thinner and longer. It looks like ribbon which is often curled or twisted. These are frequently hyaline.

vi) Fatty casts : Fatty casts are of many kinds which contain mainly fat.

Fatty degeneration of the tubular epithelium produces these fatty casts.

Clinical Significance: The presence of fatty casts indicates nephrotic syndrome and toxic renal poisoning.

vii) Fibrinous casts: These are similar to waxy casts which are yellowish in colour.

viii) Pus casts: These consist entirely of pus cells.

ix) Pseudo casts: These casts look like ribbon and have tapering ends and have less defined edges.

x) Blood casts: These will contain RBCs.

C. CRYSTALS:

Generally many of the crystals which are found in urine have little clinical significance although they may be found in calculus formation, metabolic disorders and during regular medication.

a) Crystals present in acid urine:

i) Uric Acid Crystals:

Uric acid crystals look like diamonds, rhombic or rosette form. Uric acid crystals are stained with urinary pigments as red brown or yellow. Uric acid crystals are insoluble in Hydrochloric acid or soluble in sodium hydroxide.

Clinical Significance: Presence of uric acid in urine indicates Gout, Chronic nephritis, Renal calculi.

ii) Calcium Oxalate Crystals:

These are colourless, octahedral or envelope shaped. Calcium oxalate crystals also appear as oval spheres or biconcave discs, which have dumbbell shape. Calcium oxalate crystals are frequently found in acid and neutral urine. Occasionally they are also found in alkaline urine. They are insoluble in acetic acid and soluble in hydrochloric acid.

Clinical Significance: Presence of increased number of calcium oxalates in freshly voided urine indicates diabetes mellitus, liver disease, and presence of oxalate calculi.

iii) Cystine Crystals: These are colourless hexagonal plates with unequal or equal sides. These crystals are soluble in ammonia and hydrochloric acid.

Clinical Significance: The presence of cystine crystals in urine indicates congenital cystinosis or congenital cystinuria. They can form calculi.

iv) Tyrosine Crystals: These crystals look like fine refractile needles occurring in clusters or sheaves. These are soluble in ammonium hydroxide.

Clinical Significance: Presence of tyrosine crystals in urine indicates severe liver disease and tyrosinosis.

v) Leucine Crystals: Leucine crystals are glistening yellow spheroids which may show radial or circular striations. Leucine crystals are easily soluble in alkalis and in acids but not soluble in ether. They have yellow or brown colour. These crystals are also soluble in hot alcohol.

Clinical Significance: Presence of leucine crystals in urine indicates severe hepatitis, acute yellow atrophy.

vi) Cholesterol Crystals: These crystals are in the form of transparent plates, large flat with notched corners. These are soluble in ether, chloroform and hot alcohol.

Clinical Significance: The presence of cholesterol in urine indicates Nephritis, Nephrotic conditions, Chyluria and excessive tissue breakdown.

vii) Sulpha Crystals: Most of sulpha drugs precipitate out as sheaves of needles. They may be clear or brown in colour. These drugs are soluble in ether.

b) Crystals found in Alkaline Urine:

i) Triple Phosphate: These are colourless prisms with 3 to 6 sides and frequently with oblique end. These are soluble in acetic acid. These are found in normal urine. They can form calculi.

Clinical Significance: The presence of triple phosphate crystals in urine indicates chronic cystitis, enlarged prostate etc.

ii) Amorphous Phosphates: These are present in amorphous, granular form. These amorphous phosphates are soluble in acetic acid. They have no clinical

significance.

iii) Calcium Carbonate Crystals : These crystals look small, colourless spherical dumbbell shaped or as granular type. These crystals are soluble in acetic acid. they have no clinical significance.

iv) Calcium Phosphate Crystals : These crystals are long thin and colourless. Calcium phosphate crystals look like prisms with one pointed end and appear as needles. They are arranged as rosettes or stars. They may also appear as irregular granular plates. These crystals are soluble in dilute acetic acid. They may be present in normal urine. they may also form calculi.

v) Ammonium Biurate Crystals : These are yellow brown spherical bodies. These are soluble in acetic acid and dissolve by heating.

Clinical Significance: Presence of ammonium biurates in fresh urine indicates abnormal condition.

Figures



Fig.7-6 1 & 2 Pus cells 3. Renal epithelial cells on addition of acetic acid 4. Fresh R.B.C.'s 5. Crenated R.B.C.s



Fig Fig.7-7 . Transitional epithelial cells
2. Caudate epithelial cells from pelvis of the kidney
3. Renal epithelial cells 4. Renal epithelial cells showing fatty degeneration.



Fig.7-8 1. Squamous epithelial cells of bladder
2. Vaginal epithelial cells
3. Irregular tailed forms of transitional epithelial cells from deep layers

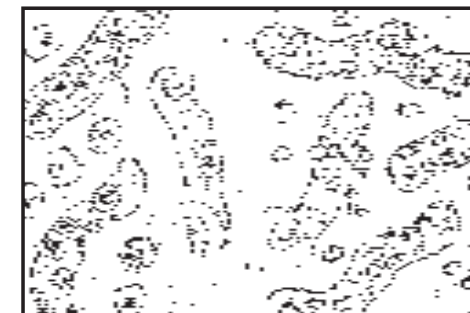


Fig 7-9 1. Epithelial casts 2. Blood casts



Fig.7-10 1. 1-a. Coarse and fine granular casts
2. Fat globules in casts 3. Hyaline casts.



Fig.7-13 Crystals of 1. Tyrosine 2. Leucine 3. Cystine

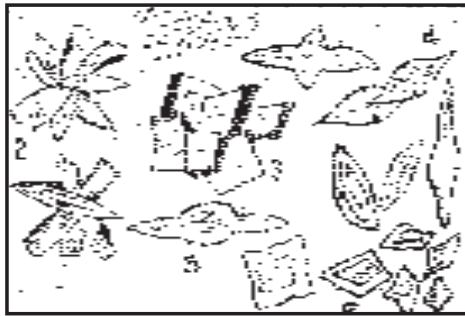


Fig.7-11 1. Amorphous form 2. Rosettes 3. Quadrates
4. Whestones 5. Prolonged into points 6. Rhombic form of uric acid

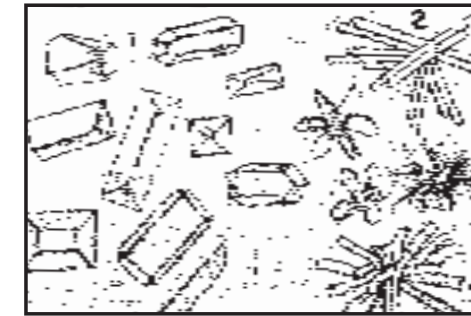


Fig.7-14 Crystals of 1. Triple phosphate 2 & 4. Calcium phosphate
3. Calcium phosphate resembling tyrosine

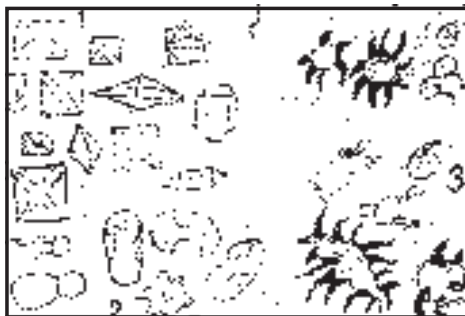


Fig. 7-12 1. Envelop shaped crystals of calcium oxalate
2. Dumb-bell shaped crystals of calcium oxalate
3. Crystals of ammonium biurate.



Fig.7-15 1. Crystals of Feathery triple phosphate
2. Amorphous phosphate with two crystals
3. Calcium carbonate

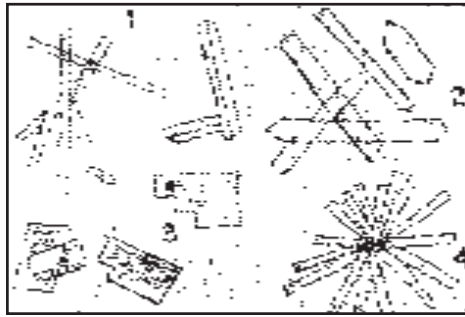


Fig.7-16 Crystals of 1. Calcium sulphate 2. Hippuric acid
3. Cholesterol 4. Sodium urate

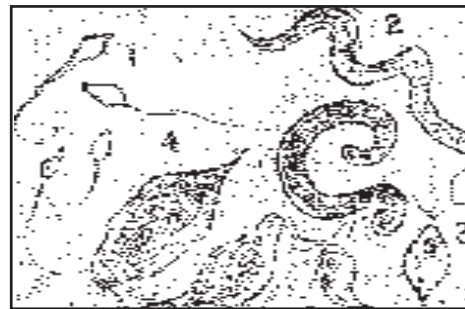


Fig.7-17 Spermatozoa 2. Micro filariae 3. Trichomonas hominis
4. Ova of schistosoma haematobium

DETERMINATION OF PROTEINS IN URINE :

Proteins in urine are quantitatively determined by

- 1) Esbach's albuminometer method
- 2) Turbidimetric method

Esbach's Albumino meter method:

Principle : Proteins are subjected to coagulation by the action of picric acid and citric acid.

Requirements : 1) Esbach's reagent(s) 33% Acetic acid.
2. Eabach's albuminometer.

Esbach reagent's Compositon : Picric acid 1 g,
Citric acid 2 g. Distilled water up to 100 ml.

Procedure : 1. Acidify urine if it is alkaline or neutral.

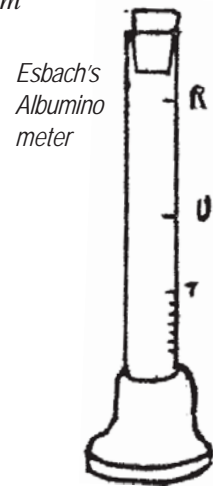


Fig.7-18

Esbach albuminometer

- 2) Take urine in Esbach's albumino meter tube upto "U" mark.
- 3) Add Esbach reagent upto "R" mark and cork.
- 4) Mix and allow to stand for 24 hours, keeping the tube vertical.
- 5) Take the reading on the tube, which gives amount of dryproteins per litre of urine.

Note : Dilution of urine is needed when specific gravity of urine is more than 1.010.

Turbidimetric procedure :

It provides accurate estimation. Turbidity produced by test is compared with standard in this method.

Interpretation with renal functioning :

Chemically detectable quantities of proteins are not found in urine of normally healthy individuals. In the process of glomerular filtration taking place at the glomeruli, only small quantity of low molecular weight proteins pass into the glomerular filtrate. Higher molecular weight proteins such as albumin and gamma globulin are not filtered at glomeruli. In the process of tubular reabsorption, taking place in the tubules, most of the protein is reabsorbed. However, Tamm-Horsfall protein, a mucoprotein secreted by renal tubules appears in urine. On the whole, <150 mg. of protein per 24 hours is generally excreted, which is not detectable by qualitative / semi quantitative testing procedures.

Proteinuria : It is a clinical condition in which chemically detectable quantities of proteins are excreted in urine.

Types of proteinuria based on quantity of protein excreted in 24 hours :Types of proteinuria based on quantity of protein excreted in 24 hours are -

1. Minimal proteinuria
2. Moderate proteinuria
3. Marked proteinuria

1. Minimal proteinuria :When excretion is less than 0.5 g. per day , it is called minimal proteinuria .

2. Moderate proteinuria : When it is between 0.5 - 3 g. per day , condition is called moderate proteinuria .

3. Marked proteinuria : When it is more than 3 g. per day , condition is called marked proteinuria .

Proteinuria occurs mainly due to two causes .

1) Glomerular damage. 2) Defective tubular reabsorption.

Glomerular damage enhances permeability of capillaries causing appearance of higher molecular weight proteins such as albumin in urine.

1. In renal tubular dysfunction , polycystic kidneys and lower urinary tract infections , proteinuria is minimal .

2. In moderate chronic glomerulonephritis , mild diabetic nephropathy and pyelonephritis proteinuria is moderate.

3. In acute glomerulonephritis , severe chronic nephritis , lipoid nephrosis , severe diabetic nephropathy , renal amyloidosis and lupus nephritis , proteinuria is marked .

DETERMINATION OF URINE CREATININE:

Method : Alkaline picrate method :

Principle : Creatinine reacts with picric acid in alkaline medium to produce reddish yellow coloured compound. Alkaline medium needed for the reaction is provided by sodium hydroxide. Concentration of creatinine is directly proportional to the colour intensity of reddish yellow colour. Concentration of creatinine in the specimen can be determined by comparing with colour intensity produced in similarly treated standard.

Requirements :

- 1 . Test tubes
- 2 . Serological pipettes
- 3 . Volumetric pipettes
- 4 . Centrifuge tubes

5 . Centrifuge

6. Photoelectric colorimeter

Reagents :

1. 0.91% w/v picric acid solution

2. Creatinine stock standard solution (100mg%)

Composition :

Creatinine 1 gm.

0.1 N HCl up to 1 litre.

Method of Preparation :

Dissolve 1 gm. of pure and dry creatinine in 0.1 N HCl and dilute to 1 litre volume with 0.1N HCl.

3. Creatinine working standard solutions (1mg% , 5mg% and 10mg%)

1 mg% solution :

Composition : Stock creatinine standard solution - 1 ml.

0.01 N HCl up to 100 ml.

5 mg% solution.

Composition : Stock creatinine standard solution - 5 ml.

0.01 N HCl up to 100 ml.

10 mg% solution

Composition : Stock creatinine standard solution - 10 ml.

0.01 N HCl up to 100 ml.

4. 10% w/v sodium hydroxide solution

Composition :

Sodium hydroxide - 10 gms.

Distilled water upto 100 ml.

5. 10% w/v sodium tungstate solution

Composition :

Sodium tungstate - 10 gms.

Distilled water upto 100 ml.

6. 2/3 N sulphuric acid

Composition :

Concentrated sulphuric acid - 2 ml.

Distilled water upto 100 ml.

7. Alkaline picrate reagent :

Composition:

Picric acid reagent - 4 parts

10% NaOH - 1 part

This reagent is stable for 1 day only.

Stability of reagents : Stock picric acid standard and 10% sodium hydroxide solution are stable at room temperature. Working standards are stable at 2 to 8°C.

Specimen : Serum

Wave length : 520 nm (green filter)

Procedure :

- 1) Pipette out 5 ml. of urine into a test tube
- 2) Add 2-3 drops of 3% sulphosalicylic acid
- 3) If turbidity is observed, deproteinise by adding 8 ml. of distilled water to 1 ml. of urine followed by 0.5 ml. of 2/3 N sulphuric acid, 0.5 ml. of 10% sodium tungstate, centrifuge for 10 minutes and separate supernatant. If urine is not turbid after adding 3% sulphosalicylic acid, dilute 10 times with distilled water.
- 4) Take 3 test tubes and label them as T (Test), S (Standard) and B (Blank).
- 5) Test : Take 4.8 ml. of distilled water, 0.2 ml. deproteinised / diluted urine and 1 ml. of Alkaline picrate reagent in the test tube labelled as 'T'.

- 6) Standard : Take 4.8 ml. of distilled water, 0.2 ml. of 10 mg% standard solution and 1 ml. of Alkaline picrate reagent in the test tube labelled as "S"

- 7) Blank : Take 5 ml. of distilled water and 1 ml. of Alkaline picrate reagent in the

- 8) Take O.D. readings of T, S against B at 520 nm wave length.

- 9) Calculate the concentration and quantity excreted in 24 hours.

$$\text{Urine Creatinine mg/dL} = \frac{\text{OD of T}}{\text{OD of S}} \times 10 \times \text{dilution factor}$$

Urine creatinine excreted over 24 hours =

Volume of 24 hours urine in ml. X urine creatinine concentration

----- mg.

100

Interpretation with renal functioning : Normal value of creatinine in serum is 1.5 - 2 mg. %. Serum creatinine concentration above 1.5 - 2 mg % is virtually diagnostic of renal disease . Pre renal conditions which have enhancing effect over blood urea have no effect over serum creatinine .Normal excretion of creatinine in 24 hours is 1 .5 - 3 g. Rate of excretion decreases in all kinds of renal diseases and also in post renal conditions .

DETERMINATION OF URINE UREA :

Method : Diacetyl monoxime method

Principle : Urea reacts with diacetyl monoxime and thiosemicarbazide in the presence of ferric ions in a hot acidic medium to give a pink coloured compound. Concentration of urea in urine is directly proportional to intensity of pink colour. Concentration of urea in urine is determined by comparing with the colour intensity of similarly treated standard.Ferric ions are provided by ferric chloride and acidic medium is provided by sulphuric acid and orthophosphoric acid present in acid reagent.

Requirements : 1. Test tubes

2. 10 ml. pipette

3. 0.1 ml serological pipette

4. 100 ml. measuring cylinder

5. Water bath
6. Colorimeter

Reagents : 1. DAM - TSC Reagent

Composition :

- Diacetyl monoxime - 1gm.
- Thiosemicarbazide - 0.2 gms.
- Sodium Chloride - 9 gms.
- Distilled water up to 100 ml

Method of Preparation :

1. Dissolve 1 gm. of Diacetyl monoxime in about 600 ml. of distilled water.
2. Dissolve 200 mg. of thiosemicarbazide in the above solution.
3. To this, add 9 gms. of sodium chloride and dissolve.
4. Dilute to 1 L with distilled water.

2. Acid Reagent :

Composition :

- Orthophosphoric acid - 10 ml.
- Sulphuric acid - 60 ml.
- 10% w/v aqueous ferric chloride solution - 1ml.

Distilled water upto 1 litre.

Method of Preparation :

1. Add 10 ml. orthophosphoric acid and 60 ml. of sulphuric acid to about 750 ml. of distilled water.
2. Cool and add 1 ml. of 10% w/v ferric chloride solution.
3. Dilute the above solution to 1 litre with distilled water.

3. Stock Urea Standard Reagent (1% w/v) :

Composition :

- Dry urea - 1 gm.

0.2 % w/v benzoic acid aqueous solution upto 100 ml.

Method of preparation :

Dissolve 1 gm. Urea in 75 ml. of 0.2% w/v benzoic acid aqueous solution and dilute to 100 ml. with 0.2% w/v benzoic acid solution.

4. Urea Working Standard (50 mg%) :

Composition :

Stock urea standard - 5 ml.

0.2% w/v benzoic acid aqueous solution up to 100 ml.

Method of preparation : Dilute 5 ml. of stock urea standard to 100 ml. with 0.2% w/v aqueous benzoic acid solution. This will be stable for 1 year in a refrigerator.

Wave length : 520 milli microns (green filter)

Specimen : 24 hours urine.

Procedure :

- 1) Dilute the urine specimen 10 times with distilled water.
- 2) Take 3 test tubes and label them as T, S and B (Test, Standard and Blank).
- 3) Take 5 ml. of working reagent, 0.05 ml. diluted urine in the tube labelled as 'T'.
- 4) Take 5 ml. working reagent and 0.05 ml. of 20mg/dl standard solution in the tube labelled as 'S'.
- 5) Take 5 ml. of working reagent and 0.05 ml. of distilled water in the tube labelled as 'B'.
- 6) Keep in boiling water bath for 20 minutes, cool and read ODs at 530 nm wave length against blank.

Calculation :

$$\text{Urine urea concentration in mg/dl.} = \frac{\text{OD of T}}{\text{OD of S}} \times 20 \times \text{dilution factor}$$

$$\text{Urine urea excreted over 24 hours} = \frac{\text{Volume of 24 hours urine in ml.} \times \text{urine urea concentration}}{100} \text{ mg.}$$

100

Interpretation with renal functioning :

Urea, creatinine, creatine, uric acid and amino acids constitute non protein nitrogen (NPN) of blood. Normal value of NPN is 25 - 50 mg %. There will be retention of these substances in blood in renal disease. About 50 % of NPN retained is urea. The condition of marked retention of NPN in blood is called uraemia.

Normal value of urinary excretion of urea in 24 hours is 10 - 15 g. There will be decrease in the excretion of urea in pre renal, renal and post renal conditions. In pre renal conditions, it is due to diminished renal plasma flow. In renal conditions, it is due to decreased glomerular filtration caused by renal disease. It is seen in acute glomerulonephritis, chronic renal failure, severe destructive renal disease and in acute tubular necrosis. In post renal conditions, obstructed urinary outflow caused by enlarged prostate gland, stones in urinary tract or tumours of urinary bladder elevate plasma urea values highly.

DETERMINATION OF URINE URIC ACID :

Method : Henry and Caraway

Principle : Uric acid reacts with phosphotungstic acid in alkaline medium to form a blue coloured complex. Concentration of uric acid is directly proportional to the colour intensity. Concentration of uric acid in urine is determined by comparing with similarly treated uric acid standard. Alkaline medium is provided by sodium carbonate.

Requirements:

1. Test tubes
2. Pipettes
3. Centrifuge tubes
4. Colorimeter

1. Sodium tungstate 10% w/v

Composition:

Sodium tungstate - 10g.

Distilled water up to 100 ml.

Method of preparation:

Dissolve 10g. of sodium tungstate in about 75 ml. of distilled water and dilute to 100 ml. with distilled water.

2. Sulphuric acid 2/3 N.

Composition :

Concentrated sulphuric acid - 2 ml.

Distilled water upto 100 ml.

Method : Add 2 ml. of concentrated sulphuric acid to about 75 ml. of distilled water with stirring and dilute to 100 ml. with distilled water and standardize.

3. Sodium Carbonate 10% w/v

Composition:

Anhydrous sodium carbonate - 10g.

Distilled water upto 100 ml.

Method of preparation :

Dissolve 10 g. of sodium carbonate anhydrous in 75 ml. of distilled water and dilute to 100 ml. with distilled water.

4. Phosphotungstic acid:

Requirements :

Sodium tungstate - 100g

Disodium hydrogen phosphate (anhydrous) - 20g.

Concentrated sulphuric acid - 25 ml.

Distilled water upto 1 litre.

Method of preparation :

1. Dissolve 100g. of sodium tungstate and 20g. of anhydrous disodium hydrogen phosphate in 200 ml. of distilled water.

2. Add 25 ml. of concentrated sulphuric acid to about 75 ml. of distilled water.
3. Pour acid solution slowly in to the solution prepared in 1st step and mix.
4. Boil for one hour with reflux condensor.
5. Cool and dilute to 1 litre with distilled water.
6. Store in amber coloured bottle

5. Stock uric acid standard (100 mg%)

Composition :

Lithium carbonate - 60 mg.

Uric acid - 100 mg.

Formalin - 2 ml.

50% acetic acid - 1 ml.

Distilled water upto 100 ml.

Method of preparation :

1. Dissolve 60 mg. of Lithium carbonate in about 40 ml. of distilled water.
2. Add 100 mg. of uric acid and warm gently.
3. Add 2 ml. formalin and 1 ml. of 50% acetic acid
4. Dilute to 100 ml. with distilled water

Store in amber coloured bottle at 2 to 8°C.

6. Working phosphotungstic acid solution

Composition:

Stock phosphotungstic acid solution - 5 ml.

Distilled water upto 100 ml.

Method of preparation :Dilute 5 ml. of stock phosphotungstic acid to 100 ml. volume with distilled water.

7. Working uric acid standard (5mg%)

Composition :

Stock uric acid standard solution - 5 ml.

Distilled water upto 100 ml.

Method of preparation :

Dilute 5 ml. of stock uric acid solution to 100 ml. with distilled water.

Wave length : 660 nm (Red filter)

Specimen : 24 hours urine

Procedure:

- 1) Dilute the urine specimen 200 times.
- 2) Dilute the stock uric acid standard solution 200 times.
- 3) Take three test tubes and label them as T,S and B.(Test,Standard and Blank)
- 4) Take 3 ml of dilute urine, 1 ml of 10% Sodium carbonate solution and 1 ml. of dilute phospho tungstic acid reagent in the tube labelled as T and mix.
- 5) Take 3 ml of dilute standard, 1 ml of 10% sodium carbonate solution and 1ml. of dilute phosphotungstic acid in the tube labelled as S and mix.
- 6) Take 3 ml of distilled water, 1 ml. of 10% Sodium carbonate solution and 1 ml of dilute phospho tungstic acid solution in the tube labelled as B and mix.
- 7) Read the intensities at 660 nm wavelength against blank.

Calculation :

$$\text{Concentration of uric acid (mg\%)} = \frac{\text{O.D. of T}}{\text{O.D. of S}} \times 100$$

$$\text{Quantity of uric acid excreted in 24 hours} = \frac{\text{Volume of 24 hours urine in ml.} \times \text{concentration of uric acid in urine}}{100} \text{mg.}$$

100

Interpretation with renal functioning : Normal excretion of uric acid is 0.6 - 1g. in 24 hours . Normal plasma uric acid level is 2 - 7 mg % . In renal failure or obstruction to excretion of urine , serum uric acid increases . Urine uric acid increases in gout and leukae mia .

SUMMARY

Kidneys are bean shaped organs in the abdominal cavity. L.S. of kidney shows fibrous capsule, cortex and medulla. Microscopic structure shows numerous nephrons. Parts of Nephron are 1) Malphigian body and 2) Renal tubule. 1) Malphigian body consists of a) Glomerulus and b) Bowman's capsule . Renal tubule consists of a) Proximal convoluted tubule b) Loop of Henle and c) Distal convoluted tubule

Main function of kidneys is excretion of waste products of cellular metabolism as part of control of internal environment .

Renal function tests are tests conducted to assess renal functioning. Different classes of renal function tests are 1) Tests performed by determining blood constituents 2) Tests related to urine analysis 3) Elimination tests and 4) Clearance tests.

Tests performed by determining blood constituents include determination of Blood urea Nitrogen, Non protein Nitrogen, Serum creatinine, serum uric acid, serum cholesterol, A/G ratio. Tests related to urine analysis include qualitative and quantitative tests with urine specimen and also quantitative tests have significance . Elimination tests include water elimination test, urea elimination test, Indigo carmine test, Iodoxyl test. Different clearance tests are Creatinine clearance test, Urea clearance test, Inulin clearance test etc.

Essay Questions

1. Write the anatomy and physiology of renal system .
2. What are renal function tests? Classify them and give their clinical significance
3. Mention different clearance tests. Write about creatinine clearance test.
4. Describe urea clearance test.

5. Give the procedure of Inulin clearance test.
6. Explain concentration and dilution tests.
7. Write about qualitative urine examination in assessing renal functioning .
8. How do you determine urine protein ? Explain interpretation with renal functioning .
9. Write the determination of urine urea . Discuss clinical interpretation.

Short answer questions

- 1) Mention different excretory organs of body.
- 2) What are the constituent organs of urinary system?
- 3) Describe L.S. of Kidney.
- 4) What are different parts of nephron?
- 5) Mention the Functions of kidney.
- 6) What are different steps in the formation of urine?
- 7) Mention the conditions affecting Renal functioning.
- 8) What are Prerenal conditions ? Mention a few.
- 9) Mention the causes of Renal conditions.
- 10) How do post renal conditions affect renal functioning?
- 11) Mention different classes of renal function tests.
- 12) What are different renal function tests performed with blood specimen?
- 13) Mention renal function tests related to urine analysis.
- 14) Name different elimination tests for assessment of renal functioning.
- 15) List the clearance tests for assessment of renal functioning.
- 16) Define a clearance test.
- 17) Write the formula of clearance test.
- 18) Mention normal values of Creatinine clearance test.

- 19) Give the normal values of urea clearance.
- 20) Write the normal values of Inulin clearance.
- 21) Write the formula of C_m and C_s .
- 30) Give the formula of Inulin clearance test.
- 31) What is uraemia ?
- 32) Expand NPN and name the substances constituting NPN
- 33) How do you interpret the values of urine proteins with renal functioning ?
- 34) Interpret the values of urine creatinine with renal functioning .
- 35) Write the normal values of urine urea and how do you interpret abnormal values .
- 36) Write the normal values of urine and serum uric acid.

VIII - GASTRIC FUNCTION TESTS

1) BASIC CONCEPTS AND INTRODUCTION

Gastric Function Tests are the tests to assess the functioning of stomach

Stomach is a baglike dilated structure of alimentary canal. Shape of normal stomach is like the letter 'J.' Stomach is divided into 3 parts.

(1) Fundus (2) Body (3) Pylorus. It consists of 4 layers.

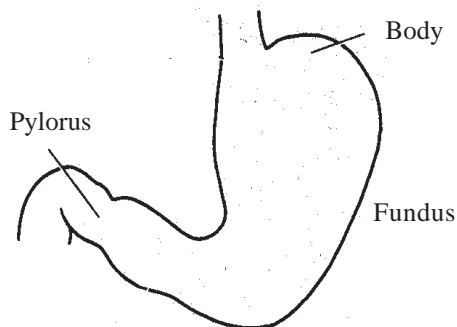


Fig.8-1 Stomach

- (1) Serous coat - outer covering of stomach.
- (2) Muscular coat - Inner to serous coat and made of muscular layers.
- (3) Submucous layer - Inner to muscular layer and made of areolar connective tissue.
- (4) Mucous layer - Inner most layer covered by columnar epithelium.

Functions of stomach:

- (1) Acting as temporary reservoir for food.
- (2) Mixing of food and digestive juices and propulsion into duodenum due to churning motility.
- (3) Secretion of gastric juice which helps in digestive process. HCl present in gastric juice kills bacteria swallowed along with food and thus provides first line of defence mechanism of body.
- (4) Digestion to some extent only.
- (5) Absorption of small quantities of water, saline, alcohol, glucose and certain drugs.

- (6) Excretion of certain toxins, certain alkaloids like morphine etc.
- (7) Stimulatory function by means of manufacture of stimulant chemical substances - Gastrin and Castle's intrinsic factor.

(8) Initiation of-

- (a) Gastro salivary reflex
- (b) Gastro- ilial reflex
- (c) Gastro -colic reflex
- (d) Pancreatic secretion
- (e) Bile expulsion

Normal Composition of gastric juice in children :

In neonates gastric juice contains small amounts of pepsin, Renin and Free acid . In first year, volume is 2-5 ml and pH-2.6-3.0

Composition of gastric juice in adults :

Total quantity : 0.5-1.3 L per meal, 1.2 -1.5 litre per day.

Organic Solids:

- | | |
|------------------------------|---|
| (1) Water - 99.02% | (a) Mucin. |
| (2) Solids - 0.98% | (b) Intrinsic factor. |
| (a) Organic solids - 0.53% | (c) Enzymes - pepsin, cathepsin, gastricin, parapepsin I and II, gastric lipase, lysozyme, gelatinase, urease, carbonic anhydrase |
| (b) Inorganic solids - 0.45% | |
| (c) pH = 0.9 - 1.5 | |
| sp. gravity = 1.006-1.009. | Inorganic solids: |
| Total acidity - 10.50 meq/L | NaCl, KCl, CaCl ₂ , Calcium phosphate, Magnesium phosphate |
| Free Hydrochloric acid | - 0.30 meq/L |
| Chlorides | - 0.5 - 0.6g % |

Total Nitrogen	-	51-75mg%
Average	-	66 mg%
Non Protein Nitrogen	-	20-30mg%
Urea Nitrogen	-	1.3-4mg%
Sulfur	-	7mg%
Phosphorous	-	5mg%
Amino acid Nitrogen	-	3-9 mg%
Ammonia nitrogen	-	2-3 mg%

Composition of gastric juice is subject to variations after seeing, smelling or tasting food.

Abnormal Constituents of gastric juice:

Blood, Food remnants, mucus or bile in excessive quantities. Pyogenic bacteria, Yeast cells, Lactobacilli, Fragments of tissues, Epithelial tissue, Parasites, Ova, Lactic acid, Mycobacterium tuberculosis (in Tb) etc.

Mechanism of gastric secretion:

Gastric juice consists of secretions of glands present in different parts of stomach. Body and fundus parts of stomach produce acidic secretions whereas pyloric part of stomach secretes alkaline juice.

Mechanism of gastric secretion is studied on animals and also to some extent on man. Direct studies on man are conducted in accidental conditions such as gastric fistula, other methods for study in man being fractional test meal. Different phases in gastric secretion of man are-

- (1) Cephalic phase
- (2) Chemical phase (Gastric and intestinal phase)
- (3) Interdigestive phase.

Cephalic phase : Cephalic or nervous phase consists of secretion of gastric juice in response to stimulation of vagal and sympathetic nerves. Hypothalamus also has influence on gastric secretion through vagal nerves. Unconditioned reflex caused by chewing and conditioned reflex caused by smell and sight also have influence.

Chemical phase : It consists of gastric and intestinal phases. Gastric phase consists of secretion of gastric juice stimulated by hormones gastrin I and gastrin II. Food also influences gastric phase.

Intestinal phase has both excitatory and inhibitory effect. Presence of certain foods in small intestine are excitatory whereas, alkalies & fats in duodenum, enterogastrone are inhibitory in nature.

Interdigestive phase : Even in fasting state, there is secretion of HCl, at regular intervals. Hormonal and cephalic mechanisms are responsible for this phase.

Hormones and substances influencing gastric secretion:

- 1) ACTH stimulates secretion of glucocorticoids by adrenal cortex.
- 2) Glucocorticoids produced by adrenal cortex increase acid and pepsin secretion and decrease mucus secretion.
- (3) Serotonin inhibits gastric secretion.
- 4) Insulin has stimulant effect on gastric secretion by causing hypoglycaemia.
- 5) Reserpine on long time administration in high doses causes enhanced secretion of acid.
- 6) Histamine stimulates gastric secretion.
- 7) Histolog also stimulates gastric secretion.
- 8) Caffeine is a stimulant of gastric secretion.
- 9) Alcohol stimulates gastric secretion.
- 10) Acetyl choline is a secretory stimulant.
- 11) Atropine is a secretory inhibitor.
- 12) Hyoscine is a secretory inhibitor.

Secretions of stomach and cells secreting :

<u>S.No.</u>	<u>Cells</u>	<u>Secretions</u>
1	Surface epithelial cells	Mucus.
2	Simple tubular glands located beneath the surface epithelium.	Gastric juice.

3	Surface mucous cells of cardiac portion.	Mucus and a few pepsinogen cells.
4	Mucous cells of fundus .	Soluble mucous intrinsic factor.
5	Chief cells/ parietal cells of body .	Pepsin, gastric renin, gelatinase.
6	Oxyntic cells	HCl.
7	Argentaffic cells of fundus	Serotonin.

Clinical significance : Gastric function tests are useful to detect different gastric disorders. When Hydrochloric acid is produced in excess than normal , the condition is called as hyperchlorohydria. When its secretion is less, it is called hypo chloro hydria. When there is no secretion of HCl, it is called as achlorohydria. When gastric enzymes are also absent along with HCl, the condition is referred to as achylia gastrica. In hyperchlorohdria, free acid exceeds 50 meq/L.

Hyperchlorohydria occurs in gastric and duodenal ulcers. In gastric ulcer, blood is also seen along with hyperchlorohydria and occasionally in gastric cancer. Volume of Residual and resting contents are high along with starch in pyloric stenosis.

Achlorohydria is present in -

- (1) Gastric tuberculosis.
- (2) Old age group.
- (3) Addison's disease.
- (4) Ulcerative colitis.
- (5) Pernicious anaemia.
- (6) Later stages of malignancies.

Occult blood in gastric specimen is indicative of ulcers , malignancies of stomach, duodenum, small intestine and large intestine. Bile is occasionally found, which has no significance. Lactic acid is due to fermentation of food remnants by the action of micro organisms. It is observed in achlorohydria, associated with retention of food & mucus. Secretion is increased in gastric cancer and gastritis.

2) TECHNIQUES OF DIFFERENT TESTS INCLUDING TUBELESS GASTRIC ANALYSIS

Different gastric function tests assessing gastric functioning are -

- a) Physical examination of gastric juice
- b) Chemical examination of gastric juice
- c) Microscopic examination of gastric juice
- d) Tubeless gastric analysis
- e) Basal gastric secretion test
- f) Augmented histamine test
- g) Histamine infusion test
- h) Augmented histalog test
- i) Insulin hypo glycaemia test
- j) Gastrin secretory test
- k) Exfoliative cytology
- l) Mycobacterial culture etc.

Physical examination of gastric juice :

(1) Colour :

Blood	Red
Acid haematin, Fresh bile, Old bile	Coffe brown , yellow, green

(2) Odour :

Normal	Sour or slightly rancid
In intestinal obstruction	Faecal
In uraemia	Ammonical

(3) volume :

Fasting (Normal)	50 - 100 ml
------------------	-------------

(4) Character - on standing :

Top	Mucus
Middle	Turbid
Bottom	Bread like residue.

(5) pH :

Normal	Acidic
--------	--------

(6) Basal rate of acid secretion (mean values):

20 - 49 yrs	2.5 meq/L.
50 -59 yrs	2.0 meq/L.
>60 yrs	1.5 meq/L.

(7) 12 hour nocturnal secretion (mean values):

Volume - 580 ml.
Free acid - 129 meq/L.

Chemical examination of gastric juice:

Chemical examination of gastric juice consists of -

- (1) Qualitative test for free HCl.
- (2) Quantitative determination of free HCl.
- (3) Quantitative determination of combined acids.
- (4) Occult blood test.
- (5) Qualitative test for starch.
- (6) Kelling's test for lactic acid.

Qualitative test for free HCl :

Requirements : (1) Evaporating dish.

(2) Topfer's reagent : (0.5%) alcoholic solution of dimethylamino azo benzene.

Specimen : gastric juice.

Procedure :

- (1) Take 5 drops of gastric juice in an evaporating dish .

(2) Add 1 or 2 drops of topfer's reagent.

(3) Observe the change.

Result :

(1) Development of cherry red colour indicates + ve response for presence of HCl.

(2) No change in case of achlorohydia.

Quantitative determination of free HCL :

Method : Titration method.

Requirements :

- (1) Evaporating dish
- (2) Topfer's reagent
- (3) Distilled water
- (4) Phenolphthalein
- (5) 0.1N NaOH.
- (6) Pipette and burette.

Specimen : Gastric juice.

Procedure :

- 1) Take 5ml of gastric juice into an evaporating dish .
- 2) Add 20ml distilled water.
- 3) Add 3 drops of Topfer's reagent and 3 drops of phenolphthalein.
- 4) Titrate with 0.1N NaOH until cherry red colour disappears.

calculation :

concentration of free HCL=value of 0.1N. NaOH X 20 meq/L.

Quantitative determination of combined acids :

Method

Requirements

Same as above.

Specimen

Procedure :- Step -1,2,3,4 : Same as above

5) Continue titration until the red colour of phenolphthalein reappears.

Calculations :-

concentration of

combined acids = volume of NaOH (in both titrations) X 20 meq/L.

Occult blood test :

Benzidine test is conducted to detect occult blood in the gastric juice. Peroxidase like activity of haemoglobin cleaves Hydrogen peroxide into water and nascent oxygen. Reaction between benzidine and nascent oxygen gives blue colour.

Qualitative test for starch:

Requirments : Ioidne solution .

Specimen : Gastric juice .

Procedure : (1) Add a few drops of Iodine Solution to a few ml of gstric juice

(2) Observe for change.

Result : Change of colour to blue indicates the presence of starch in gastric juice.

Kelling's test for lactic acid :

Nature of the test : Qualitative.

Requirements :

(1) Test tubes.

(2) 10% ferric chloride solution

Specimen :Gastric juice.

Procedure :

- (1) Take one test tubeful of distilled water.
- (2) Add 2 drops of 10% ferric chloride solution .
- (3) Transfer half of it into another test tube.
- (4) Add 1ml of gastric juice to one tube.

(5) Compare gastric juice added tube with gastric juice not added tube.

Result : Gastric juice added tube develops canary yellow colour in presence of lactic acid .

Microscopic examination of gastric juice:

Requirements : Slide, Coverslip, Centrifuge, Gastric juice,

- 1) Centrifugate gastric juice.
- 2) Take one drop of sediment on a slide .
- 3) Place a coverslip over it
- 4) Observe under microscope.

Observe for bacteria , undigested food particles, blood , yeasts, sarcinae, Lacto bacili. For investigation of gastric neoplasm, exfoliative cytologic preparation of gastric washings has to be used.

Various methods of analysis requiring and not requiring intubation:

They are - Tubeless gastric analysis (TGA)

Basal gastric secretion test (BGST)

Augmented histamine test (AHT)

Histamine Infusion Test (HIT)

Augmented Histalog Test (AHIT)

Insulin hypoglycaemia Test (IHT)

Gastrin Secretory Test (GST)

Tubeless gastric analysis (TGA): Principle : This test is based upon dissociation of azure A carbacrylic resin in presence of a free acid in stomach to give free Azure A. Azure A dissociated from Azure A carbacrylic resin appears in urine. In the absence of free acid in stomach, Azure A will not appear in urine.

Procedure :

If the test is performed in the morning, patient should not be given anything after previous night meals. Morning urine also has to be discarded.

Until completion of the test, anything should not be given orally.

- (1) Administer 500mg of caffeine with sodium benzoate or 50mg Histalog with a glassful of water orally. Alternatively administer histamine or histalog subcutaneously.
- (2) Instruct the patient to discard urine passed after one hour.
- (3) Immediately administer 2g of Azure A resin with half glass of water.
- (4) Save the sample of urine passed by the patient two hours following ingestion of azuresin.
- (5) Dilute the samples to 300ml with water.
- (6) Take 10ml of diluted urine in to each of three test tubes. Two of the tubes serve as colour controls.
- (7) Add 300mg of ascorbic acid to each of the tubes.
- (8) Place the tube in comparator block which contains standard Azure A solution of concentrations - 0.3 mg% and 0.6 mg%.
- (9) If colour of test urine is more intense than 0.6 mg% standard azuresin solution, patient is a secretor of HCl. If colour is less intense than 0.6 mg% standard azuresin solution, add a drop of diognex blue reagent (195 mg. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 100 ml. 18% HCl) to each of three tubes.
- (10) Place the three tubes in boiling water bath for 10 minutes.
- (11) Compare the colour again after 2 hours with standard solution.

Report : Report as

- (1) Less than 0.3mg % Azure A solution.
- (2) Between 0.3 mg% and 0.6 mg%.
- (3) Greater than 0.6 mg% Azure A soln.

Interpretation :

- (1) If excretion of azure A is less than 0.3 mg% of azure A standard, it is indicative of absence of acid secretion.
- (2) If excretion is in between 0.3 mg% and 0.6 mg% it is indicative of border line secretion of acid.

(3) If excretion of Azure A is more than 0.6mg% of Azure A standard, it is indicative of acid secretion.

Basal gastric secretion test (BGST) : This test is useful to study the response of stomach to endogenous stimuli in the fasting state.

Instructions to be followed by patient :

- (1) Patient should be on fasting.
- (2) Sight and odour of food should be avoided.
- (3) Medicines causing gastric stimulation should be avoided for 24 hours.
- (4) Psychological conditions such as fear, anger, depression etc should be avoided.
- (5) Water can be taken up to 8 hours prior to intubation.

Procedure :

- (1) After 12 hours over night fasting intubate the patient.
- (2) Measure and examine the residual volume of gastric secretion qualitatively.
- (3) Conduct continuous aspiration manually with a syringe.
- (4) Segregate the aspirate into 15 minute samples.
- (5) Discard the first one or two samples to make the patient adjusted to intubation.
- (6) Take four 15 minute samples subsequent to adjustment to intubation.
- (7) Measure pH, volume, titrable acidity of the samples collected at 15 minute intervals and calculate acid out put.

Calculation :

Basal acid out put =Sum of acid outputs of 4 samples (meq).

Normal range : (1) 1.3 to 4meq/hour in males.

- (2) Lower values in females.
- (3) Lower values with increasing age.

Clinical Significance:

- (1) Lower values are observed in gastric carcinoma and benign gastric ulcer.

- (2) Higher values are observed in duodenal or jejunal ulcers following partial gastrectomy with gastrojejunostomy.
- (3) Very high values are present in patients with zolinger - ellison syndrome.

Augmented histamine test (AHT) : If augmented histamine test has to be done following basal gastric secretion test, antihistamine should be administered parenterally 30 minutes prior to collection of basal secretion.

- (1) Histamine acid phosphate is administered subcutaneously in a dose of 0.04 mg per kg of body weight.
- (2) Gastric contents are collected at intervals of 15 minutes for one hour.
- (3) For each of the four samples, volume, pH and titrable acidity are measured and acid output /hour is calculated.

Normal values :- Not more than 30 meq/half an hour (sum of 2nd and 3rd sample values)

Clinical significance :

- (1) Higher values are found in duodenal ulcer and Zolinger-Ellison syndrome.
- (2) Non secretion of acid is found in:
 - a) Pernicious anaemia. b) Gastric carcinoma.

It is also reported in a) Hypochromic anaemia. b) Rheumatoid arthritis.

- c) Steatorrhoea. d) Aplastic anaemia . e) Myxoedema.
- f) Nutritional megaloblastic anaemia etc.

Contraindications : Urticaria, severe cardiac, pulmonary or renal disease, paroxysmal hypertension, symptoms of pheochromocytoma .

Histamine Infusion Test (HIT) : Histamine infusion test is conducted by injecting Histamine as IV infusion and measuring acid output. IV infusion achieves steady acid output and need for doing both basal and augmented tests is avoided.

It has high reproducibility and least side effects.

Procedure :

- (1) Intubate the patient following overnight fasting .

- (2) Obtain basal hour collection .
- (3) Administer anti histamine by intramuscular route half an hour before completion of basal hour.
- (4) After collecting basal hour secretion, start I.V infusion of histamine in normal saline. Adjust dose rate to 0.04 mg of histamine phosphate per kg body weight per hour.
- (5) Collect four 15 minute steady state samples while continuing infusion.
- (6) Analyse each sample for volume, pH and titrable acidity.

Normal values :-

16 - 32 meq/hr (in males)

18 - 25 meq/hr (in females)

Values are higher in duodenal ulcer.

Augmented Histalog Test (AHT) : Lesser side effects than with histamine make histalog alternative to histamine. Antihistamine is also not needed for this test.

- (1) Dose : 1.7 mg/kg by i . m . route.

- (2) 8 fifteen minute samples following histalog administration are needed.

Insulin Hypoglycaemia Test (IHT) : Insulin hypoglycaemia test is test for acid secretion stimulated by hypoglycaemic condition evoked by administration of insulin.

Procedure :-

- (1) Intubate the patient after overnight fasting.
- (2) Collect 2 hour basal secretion in 15 minute samples.
- (3) Insulin is administered by i.v.route . Dose can be fixed 15 or 20 units or calculated dose of 0.2 units per kg body weight .
- (4) Collect 3 blood samples for blood glucose determination at 30 minute intervals following insulin administration.
- (5) Collect gastric secretion at 15 minutes for 2 hours after insulin.
- (6) Determine volume and titrable acidity for basal and post insulin gastric samples.

Precaution :- Keep ready - 50ml syringe filled with 50% w/v glucose solution in case of any serious hypoglycaemic effects.

Note :- (1) Test is valid in case blood glucose level is below 50mg% at some point of the test.

(2) Validity also depends on ability of stomach to secrete HCl.

(3) Augmented histamine test has to be conducted if there is no acid secretion during basal and post insulin periods.

Gastrin Secretory Test (GST) : Gastrin secretory test is conducted by giving gastrin. Gastrin is given as 50g. i.v. injection. Maximum output is got 20 minutes after gastrin injection. Pentagastrin can also be given instead of gastrin .

Exfoliative cytology : Exfoliative cytology of gastric secretions is useful in the diagnosis of gastric carcinomas.

Mycobacterial culture: Aspiration of gastric contents for mycobacterial culture is needed in cases when patient cannot produce sputum. For children, not able to expectorate also this procedure is conducted. Gastric contents are collected in the early morning prior to eating and drinking. This sample is sent for culturing.

SUMMARY

Gastric function tests assess the functioning of stomach. HCl produced by oxyntic cells of stomach is useful for providing first line of defence and also acidic medium for digestive process taking place in stomach. Different gastric function tests are :a) Physical examination of gastric juice b) Chemical examination of gastric juice c) Microscopic examination of gastric juice d) Tubeless gastric analysis e) Basal gastric secretion test f) Augmented histamine test g) Histamine infusion test h) Augmented histalog test i) Insulin hypoglycaemia test j) Gastrin secretory test k) Exfoliative cytology l) Mycobacterial culture etc.

Essay Questions

- 1) Write the functions of stomach.
- 2) Give normal and abnormal composition of gastric juice.
- 3) Write the clinical significance for performing gastric function tests.

- 4) Give a note on physical examination of gastric juice.
- 5) Give a note on chemical and microscopic examination of gastric juice.
- 6) What is tubeless gastric analysis ? Write the principle , procedure, reporting and interpretation.
- 7) Describe basal gastric secretion test.
- 8) Write about augmented histamine test.
- 9) Give the procedure of histamine infusion test.
- 10) Write the procedure of insulin hypoglycaemia test.

Short Answer Questions

- 1) Mention different parts of stomach.
- 2) What are different layers of stomach.
- 3) Mention the substances absorbed in stomach.
- 4) What are the reflexes initiated by stomach?
- 5) Name the cells of stomach secreting a) HCl. b) Serotonin.
- 6) Define hyperchlorhydria and mention diseases in which hyperchlorhydria occurs.
- 7) What is achlorhydria?
- 8) Mention the conditions in which achlorhydria occurs.
- 9) How do you conduct occult blood test ?
- 10) Write the principle of tubeless gastric analysis.
- 11) Write about augmented histalog test.
- 12) What is gastrin secretory test ?

IX. THYROID FUNCTION TESTS

1) BASIC ONCEPTS

Thyroid is an endocrine gland situated at the root of the throat. It has two fairly lateral lobes, which are symmetrical. Each measures 5 x 2 x 2 cm³ approximately. These lobes are present one on either side of trachea. They are connected by a thin portion of thyroid tissue called as isthmus. Pyramidal lobe extends upwards from isthmus.

Hormones of Thyroid gland :

Thyroid gland produces three hormones.

- 1) Thyroxine
- 2) Triodo thyronine
- 3) Calcitonine

Functions of hormones of Thyroid are :

- a) Acceleration of Basal metabolic rate.
- b) Metabolism of carbohydrates, proteins, lipids, calcium and phosphorous.
- c) Increase of Nitrogen excretion, volume of urine and creatinine excretion.
- d) Maintenance of normal growth.
- e) Increase of output of milk from mammary gland.
- f) Increase of heart rate.
- g) Increase of tolerance to some drugs like morphine and digoxin.
- h) Cerebral activity, emotional responsiveness and sensory activity require these hormones.
- i) Influence on central, autonomic peripheral nervous system and voluntary muscular activity.

Hypothyroidism : It is a clinical condition in which there is hypo secretion of thyroid hormones. Hypothyroidism causes cretinism in the young and Myxoedema in the adults. Cretinism is characterized by

- a) Delay in the milestones of child's development.

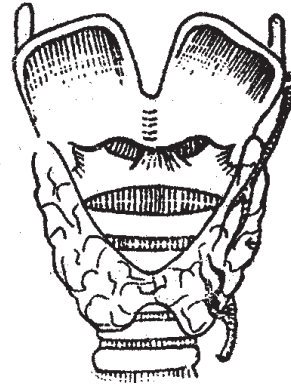


Fig.9-1 Thyroid gland

- b) Stunted skeletal growth, deformation in teeth and bones.
- c) Rough thick and wrinkled skin.
- d) Scanty hair.
- e) Idiocy in looks.
- f) Protruding large tongue.
- g) Stunted mental growth etc.,

Myxoedema in the adults occurs more frequently in women than in men and is characterised by

- a) Mongoloid appearance due to deposition of Myxomatous tissue, which is rich in proteins and mucopolysaccharides.
- b) Sexual degeneration, Impotency, Amenorrhoea etc.
- c) Impaired mental condition.
- d) Slow heart rate.
- e) Lethargy, apathy.
- f) Lowered blood sugar and Iodine
- g) Raised serum cholesterol, plasma albumin etc.

Goitre : Goitre is enlargement of thyroid. Simple goitre is caused by Iodine deficiency, presence of goitrogenic substances, some drugs, trypanosome infection etc. Toxic goitre is enlargement of thyroid accompanied by hyper thyroidism. Hokkaido goitre is due to high concentration of Iodine.

Hyper thyroidism is a clinical condition in which there is hyper secretion of thyroid hormones. It is also called as thyrotoxicosis. Grave's disease is caused by hyper thyroidism. Grave's disease is also called as exophthalmic goitre or Basedow's disease. It is characterised by :

- a) Enlarged thyroid
- b) Increase in Basal metabolic rate.
- c) Exophthalmos
- d) Restless, irritable mental condition
- e) Increased heart rate etc.

THYROID FUNCTION TESTS: There are the tests done to assess the functioning of thyroid.

1) Tests for determining the concentration of thyroid hormones in blood.

a) Free thyroxine in serum (T_4)

Normal mean value - 2.76 ng%

b) Thyroxine (T_4) in bound form with Thyroid binding globulin (TBG).

Normal range - 4-11 mg%

c) T_4 Iodine.

(Serum T_4 x 0.6)

d) Protein bound Iodine (PBI)

Normal range - 5-8 mg%

2) Radio active Iodine¹³¹I uptake

3) ¹³¹I - T_3 Red cell uptake test.

4) TSH stimulation test.

5) TSH secretion suppression test

6) Anti thyroglobulin antibody titres.

2) ESTIMATIONS OF VARIOUS THYROID HORMONES , THEIR INTERPRETATIONS

Various Thyroid hormones estimated for assessment of Thyroid functioning are -

1) Tri-Iodo Thyronine T_3

2) Thyroxine T_4

3) Thyroid stimulating hormone (TSH)

Tri-Iodo Thyronine T_3 : Refer to IMMUNO ASSAYS

Thyroxine T_4 : Refer to IMMUNO ASSAYS

TSH - Thyroid stimulating hormone:

Thyroid stimulating hormone is secreted by thyrotrophic cells of adenohypophysis, which are Periodic acid - schiff (PAS) positive. Thyroid

stimulating hormone is also called as Thyrotrophin or Thyrotrophic hormone. TSH is a glycoprotein with molecular weight of about 25,000.

Determination of TSH:

1) Injection of TSH preparation to guineapig and subsequent determination of number of colloid droplets in its thyroid cells.

2) Hypophysectomy of rat and measuring ¹³¹I uptake by its thyroid.

3) Injection of TSH preparation to starved, non metamorphosing tadpole and measurement of thyroid acinar cell height and extension of hind limb.

4) Iodine depletion in thyroid gland of one day old chick.

Normal range - 0-10 mIU/ml. (adults), < 25 mIU/ml. (neonates by third day)

Interpretation :

1) TSH values are elevated in hypothyroidism.

2) They are decreased in primary hypothyroidism, secondary and tertiary hyperthyroidism. Its determination provides useful to avoid a) under treatment of hypothyroidism. b) over treatment of hyperthyroidism.

3) RECENT METHODS OF THYROID FUNCTION TESTS

1) Thyrocalcitonin

Normal range - 50-100 pg/ml

2) Free thyroxine (F T_4)

Normal range 1-2.3 mg%

3) Free triiodothyronine (F T_3)

Normal range 250-390 pg%

4) Free thyroxine index (F T_4 I)

Normal range - 5.8 - 10.6 arbitrary units.

5) Thyrotrophin stimulating hormone in neonates.

Normal range by third day < 25.I.U./ ml.

6) Thyroxine (T_4) in neonates

Normal range by 5 days < 4.9 mg%

7) Thyroglobulin (Tg)

Normal range upto 50 ng/ml.

- 8) Thyroid stimulating hormone (TSH)
- 9) TRH (Thyrotropin releasing hormone) stimulation test.
- 10) Thyroxine binding globulin (TBG)
Normal - 12-28 mg/ml.
- 11) Determination of thyroxine by RIA
Normal 5-12 mg%
- 12) Long acting thyroid stimulator (LATS).
- 13) Total Thyroxin (T₄).
Normal 5-12.5 m%
- 14) Determination of Tri Iod Thyronine by RIA
Normal 110-230 ng%

SUMMARY

Thyroid function tests help to assess the condition of thyroid gland. Thyroid gland produces Thyroxine, Tri Iodothyronine and Calcitonine. In hypothyroidism, thyroid hormones are produced in quantities less than normal. In Hyperthyroidism, they are produced in quantities more than normal. Different thyroid function tests are available.

- There are
- 1) Tests for determining conc. of thyroid hormones in blood.
 - 2) Radio active Iodine uptake test.
 - 3) ¹³¹I - T₃ Red cell uptake test.
 - 4) TSH stimulation test
 - 5) TSH secretion suppression test
 - 6) Antithyroglobulin anti body titre and recent methods for study of thyroid functioning.

ESSAY QUESTIONS

- 1) Write the anatomy & physiology of thyroid gland. Give the significance of Thyroid function tests.
- 2) List out various thyroid function tests.
- 3) Explain Radio immuno assay of Triiodo thyronine.
- 4) Write about Radio immuno assay of thyroxine.
- 5) Write about TSH.

Short Answer Questions

- 1) Write the location and dimensions of thyroid.
- 2) Name the hormones produced by Thyroid.
- 3) What are the functions of Thyroid hormones? Write any four.
- 4) Define a) Hypo thyroidism b) Hyper thyroidism.
- 5) Write the normal values of a) Thyroxine (T₄) b) Tri Iodothyronine (T₃).

X - PANCREATIC FUNCTION TESTS

1) BASIC CONCEPTS AND INTRODUCTION

Pancreas is a soft greyish-pink coloured gland . It is 12-15 cm in length. It has a broad head and narrow tail. Head of the pancreas lies with in the curve of duodenum and tail extends as far as the spleen. Body lies between the two. Pancreas consists of lobules. These lobules are further made up of small alveoli. Walls of these alveoli contain secretory cells. Each lobule is drained by a small duct. These small ducts unite to form pancreatic duct. Pancreatic duct extends along the whole length of the gland and opens into duodenum. Between acini or alveoli, group of solid cells called as islets of langerhans exist. Though these islets are present all throughout pancreas, they are more numerous in the tail portion. Two main types of cells in these islets are Alpha cells and Beta cells. There are also a few δ cells in these islets. δ cells can be classified into δ_1 and δ_2 cells.

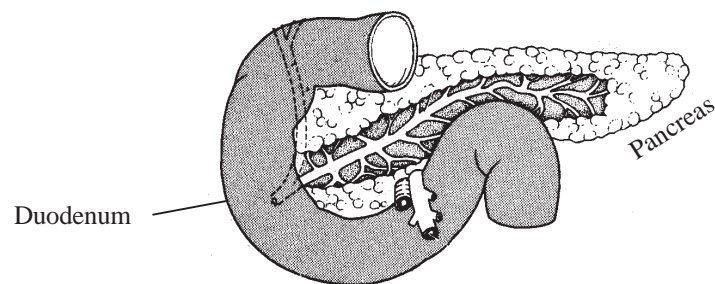


Fig.10-1 Pancreas

Functions of pancreas:

Pancreas is both endocrine and exocrine in its functions.

- 1) α -cells of islets of langerhans of pancreas produce glucagon hormone.
- 2) β -cells of islets of langerhans of pancreas produce insulin hormone. Glucagon and insulin are responsible for carbohydrate metabolism. Glucagon increases blood glucose levels whereas insulin decreases.

- 3) Pancreatic juice produced by the pancreas helps in the digestive process. It is useful in digestion of carbohydrates, fats and proteins. It neutralises gastric contents.

Physical properties & Chemical Composition of Pancreatic juice:

- 1) Quantity : Approximately 0.5 L per Meal.

Approximately 1.5 L per 24 hours.

- 2) Reaction - alkaline.
- 3) pH - 8.0 - 8.3
- 4) Specific gravity - 1.010 to 1.030

Composition of pancreatic juice :

Total solids - 1.5 - 2.5% w/v Sodium - 100 - 150 meq/L.

Potassium 2-8 meq/L. Chlorides - 50 - 95 meq/L.

Bicarbonate - 70 - 100 meq/L.

Enzymes in pancreatic juice:

Digestive Enzymes present in pancreatic juice are -

- I) Proteolytic enzymes: These are enzymes which break down proteins into peptides.
- II) Peptidases : These enzymes hydrolyse peptides into amino acids.
- III) Nucleases: They hydrolyse nucleic acids.
- IV) Amylolytic Enzymes : for hydrolysis of starch.
- V) Lipolytic Enzymes : They hydrolyse fats.

I . PROTEOLYTIC ENZYME	ACTION
1) Trypsin (Self activated or activated by enterokinase).	1) Hydrolysis of proteins into peptides.
2) Chymotrypsin. (Activated by trypsin)	2) Hydrolysis of proteins into peptides.

3) Collagenase. 4) Elastase	3) Digestion of collagen 4) Digestion of elastin
II .PEPTIDASE ENZYME	ACTION
1) Carboxy peptidase 2) Amino peptidase	1)Lysis of peptide chains containing carboxyl ends into amino acids. 2)Lysis of peptide chains containing free amino groups into amino acids.
III)NUCLEASE ENZYME	ACTION
1)Ribonuclease 2) Deoxyribonuclease.	Hydrolysis of respective nucleic acids.
IV) AMYLOLYTIC ENZYME	ACTION
Amylase.	Hydrolysis of starch into disaccharides.
V)LIPOLYTIC ENZYME	ACTION
1)Lipase 2)Phospholipase A and 3)Phospholipase B	Partial hydrolysis of neutral fats into diglycerides and monoglycerides. Hydrolysis of lecithin Hydrolysis of Cephalin

Mechanism of pancreatic secretion : Pancreatic secretion occurs in two phases. They are-1) Nervous phase. 2) Chemical phase.

Nervous Phase:Nervous phase of pancreatic secretion occurs through

vagus nerve just after taking food. It is an unconditioned reflex mechanism to evoke pancreatic secretion when food is in mouth during mastication and when food enters stomach. The reflex which evokes pancreatic secretion when food enters stomach is called as gastro pancreatic reflex. There is a local cholinergic reflex mechanism in pancreas which is independent of vagal mediation, evoking pancreatic secretion.

Chemical Phase: Chemical phase is initiated when gastric contents enter duodenum. Rate of secretion of pancreatic juice rises sharply at this point. It is proved that pancreatic secretion of this phase is due to secretion of pancreaticozymin, which is also responsible for stimulation of bile by liver. Various food stuffs have influence on pancreatic secretion.

2) VARIOUS TESTS DONE AND METHODS INCLUDING SERUM AMYLASE DETERMINATION

Pancreatic function tests are useful for diagnosis of pancreatic disorders. Different pancreatic disorders are acute pancreatitis, chronic pancreatitis, carcinoma of pancreas, mucoviscidosis etc. Different tests done for diagnosis of pancreatic disorders are:

- 1) Serum amylase determination.
- 2) Serum lipase determination.
- 3) Secretin test.
- 4) Sweat testing etc.

Determination of serum amylase is useful for diagnosis of acute pancreatitis. Diagnosis of chronic pancreatitis depends partly on determination of serum and urine amylase. Though serum amylase is elevated in pancreatic carcinoma, it has less diagnostic significance. Serum lipase rises slower than amylase. It remains elevated longer than serum amylase. It is a confirmatory diagnostic test for acute pancreatitis. Elevation of serum lipase during mumps indicates - pancreatic as well as salivary gland infection. Serum lipase determination is not of much diagnostic importance in chronic pancreatitis. Serum lipase is elevated in pancreatic carcinoma also. Tumour of head of pancreas depresses overall volume flow in secretin test. In carcinoma of body of pancreas, 50% of patients show normal volume whereas in carcinoma of tail of pancreas, volume is not affected. Duodenal contents

containing cholesterol crystals or calcium bilirubinate crystals and pus suggest gallstone etiology. Cytological examination of duodenal aspirate is helpful in diagnosis of cancer.

Other tests for diagnosis of pancreatic disorders:

No	Disorder	Test	Condition
1)	Acute “Pancreatitis	Total Leucocyte count	Leukocytosis count upto 30,000 per cubic mm
		Hb estimation	Raised Hb -Heamo concentration.
		Serum lecithinase A, trypsin, deoxyribonuclease.	elevated.
		Serum calcium	Falling
		Blood glucose	Lowered (transient hypoglycaemia)
	Serum bilirubin	Elevated in alcohol related pancreatitis.	
2)	Chronic Pancreatitis and Carcinoma of pancreas	a) Serum Carotenoids b) GTT c) Three day faecal fat determination. d) Gross and microscopic faecal examination. e) 131-I Triolein test. f) D- xylose test	
3)	Mucoviscidosis		Sweat chloride increased, sweat sodium increased.

Serum amylase

Amylases are group of hydrolytic enzymes which cleave polysaccharides like starch and glycogen into simpler sugars ex. maltose.

Determination of serum amylase: It is significant in diagnosis of pancreatic disorders, pancreatic carcinoma, mumps and renal failure.

Name of the method: Amylocalastic / colorimetric / Iodometric.

Principle : Concentration of amylase is determined by its activity on starch to hydrolyse into maltose and dextrins. After incubation, end products are treated with iodine reagent and difference in colour intensity of blue colour is measured. It gives concentration of Amylase.

Specimen: Serum

Requirements :

- 1) Test tubes.
- 2) Serological pipettes.
- 3) Water bath (Thermostatic)
- 4) Photo electric colorimeter.

Reagents:

1) Buffered substrate:

Composition: Anhydrous disodium hydrogen phosphate - 2.66 g.
Benzoic acid - 0.86 g.
Starch - 0.04 g.
Distilled water - up to 100 ml.

Method of preparation:

Dissolve 2.66 g of anhydrous disodium phosphate, 0.86 g of benzoic acid and 0.04 g of starch in 75 ml distilled water and dilute to 100 ml with distilled water.

Stability: This reagent is stable at 2-8°C for 3 months.

2) Stock colour reagent:

Composition: Potassium iodate - 0.36 g.
 Potassium iodide - 4.5 g
 Conc. Hydrochloric acid - 0.9 ml.
 Distilled water up to 100 ml.

Method of preparation:

Dissolve 0.36 g of potassium iodate, 4.5 g of potassium iodide, 0.9 ml of conc. hydrochloric acid in 75 ml of distilled water and dilute to 100 ml with distilled water.

Stability:- This reagent is stable at 2-8° C for one year.

3) Working colour reagent:

Composition:- Stock colour reagent - 10ml.
 Distilled water up to - 100 ml.

Method of preparation:- Dilute 10 ml of stock colour reagent to 100 ml volume with distilled water. This reagent should be made by dilution from stock colour reagent as and when needed.

Specimen: Serum

Wavelength : 660 - nm.

Procedure :

1) Pipette into the tubes labelled as T and B as follows:

S.No.	Reagent	Test	Blank
1)	Buffered substrate	2.5 ml	2.5 ml
	Keep at 37°C for five minutes.		
2)	Serum	0.1 ml	-
	Mix and incubate for 8 minutes at 37°C		
3)	Working colour reagent	2.5 ml	2.5 ml
4)	Serum	-	0.1 ml.
5)	Distilled water	20 ml	20 ml

- 2) Mix thoroughly.
- 3) Read optical densities at 660 nm wave length (Red filter).
- 4) Calculate the concentration with formula.

$$\text{Serum amylase} = \frac{\text{OD of B} - \text{OD of T}}{\text{OD of B}} \times 400$$

Caraway units.

5) If serum value of amylase is more than 400 Caraway units, use 5 times diluted serum as specimen instead of undiluted serum.

6) Multiply the value with 5.

Normal values:- 60 - 180 caraway units/dL.

Serum lipase

Determination of serum lipase is significant in diagnosis of acute pancreatitis, mumps and pancreatic carcinoma etc.

Determination of serum lipase : Method : Cherry and Crandoll

Principle : In this method olive oil as substrate, over night incubation is conducted. Due to the activity of lipase, fatty acids are liberated. These fatty acids are titrated with sodium hydroxide using phenolphthalein as indicator.

Normal values : 1.5 units.

Requirements :

- 1) Test tubes
- 2) Pipettes
- 3) Boiling water bath
- 4) Burette

Reagents:

1) Phosphate buffer - pH-7 :

Composition :

M/3 Di-sodium hydrogen phosphate solution - 100 ml.

M/3 Potassium dihydrogen phosphate solution - 30 ml.

2) Emulsion of olive oil :

Composition :

Olive Oil - 50 ml.
Acacia - 2.5 g.
Water - upto 100 ml.
Sodium benzoate - 0.1 g.

- 3) 95% Ethyl alcohol
- 4) 1% Alcoholic solution of Phenolphthalein.
- 5) 0.05 N Sodium hydroxide solution.

Procedure :

- 1) Take two test tubes.
- 2) Add 3 ml. of distilled water and 1 ml. of patient's serum in each tube
- 3) Transfer one tube to boiling water bath and inactivate lipase by keeping for 5 minutes.
- 4) Add 0.5 ml. of phosphate buffer and 2 ml. of emulsion of oliveoil to each tube.
- 5) Mix and incubate at 27°C for 24 hours.
- 6) Add 3 ml. of 95% ethanol and 2 drops of phenolphthalein indicator to each.
- 7) Titrate with 0.05N NaOH solution until pink shade is obtained.

Calculation: Lipase activity per ml. =

Titration value of unknown in ml. - Volume of control in ml.

Secretin test

Secretin test is conducted by injecting secretin by I.V route and collecting pancreatic secretion with the help of a double lumen tube passed into the duodenum with fluoroscopic guidance. Secretin is given in the dose of one unit per kg of body weight. Aspirate is examined for volume, bicarbonate content and amylase activity.

Contraindication:- Acute pancreatic necrosis.

Sweat testing

Sweat testing is done by pilocarpine iontophoresis and collecting resulting sweat with a filter paper. Sweat absorbed by filter paper is diluted with distilled water and this is quantitatively tested for sodium and chloride.

Normal values:-

Chloride - up to 70 meq/ L in males.

up to 65 meq / L in females.

Summary

Pancreas is a gland with both endocrine and exocrine functions. Endocrine part of secretion of pancreas are insulin and glucagon hormones. Exocrine part of secretion is pancreatic juice, useful in digestive process. Pancreatic function tests help in diagnosis of different pancreatic disorders such as acute pancreatitis, chronic pancreatitis, carcinoma of pancreas etc. Different tests conducted are serum amylase, lipase, secretin test, sweat testing etc.

Essay Questions

- 1) Write about anatomy & physiology of pancreas.
- 2) Write the composition and mention the enzymes of pancreatic juice.
- 3) Describe the mechanism of pancreatic secretion.
- 4) What are different pancreatic function tests? Write the clinical significance of pancreatic function tests.
- 5) Write about determination of serum amylase.
- 6) Describe serum lipase and secretin tests.

Short Answer Questions

- 1) Mention proteolytic enzymes in pancreatic juice.
- 2) What are the peptidases of pancreatic juice and their actions ?
- 3) Mention different phases of pancreatic secretion .
- 4) Exemplify some pancreatic disorders.
- 5) Give examples of pancreatic function tests.
- 6) Write the principle of serum amylase determination by amyloclastic method.
- 7) What is secretin test ?
- 8) Write about sweat testing .
- 9) Write the normal values of sweat chloride in a) Males b) Females .

XI - CLINICAL ENZYMOLOGY

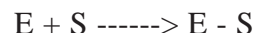
1) BASIC CONCEPTS OF ENZYMES , COENZYMES AND ISOENZYMES

ENZYMES :

Enzymes are biocatalysts which are chemically proteins in nature. They bring about catalytic effect in dilute aqueous solutions at body pH, body temperature etc. This is in contrast to the extreme conditions required for conducting similar type of reactions in a chemical laboratory. They are also characterised by their specificity. Specificity property of enzymes is responsible for avoiding side reactions and there by their by products.

Mechanism of enzyme specificity :

Mechanism of enzyme specificity is believed to be formation of enzyme - substrate complexes.



enzyme substrate enzyme -substrate

This is believed to occur by substrate molecule fitting in to the active site of enzyme in lock and key model. Enzyme specificity is variable for different enzymes. Requirement of a substrate is that it should have a structural feature which binds it to the active site of enzyme.

Factors influencing enzyme action :

- 1) Contact between enzyme and substrate
- 2) Concentration of the substrate
- 3) Concentration of enzyme
- 4) Concentration of product
- 5) Effect of pH
- 6) Effect of temperature
- 7) Effect of oxidising temperature

1) Contact between enzyme and substrate:

Contact between enzyme and substrate is essential for enzyme action to

take place. Enzyme being a protein forms colloidal solution. Hence substrate also must be a water soluble substance.

2) Concentration of substrate:

Keeping the concentration of enzyme constant, enzyme action is directly proportional to the concentration of substrate.

3) Concentration of enzyme:

Enzyme action is directly proportional to the concentration of enzyme.

4) Concentration of product :

Product being accumulated lowers enzyme activity. Thus there exists inverse relation between enzyme and product.

Prompt removal of the product prevents this effect.

5) Effect of pH:

There is optimum pH for maximum activity of any enzyme above and below which enzyme activity declines.

6) Effect of temperature :

There will be optimum temperature for maximum activity of any enzyme above and below which enzyme activity declines or becomes nil.

7) Effect of oxidising substances:

Activity of enzymes depends on some functional group. ex : -SH group present in dehydrogenases and many other enzymes. If such group is oxidised, enzyme activity is lost.

Enzyme inhibition: Enzyme activity is inhibited by certain substances called as enzyme inhibitors and the process is called as enzyme inhibition.

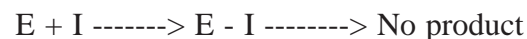
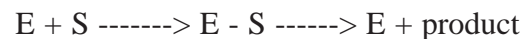
There are two types of enzyme inhibition.

1) Competitive inhibition.

2) Non competitive inhibition.

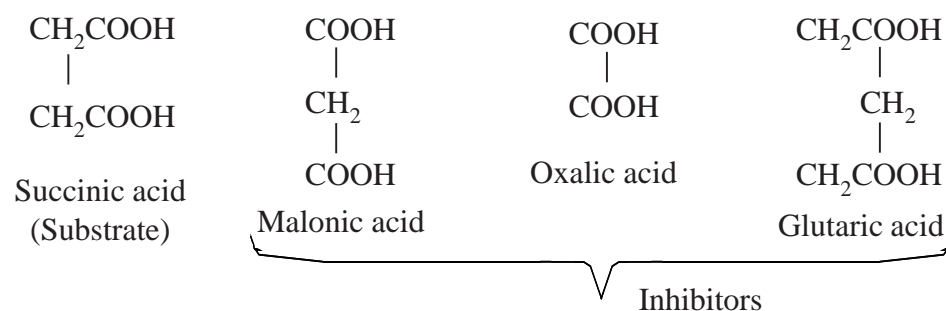
1) Competitive inhibition: Inhibitor substance competes with substrate for the

active site of enzyme. This is due to structural similarity of inhibitor with substrate. This yields enzyme inhibitor complex instead of enzyme-substrate complex. Enzyme - inhibitor complex does not yield any product and prevents further enzyme activity.



It can be reversed by enhancing the concentration of substrate.

Ex: Succinate dehydrogenase is an enzyme whose substrate is succinic acid. Malonic acid, oxalic acid and glutaric acid with structural similarity to succinic acid can inhibit the activity of the enzyme succinate dehydrogenase.



Non -Competitive inhibition: In this type, inhibition can not be reversed by increasing the concentration of substrate.

Classification of enzymes:

Enzymes are classified as per the reaction, catalysed by them.

They are -

- 1) Oxido-reductases.
- 2) Transferases.
- 3) Hydrolases.
- 4) Lyases.
- 5) Isomerases.

6) Ligases (Synthetases)

1) Oxido-reductases :

This class of enzymes bring about oxidation / reduction reactions.

Ex: Succinate dehydrogenase

Amino acid oxidase etc.

2) Transferases:

This class of enzymes bring about transfer of a group from one substrate to another substrate.

Ex: SGOT, SGPT, Hexokinase

3) Hydrolases :

This class of enzymes bring about hydrolysis reaction.

Ex: Amylase.

4) Lyases:

This class of enzymes bring about removal of groups.

Ex: Aldolase, Fumarase, Pyruvate decarboxylase etc.

5) Isomerases:

This class of enzymes bring about conversion of isomers into other forms.

Ex: Triose phosphate isomerase.

Glucose Phosphate isomerase etc.

6) Ligases:

This class of enzymes bring about linking of two compounds together.

Ex: Glutamine synthetase.

Succinate thokinase.

Acetyl Co-A carboxylase etc.

Nomenclature of enzymes :

1) Enzymes are named by adding 'ase' as suffix to the name of the substrate on which they act.

<u>Name of substrate</u>		<u>Enzyme</u>
Lipid	-	Lipase
Amylum	-	Amylase
Sucrose	-	Sucrase.
Maltose	-	Maltase
Protein	-	Protease

2) They are also named by adding 'ase' as suffix to the activity brought about by them.

Isomerisation	-	Isomerases
Decarboxylation	-	Decarboxylases
Transamination	-	Transminases
Dehydrogenation	-	Dehydrogenases

3) Some enzymes are named without any relation to name of substrate or activity brought about by them.

Ex: Pepsin, Trypsin, Chymotrypsin, Renin etc.

Units of measurement of enzyme activity :

Widely used unit of enzyme activity is the amount which transforms 1 micro mol. of substrate per minute, temperature maintained at 25°C and thus maintaining optimal conditions of measurement.

Katal (Kat) : It is the amount of enzyme activity transforming 1 mol. S⁻¹ of substrate. It is the new international unit for enzyme activity.

Microkatal (m Kat) : It is 10⁻⁶ times the activity of Katal.

Nanokatal (nKat) : It is 10⁻⁹ times the activity of Katal.

Picokatal (pKat) : It is 10⁻¹² times the activity of Katal.

CO-ENZYMES :

Co-enzymes are organic cofactors.

Cofactors : Some enzymes depend on one or more non protein compounds called as cofactors.

Principal coenzymes and the enzymatic reactions in which they participate are as follows.

- 1) Nicotinamide adenine dinucleotide (NAD), Nicotinamide adenine dinucleotide Phosphate (NADP), Flavin mononuceotide (FMN), Flavin adenine dinucleotide (FAD), Coenzyme P participate in transfer of hydrogen atoms (electrons).
- 2) Thiamine pyrophosphate (TPP) participates in transfer of aldehydes.
- 3) Co enzyme A and Lipoamide participate in transfer of acyl groups.
- 4) Cobamide Co enzymes participate in transfer of alkyl groups.
- 5) Biocytin transfers carbon dioxide.
- 6) Pyridoxal phosphate (PP) transfers aminogroups.
- 7) Tetra hydro folate coenzymes transfer methyl, methylene, formyl or formimino groups.

Each of these coenzymes contain a molecule of vitamin. Vitamins are organic substances required in trace quantities and are vital for functioning of all cells.

Coenzyme forms of Vitamins :

Vitamin	Co enzyme form
1) Thiamin	Tiamin pyrophosphate (TPP)
2) Riboflavin	Flavin mononucleotide (FMN)
3) Nicotinic acid	Nicotinamide adenine dinucleotide (NAD) Nicotinamide adenine dinucleotide Phosphate (NADP)
4) Pantothenic acid	Coenzyme A
5) Pyridoxine	Pyridoxal phophate
6) Folic acid	Tetra hydrofolic acid
7) Vitamin B ₁₂	Coenzyme B ₁₂

- 8) Vitamin-A 11 Cis-Retinal
 f) Vitamin-D 1, 25 Dihydroxycholecalciferol.

ISOENZYMES :

Iso-enzymes are different forms of same enzyme having same enzymatic activity . They are from different sources . They may differ in physical , biochemical and immunological properties. They also differ in molecular weights and hence their electrophoretic migration rates also differ . They also differ in their stability to denaturation by heat , resistance to chemical binding . They also differ in affinity for substrates and coenzymes .

Examples : 1) Lactate dehydrogenase has five forms they are LD1, LD2 , LD3 , LD4 , LD5 . Out of them , LD1 is the fastest in migration towards anode and LD2 - LD5 are slow successively .

<u>Isoenzyme</u>	<u>Source</u>
LD1	Cardiac muscle (Rich source)
LD2	Kidneys (Rich source)
LD3 & LD4	Cardiac muscle , liver , spleen , pancreas and leukocytes
LD5	Liver (Rich source)

2)Creatine phospho kinase has three forms . It is a dimer . Two units of CPK recognised are M (Muscle) and B (Brain).

<u>Isoenzyme</u>	<u>Units</u>
Skeletal muscle CPK	MM
Brain CPK	BB
Cardiac muscle CPK	MB

3) Source of alkaline phosphatase is mostly liver in adults .In children it is a mixture of liver and bone enzymes .Other forms originate from kidney , small intestine and placenta .

Alkaline phosphatase has three isoenzymes.They are-

- 1) AP-1, Alpha -2
 - 2) AP-2, Beta -1
 - 3) AP-3, Beta-2
- 1) AP-1, Alpha -2 is heat labile and is produced in liver and by proliferating blood vessels.
- 2) AP-2, Beta-1 is heat stable and is produced by bone and placenta.
- 3) AP-3, Beta-2 is present in group ‘O’ and ‘B’ individuals.

2) IMPORTANCE OF ENZYMES

Medicinal and pharmaceutical significance:

- 1) Sulphonamides kill pathogenic organisms by inhibiting folic acid synthetase enzyme.
- 2) Allopurinol, used in the treatment of gout acts by inhibiting the enzyme xanthine oxidase.
- 3) Enzyme asparaginase is used in the treatment of tumours.
- 4) Enzyme Galactosidase is used in the treatment of lactose intolerance in children.
- 5) Pencillin acylase is used in manufacture of 6-amino penicillanic acid.
- 6) Aminoacylase is used in preparation of alpha-amino acid.
- 7) Glucose isomerase is used in manufacture of fructose syrup.
- 8) Papain is used in the production of protein hydrolysate.
- 9) Amylase is also used in manufacturing process.

Diagnostic significance:

I. Determination of enzymes useful in diagnosis of various conditions:

Determination of different enzymes is useful in diagnosis of various conditions.

Some of them are -

- 1) Determination of lipase in plasma is useful in diagnosis of pancreatitis, pancreatic carcinoma, liver disease, diabetes miletus and vit-A defficiency. Values are elevated in pancreatitis and pancreatic carcinoma whereas they are decreased in liver disease, vit-A defficiency and diabetes miletus.
- 2) Trypsin is elevated in pancreatic disease.
- 3) Low plasma levels of cholinesterase are seen in liver disease, malnutrition and anaemia. Infection in cardiac muscle causes lowering of plasma activity of this enzyme.
- 4) Determination of LDH is significant for diagnosis of myocardial infarction.
- 5) Serum acid phosphatase is elevated in males with prostatic cancer with metastases. Its elevation is moderate in paget's disease and hyperpara thyroidism.
- 6) Serum alkaline phosphatase is elevated moderately in hepatic conditions. Elevation is more marked in post hepatic conditions. In paget's disease, elevation of serum alkaline phosphatase is high. In osteomalacia, elevation of serum ALP is moderate. In rickets, this rise is two to four times the normal, which returns to normal after treatment with vitamin D. Elevations of serum ALP are very high in bone cancer. In hyperparathyroidism, elevation of this enzyme is slight to moderate.
7. SGOT is increased rapidly in myocardial infarction. It starts increasing by the second day and reaches peak value by fifth day. It reaches normal by eighth day of infarction.
8. SGPT is slightly elevated in cardiac necrosis. Its elevation is high in both hepatic and post hepatic conditions.
9. Determination of CPK (creatine phosphokinase) is significant in diagnosis of

myocardial infarction. CPK is elevated in myocardial infarction, muscular dystrophy, polymyositis, motor neurone disorders and acute cerebrovascular accidents.

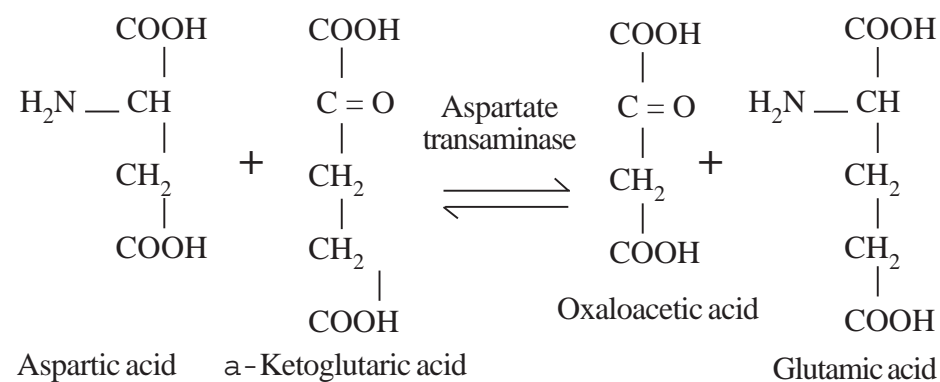
II. Use of enzymes as reagents:

Enzymatic methods are advantageous owing to their reasonable cost, time saving, avoidance of corrosive and multiple reagents.

- Ex: 1) Glucose oxidase and peroxidase enzymes are used in the determination of glucose by glucose oxidase peroxidase (God-Pod) method.
- 2) Uricase is used in enzymatic method of determination of uric acid.
 - 3) Urease is used in enzymatic method of determination of urea.
 - 4) Lipase, Glycerol kinase, Glycerol Phosphate oxidase and peroxidase are used in Enzymatic method of determination of triglycerides.
 - 5) Cholesterol ester hydrolase, cholesterol oxidase and peroxidase are used in enzymatic method of determination of cholesterol.

3) TRANSAMINASES

Transaminases are the enzymes which catalyse the transamination reaction. Transamination is the enzymatic process of transfer of alpha-amino group of an amino acid (under going catabolism by the process of transamination) to the alpha-carbon atom of an alpha-Keto acid, leaving behind corresponding alpha-keto acid analog of the amino acid. In most cases, alpha-keto acid transferring the alpha-amino group is alpha-keto glutaric acid.



About 12 amino acids undergo 1st stage of catabolism (removal of alpha-amino group) by the process of transamination. They are Alanine, Arginine, Asparagine, Aspartic acid, Cysteine, Isoleucine, Leucine, Lysine, Phenyl alanine, Tryptophan, Tyrosine and Valine. Enzyme catalysing the transfer of α -amino group of Aspartate to α -keto glutarate is Aspartate transaminase (Glutamate oxaloacetate transaminase-GOT). This enzyme is prominent transaminase in the tissues of animals. Some other transaminases present in animal tissues are alanine transaminase (Glutamate pyruvate transaminase -GPT), Leucine transaminase and Tyrosine transaminase. Transaminases are found both in mitochondria and in cytosol of eukaryotic cells. Transamination reactions are freely reversible. Glutamate is the end product of most transamination reactions. It serves as amino group donor in final series of reactions leading to formation of nitrogenous excretory products. All the transaminases have same prosthetic group pyridoxal phosphate and share a common reaction mechanism.

Determination of transaminases is clinically significant in diagnosis of liver dysfunction and myocardial infarction. Serum transaminase activity is increased in liver dysfunction. Greater activity of GOT over GPT is typically indicative of myocardial infarction.

Determination of serum glutamate oxaloacetate transaminase - SGOT (Aspartate transaminase):

Name of the method : Reitman & Frankel method.

Principle: Aspartate transaminase catalyses the reaction.

Aspartic acid + α -ketoglutaric acid \rightleftharpoons oxaloacetic acid + glutamic acid

GOT enzyme present in serum catalyses the reaction between aspartic acid and α -keto glutaric acid in alkaline medium provided by NaOH and buffered by phosphate buffer and yields oxaloacetic acid. Oxaloacetic acid on reaction with dinitrophenyl hydrazine produces hydrazone. Which is highly coloured. Colour intensity of hydrazone is directly proportional to the concentration of GOT enzyme in serum.

Requirements:

- 1) Test tubes

- 2) Serological pipettes
- 3) Thermostatic water bath
- 4) Stop watch
- 5) Photo electric colorimeter

Reagents:

- 1) GOT substrate:

Composition: Aspartic acid - 2.66 g.

α -Keto glutaric acid - 30 mg.

1-N Sodium hydroxide in phosphate buffer - 20 ml.

Phosphate buffer (7.45 pH and M/15 conc.) - up to 100 ml.

Method of Preparation :

Dissolve aspartic acid, α -ketoglutaric acid in about 60 ml. of phosphate buffer.

Add 1 N Sodium hydroxide and make the volume to 100 ml. with phosphate buffer.

Stability of the reagent: This reagent is stable at 2-8°C.

- 2) DNPH Colour developer :

Composition:

2,4 Dinitrophenyl hydrazine - 200 mg.

Concentrated hydrochloric acid - 85 ml.

Distilled water - upto 1000 ml.

Method of preparation :

Dissolve 200 mg. of 2, 4 DNPH and 85 ml. conc. HCl in about 700 ml. of distilled water and dilute to 1 litre with distilled water.

Stability : This reagent is stable at 2-8°C.

- 3) 0.4 N Sodium hydroxide solution:

Composition:

Sodium hydroxide - 16 g.

Distilled water - upto 1 litre.

Method of preparation:

Dissolve 16g. of sodium hydroxide in about 750 ml. of distilled water. Dilute to 1 litre with distilled water. Standardise and adjust normality if necessary.

Stability : It is stable for several months at room temperature.

4) Sodium pyruvate stock standard (220 mg./dl conc.)

Sodium pyruvate - 220 mg.

Phosphate buffer - upto 100 ml.

Method of preparation: Dissolve 220 mg. of sodium pyruvate in 75 ml. of phosphate buffer and dilute to 100 ml. with phosphate buffer.

5) Pyruvate working standard :

Composition:

Pyruvate stock standard-1 part

Pyruvate working standard-upto 10 parts.

Method of preparation:

Dilute 1 part of pyruvate stock standard to 10 parts with phosphate buffer.

Stability: This reagent is stable at 2-8°C.

6) Phosphate buffer : (pH - 7.45, conc-M/15)

Composition :

a) Disodium hydrogen phosphate dihydrate solution -210 ml.

b) Mono potassium dihydrogen phosphate anhydrous solution- 40 ml.

Method of Preparation :

Add 40 ml. of Monopotassium dihydrogen phosphate solution to 210 ml. of Disodium hydrogen phosphate dihydrate solution.

a) Disodium hydrogen phosphate dihydrate solution:

Composition:

Disodium hydrogen phosphate dihydrate - 4.45 g.

Distilled water upto 250 ml.

Method of Preparation:

Dissolve 4.45 g of disodium hydrogen phosphate dihydrate in about 180 ml. of water and dilute to 250 ml. with water.

b) Mono potassium dihydrogen phosphate solution :

Composition: Mono potassium dihydrogen phosphate anhydrous - 1.36 g.

Distilled water upto - 100 ml.

Method of Preparation :

Dissolve 1.36 g. of Monopotassium dihydrogen phosphate anhydrous in about 75 ml. of water and dilute to 100 ml. with distilled water.

Procedure :

1) Take test tubes labelled as Test and Blank and Pipette into them as follows.

	Test	Blank
1. GOT substrate	0.5 ml.	0.5 ml.
Incubate for 5 minutes at 37°C		
2. Serum	0.1 ml.	-
Incubate for 60 minutes at 37°C		
3. DNPH colour developer	0.5 ml.	0.5 ml.
4. Serum	0.1 ml.	-
Mix thoroughly and keep at room temperature for 2 minutes		
5. 0.4 N NaOH solution	5 ml	5 ml.

2) Mix thoroughly.

- 3) Keep at room temperature for 10 minutes.
- 4) Read the O.D.s of 'T' against blank using photo electric colourimeter at 540nm wavelength.
- 5) Calculate the conc. of test by referring to a standard graph.

Procedure to prepare standard graph :

a) Pipette in to tubes as follows .

- | | | | | | |
|---------------------------------|----------|---------|----------|---------|---------|
| 1) GOT substrate | 0.45 ml. | 0.4 ml. | 0.35 ml. | 0.3 ml. | 0.5 ml. |
| 2) Sod. pyruvate (standard) | 0.05ml | 0.1 ml. | 0.15 ml. | 0.2 ml. | - |
| 3) Distilled water | 0.1 ml. | 0.1 ml. | 0.1 ml. | 0.1 ml. | 0.1 ml. |
| 4) 2,4 D.N.P.H colour developer | 0.5 ml. | 0.5 ml. | 0.5 ml. | 0.5 ml. | 0.5 ml. |

Mix and keep at R.T. for 20 minutes.

- | | | | | | |
|----------------|-------|-------|--------|--------|--------|
| 5) 0.4 N NaOH | 5 ml | 5 ml | 5 ml | 5 ml | 5 ml |
| Concentrations | 27 KU | 61 KU | 114 KU | 190 KU | 0 K.U. |

b) Mix the above and read the O.D.s of S₁, S₂, S₃ and S₄ against blank

c) Prepare graph by plotting O.D. values on Y-axis and concentrations on X-axis.

- 6) If the determined value is more than 190, dilute the serum 10 times and multiply the value with 10.

Normal Values: 8-40 Karamen units.

Determination of SGPT : (Alanine transaminase):

Name of the method: Reitman & Frankel

Principle:

Alanine transaminase catalyses the reaction. Alanine + a-ketoglutaric acid ⇌ pyruvic acid + glutamic acid.

GPT enzyme present in serum catalyses reaction between Alanine and a-keto glutaric acid in alkaline medium provided by sodium hydroxide and buffered by phosphate buffer yielding pyruvic acid .Pyruvic acid on reaction with

Dinitrophenyl hydrazine produces hydrazone, which is highly coloured. Colour intensity of hydrazone is directly proportional to the concentration of GPT enzyme in serum.

Requirements: Same as for GOT determination.

Reagents :

1) GPT substrate :

Composition :

- | | | |
|------------------------------|---|--------------|
| Alanine | - | 1.78 g. |
| a-Keto glutaric acid | - | 30 mg. |
| 1N NaOH in Phosphate buffer- | | 0.5 ml. |
| Phosphate buffer | - | upto 100 ml. |

(7.45 pH and M/15 conc.)

Method of Preparation:

Dissolve Alanine, a-keto glutaric acid in about 75 ml. of phosphate buffer. Add 1N NaOH and make the volume to 100 ml. with phosphate buffer.

Adjust pH to 7.45

Stability of the reagent :

This reagent is stable at 2-8°C

Reagents : 2,3, 4,5 and 6 same as for GOT determination.

Specimen : Serum

Wave length : 540 nm (green filter)

Procedure :

- 1) Take test tubes labelled as Test and Blank and pipette into them as follows.

S.No.	Reagent/sample	Test	Blank
1.	GPT substrate	0.5 ml.	0.5 ml.

Incubate at 37°C for 5 minutes.

2. Serum	0.1	–
Incubate at 37°C for 30 minutes.		
3. DNPH colour developer	0.5 ml.	0.5 ml.
4. Serum	-	0.1 ml.
Mix thoroughly and keep at room temperature for 20 minutes.		
5. 0.4 N NaOH solution	5 ml	5 ml.

- 2) Mix thoroughly.
- 3) Keep at room temperature for 10 minutes.
- 4) Read the O.D. of 'T' against blank using photoelectric colorimeter.
- 5) Calculate the concentration of test by referring to a standard graph prepared as follows.

a) Pipette into the tubes as follows:

S.No.	S ₁	S ₂	S ₃	S ₄	Blank
1) SGPT substrate	0.45 ml.	0.4 ml.	0.35 ml.	0.3 ml.	0.5 ml.
2) Std. Sodium pyruvate solution.	0.05 ml.	0.1 ml.	0.15 ml.	0.2 ml.	-
3) Distilled water	0.1 ml.	0.1 ml.	0.1 ml.	0.1 ml.	0.1 ml.
4) DNPH colour developer	0.5 ml.	0.5 ml.	0.5 ml.	0.5 ml.	0.5 ml.
Mix thoroughly and keep at room temperature for 20 minutes					
5) 0.4N NaOH	5 ml.	5 ml.	5 ml.	5 ml.	5 ml.
Concentration,	28KA	57KA	97KA	150KA	0KA

- b) Mix above and read the O.Ds of S₁, S₂, S₃ and S₄ against blank.
- c) Prepare graph by plotting O.D. values on Y-axis and concentrations on X-axis.
- 6) If the determined value is more than 150, dilute serum 10 times and multiply the value with 10.
Normal values: 5-35 K.U.

4) CARDIAC ENZYMES

Enzymes of significance for diagnosis of cardiac conditions are called as cardiac enzymes. GOT, CPK (Creatine phosphokinase), LDH (Lactate dehydrogenase) and SHBD are enzymes of significance in diagnosis of myocardial infarctions. Their determinations constitute cardiac injury panel tests. CPK values raise 4 to 6 hours after infarction. They reach peak in 24 to 36 hours. GOT values start raising at this time and reach peak values in 2 to 3 days. They stay elevated for about 14 days. CPK and GOT levels do not alter in other cases of chest pain as caused in pneumonia, pleurisy and pulmonary infarction etc.

LDH may raise in such conditions. Combination of LDH - CPK isoenzyme analysis by electrophoresis gives greater accuracy in diagnosis of myocardial infarction. Elevation of SHBD is more specific for diagnosis of myocardial infarction than either LDH or GOT. It also remains elevated for a longer period after infarction.

Determination of SGOT :

Refer to Transaminases.

Determination of CPK :(Creatine Phosphokinase):

Name of the method : Colorimetric method.

Principle :

CPK enzyme catalyses the reactions



Creatine formed in this reaction reacts with diacetyl and α -naphthol in presence of alkaline medium, giving coloured compound. Colour intensity is proportional to the concentration of enzyme.

Requirements:

- 1) Test tubes
- 2) Graduated pipettes
- 3) Thermostatic water bath

4) Photoelectric colorimeter.

Reagents:

1) Tris buffer (conc. 0.1 M, pH - 7.4)

Composition :

Tris (Hydroxymethyl) amino methane - 12.1 g.

Magnesium sulphate heptahydrate - 3.7 g.

Distilled water - upto 1 litre

Method of preparation :

a) Dissolve 12.1 g. of Tris (hydroxymethyl) amino methane and 3.7 g. of magnesium sulphate heptahydrate in approximately 600 ml. of distilled water.

b) Adjust volume to 1 litre with distilled water.

c) Adjust pH to 7.4

Stability : This reagent is stable at 2-8 °C

2. Buffer-Cysteine solution:

Composition:

Cysteine - 0.7 g.

Tris Buffer - 100 ml.

Method of preparation:

Dissolve 0.7 g. cysteine in enough quantity of Tris buffer and dilute to 100 ml. with tris buffer.

Stability: This reagent should be prepared freshly on the day of use.

3. Phosphocreatine solution (0.012 M) (pH-7.4):

Composition :

Phosphocreatine - 0.25 g.

Distilled water - upto 100 ml.

Method of preparation :

Dissolve 0.25 g. of Phosphocreatine in 75 ml. of distilled water, adjust pH to 7.4 and dilute to 100 ml. with distilled water.

Stability : This reagent is stored in refrigerator and stable for two weeks. Hence it should be prepared freshly every two weeks.

4. Adenosine diphosphate solution :

Composition :

Distilled water upto 100 ml.

Method of Preparation :

Dissolve 0.17 g. of ADP in about 75 ml. of distilled water and dilute to 100 ml. with distilled water.

Storage: Store the solution frozen.

5. Substrate solution:

Composition:

Buffered cysteine solution - 1 part

Phosphocreatine solution - 1 part

ADP solution - 1 part

Distilled water - 1 part

Method of preparation :

Mix equal volumes of buffered cysteine solution, phosphocreatine solution, ADP solution and distilled water.

Storage : Store the solution in refrigerator.

6. P-Chloromercuri benzoic acid:

Composition:

P-Chloromercuri benzoic acid - 1.1g

1 N NaOH - 25 ml.

1N HCl - quantity sufficient to form precipitate.

Distilled water upto 100 ml.

Method of preparation:

Dissolve 1.1g of P-chloromercuri benzoic acid in 25 ml. of 1N NaOH. Add 1N HCl until precipitation takes place. Dilute to 100 ml. with distilled water. Solution becomes clear on dilution to 100 ml.

Stability : this reagent is stable at room temperature for several months.

7. Barium hydroxide solution (4.5% w/v) :

Composition :

Barium hydroxide octahydrate - 45 g.

Distilled water upto 1 litre

Method of preparation:

Dissolve 45 g. of barium hydroxide octahydrate in 750 ml. of distilled water and dilute to 1 litre with distilled water.

Note: Equal volumes of barium hydroxide and zinc sulphate solutions should neutralize each other.

8. Zinc sulphate solution (5% w/v) :

Composition :

Zinc sulphate heptahydrate - 50 g.

Distilled water - upto 1 litre

Method of preparation :

Dissolve 50 g. of zinc sulphate heptahydrate in 750 ml. of distilled water and dilute to 1 litre with distilled water.

9. Stock solution of alkali :

Composition :

Sodium hydroxide - 6 g.

Sodium carbonate - 12.8 g.

Distilled water upto- 1 litre

Method of preparation :

Dissolve 6 g. of sodium hydroxide and 12.8 g. of sodium carbonate in 75 ml. of distilled water and dilute to 1000 ml. with distilled water.

Stability : This reagent is stable at room temperature for several months.

10. a-Naphthol solution :

Composition :

a-Naphthol - 0.8 g.

Stock alkali - upto 10 ml.

Method of preparation :

Dissolve 0.8 g. of a-Naphthol in 7 ml. of stock alkali and dilute to 10 ml. with stock alkali.

Stability : It should be prepared fresh just before use.

11. Creatine standard (1.7 mmol. / ml.) :

Composition :

Creatine : 11 - 15 mg.

Distilled water upto 50 ml.

Method of preparation :

Dissolve 11.15 mg. of creatine in about 35 ml. of distilled water and dilute to 50 ml. with distilled water.

Stability : Store in frozen condition in 1 ml. aliquots. This solution is stable for about one month.

Sample : Serum

Wavelength : 540 nm.

Procedure:

1) Take three tubes and label them as "T" "S" and "B".

2) Pipette into them as follows.

S.No. Reagent / Sample	T	S	B
1. Substrate (in ml.)		1	1

Keep the tubes at 37°C in thermostatic water bath.

2. Serum (in ml.)		0.1	-
3. Standard (in ml.)		-	0.1
4. Distilled water (in ml.)	-	-	0.1

Continue in thermostatic water bath at 37°C for 30 minutes

5. P-Chloromercuri benzoic acid (in ml.)	0.5	0.5	0.5
6. Barium hydroxide solution (in ml.)	0.5	0.5	0.5
7. Zinc sulphate solution	0.5	0.5	0.5

Centrifuge covering the tubes with plastic cap at 2500 RPM for 15 minutes

3) Take supernatants of above tubes into respectively labelled tubes (0.5 ml. each) and treat them as follows

	T	S	B
1. α-Naphthol (in ml.)	1	1	1

Keep the tubes in water bath at 37°C for 20 minutes.

2. Distilled water (in ml.)	2.5	2.5	2.5
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Centrifuge for few minutes.

4) Take supernatants of above tubes into respectively labelled tubes and read O.D. values of 'T' and 'S' against 'B'.

5) Calculate the concentration of enzyme in the specimen using the formula.

Concentration (activity) of CPK =

$$\frac{\text{O.D. of Test}}{\text{O.D. of standard}} \times \frac{1}{30} \times 0.17 \times \frac{1000}{0.1} \text{ units / L}$$

1/30 = Conversion to 1 minute

1000/0.1 = Conversion to 1 litre of serum

Normal Values:

Men : 20 -50 I.U.

Women : 10-37 I.U.

Determination of Lactate dehydrogenase:

Name of the method: Colorimetric method

Principle :

LDH enzyme catalyses the reaction.



2,4 Dinitro phenyl hydrazine reacts with pyruvate in the alkaline medium giving brown coloured 2,4 dinitro phenyl hydrazone. Colour intensity is proportional to the concentration of pyruvate which gives measure of LDH concentration.

Requirements:

- 1) Test tubes
- 2) Serological pipettes
- 3) Thermostatic water bath
- 4) Photoelectric colorimeter

1) Substrate solution :

Composition :

Glycine buffer - 120 ml.

0.1 N Sodium hydroxide solution - 20 ml.

35% w/v Sodium lactate solution - 10 ml.

Method of preparation :

Take 120 ml. of glycine buffer, 20 ml. of 0.1 N sodium hydroxide solution

and 10 ml. of 35% w/v sodium lactate solution in 250 ml. volumetric flask. Dilute to 250 ml. Adjust pH to 10 with 0.1N NaOH solution.

Storage : Store in a refrigerator.

2) Glycine buffer (0.1M) :

Composition :

Glycine - 3.753 g.

Sodium chloride (A.R) - 2.922 g.

Distilled water - upto 500 ml.

Method of preparation:

Dissolve 3.753 g. of glycine and 2.922 g. of sodium chloride (A.R) in about 350 ml. of distilled water and dilute to 500 ml. with distilled water.

Storage: Store this solution in a refrigerator.

3) 35% w/v Sodium lactate solution :

Composition :

Lactic acid (AR) - 52 ml.

Sodium hydroxide - 21.5 g.

Distilled water - upto 200 ml.

Method of preparation :

React 52 ml. of lactic acid and 21.5 g. of sodium hydroxide dissolved in 120 ml. of distilled water by boiling for about 30 minutes. Avoid bumping using broken glass pieces. Cool and make the volume to 200 ml. with distilled water.

Storage : Store the solution in a refrigerator.

4) 0.1N sodium hydroxide solution :

Composition :

Sodium hydroxide - 0.4 g.

Distilled water - upto 100 ml.

Method of preparation :

Dissolve 0.4 g. of sodium hydroxide in about 75 ml. of distilled water and dilute to 100 ml. with distilled water. Standardise the solution and adjust normality if necessary.

Stability : This reagent is stable at room temperature for several months.

5) NAD Solution :

Composition :

Nicotinamide adenine dinucleotide - 10 mg.

Distilled water - 2 ml.

Method preparation:

Dissolve 10 mg of NAD in 2 ml. of distilled water.

Storage : Store this solution in refrigerator. Store solid NAD also in refrigerator.

6) Sodium pyruvate concentrated solution:

Composition :

Sodium pyruvate - 220 mg.

Glycine buffer upto 100 ml.

Method of preparation:

Dissolve 220 mg. of sodium pyruvate in 75 ml. of glycine buffer and dilute to 100 ml. with glycine buffer.

7) Sodium pyruvate working standard solution (1mmol/L) :

Composition :

Sodium pyruvate concentrated solution - 5 ml.

Glycine buffer - upto 100 ml.

Method of preparation:

Dilute 5 ml of sodium pyruvate concentrated solution to 100 ml. with glycine buffer.

Stability : This reagent is stable at 2-8°C.

8) 2,4 dinitro phenyl hydrazine :

Composition:

2,4 DNPH - 200 mg.

Conc. HCl - 85 ml.

Distilled water upto 1 litre.

Method of preparation :

a) Dilute 85 ml. of conc. HCl to about 750 ml. with distilled water b) Dissolve 200 mg. of 2,4 DNPH in this diluted acid solution. c) Dilute the solution prepared in step (b) to 1000 ml. with distilled water.

Stability : This reagent is stable at 2-8°C.

9) 0.4 N Sodium hydroxide solution :

Composition :

Sodium hydroxide - 1.6 g.

Distilled water upto 100 ml.

Method of preparation :

Dissolve 1.6 g. of sodium hydroxide in 75 ml. of distilled water and dilute to 100 ml. with distilled water. Standardise and adjust normality if necessary.

Stability : This reagent is stable at room temperature for several months.

Sample : Serum

Note: Red cells contain 150 times more activity of LDH than in serum. Hence haemolysed serum should not be used for LDH determination.

wavelength : 505-510 nm (green filter)

1. Label two tubes as 'T' and 'B' and pipette into them as follows:

S.No.	Reagent / sample	T	B
1)	Substrate solution	1 ml.	1 ml.
2)	NAD solution	0.2 ml.	0.2 ml.

Keep in a water bath at 37°C for five minutes.

	T	B
3. Serum	0.1	-
4. After 15 minutes of incubation at 37°C.		
2,4 DNPH	1.0 ml.	1.0 ml.
5. Serum	-	0.02 ml.

After 15 minutes incubation at room temperature,

6. 0.4 N NaOH	10 ml.	10 ml.
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2. Mix thoroughly and read O.D. value of T and B against water.

3. Subtract O.D. of serum blank from O.D. of test.

4. Determine O.D. of standard as follows.

a) Take two tubes and label them as 'S' and 'B'

b) Pipette into them as follows.

S.No.	Reagent	S	B
1)	Substrate	1 ml.	1 ml.
2)	Water	0.2 ml	0.3 ml.
3)	Pyruvate standard	0.1 ml.	-
4)	DNPH solution	1 ml.	1 ml.
Keep in 37°C for 15 minutes.			
5)	0.4 N NaOH	10 ml.	10 ml.

Determine O.D. of 'S' against blank.

5. Calculate with the formula

$$\text{Activity of LDH in serum} = \frac{\text{O.D. of T} - \text{O.D. of B}}{\text{O.D. of 'S'}} \times 333 \text{ U/L}$$

6. If enzyme activity is more than 1000 U/L, dilute the specimen and multiply the determined value with dilution factor.

Note: 1) Do not handle the specimen improperly which can inactivate the enzyme.

2) Do not resort to repeated freeze thawing which will cause harm to enzyme.

Normal Values :

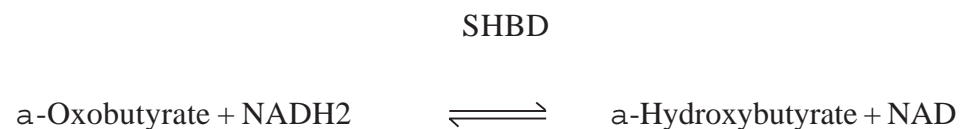
Adults - 70-240 U/L

Children - 150 - 590 U/L

Determination of SHBD (Serum Hydroxybutyrate - dehydrogenase):

Name of the method : UV Kinetic method :

Principle : SHBD enzyme catalyses the reaction between 2 oxobutyrate and NADH₂ to yield 2 hydroxybutyrate and NAD. In this reaction, NADH is dehydrogenated to NAD by the action of this enzyme. Measure of difference in absorbance gives the activity of the enzyme.



Reagents :

1) Substrate solution (pH - 7.45):

Composition :

Sodium salt of alpha oxobutyric acid - 124 mg.

Phosphate buffer - up to 1 litre.

Method of preparation :

It is prepared by dissolving 124 mg. of sodium salt of 2 oxobutyric acid in about 750 ml. of water and diluting to 1 litre with phosphate buffer.

Stability : This reagent is stable at 2-8°C.

2) Phosphate buffer (pH = 7.45)

Composition and method of preparation:

Refer to SGOT determination

3) NADH₂ solution :

Composition

NADH₂ - 0.017 g.

Distilled water- upto 100 ml.

Method of preparation:

It is prepared by dissolving 0.017 g. of NADH₂ in 75 ml. of distilled water and diluting to 100 ml. with distilled water.

Stability : This reagent is stable at 0-4°C for one week.

Sample : Serum

General system parameters:

Reaction type : Kinetic

Wavelength : 340 nm

Path length : 1 cm.

Flow cell temperature : 30°C

Reagent volume : 3.1 ml.

Sample volume: 0.1 ml.

Procedure :

- 1) Pipette into a cuvette as follows-
 - Substrate 3 ml.
 - NADH₂ solution 0.1 ml
 - serum - 0.1 ml.
2. Mix well. Read absorbance immediately and also at 1 minute intervals three more times.
3. Determine mean absorbance change.
4. Calculate activity of enzyme with formula

$$\text{ALPHA HBDH} = 5079 \times \text{mean absorbance change I.U.}$$
5. If mean absorbance change exceeds 0.1, dilute serum 10 times and multiply the determined value with dilution factor i.e. 10.

5) ACID PHOSPHATASE

Phosphatases are the enzymes catalysing the hydrolysis of esters of different organic phosphates such as phenyl phosphate, p-nitro phenyl phosphate and sodium beta-glycerophosphate. Phosphatases are group of related enzymes. Clinically identified phosphatases are-

- 1) Acid phosphatase
- 2) Alkaline phosphatase and
- 3) Red cell phosphatase

Two isoenzymes of Acid phosphatase are known. Optimum pH is 4.9.

Determination of serum acid phosphatase :

Name of the method: PNP method

Principle : Acid phosphatase enzyme acts on paranitrophenyl phosphate and breaks it down to paranitrophenol. p -Nitrophenol is colourless. It turns yellow in alkaline medium. Colour intensity is proportional to the concentration of the enzyme in serum.

Requirements :

- 1) Test tubes
- 2) Serological pipettes
- 3) Thermostatic water bath
- 4) Stop watch
- 5) Photo electric photometer

Reagents:

- 1) Citrate buffer (pH - 4.9)

Composition :

- Citric acid, monohydrate - 18.91 g.
- 1 N. NaOH - 180 ml.
- 0.1 N. HCl - 100 ml.
- Distilled water- upto 1 litre.

Method of preparation :

Mix 18.91 g. of citric acid, 180 ml of 1 N. NaOH and 100 ml. of 0.1 N.HCl to 500 ml. of distilled water and dilute to 1 litre with distilled water. Adjust pH to 4.9

Stability : This reagent is stable at 2-8⁰C for one year.

2) Stock PNP solution : (Stock substrate)

Composition:

- p- Nitrophenyl phosphate - 0.4 g.
- Distilled water - 100 ml.

Method of preparation :

Dissolve 0.4 g. of p-Nitro phenyl phosphate in 100 ml. of distilled water.

Stability : This reagent is stable at 0-4⁰C for six months.

4) 0.1 N NaOH solution :

Composition :

Sodium hydroxide - 0.4 g.

Distilled water upto - 100 ml.

Method of preparation:

Dissolve 0.4 g. of sodium hydroxide in about 75 ml. of distilled water and dilute to 100 ml. with distilled water. Standardise and adjust normality if necessary.

Stability: This reagent is stable at room temperature for 2 years.

3) Working PNP solution (working substrate) :

Composition :

Stock PNP solution - 1 part

Citrate buffer - 1 part

Method of preparation :

Mix equal parts of stock PNP solution and citrate buffer.

Stability : It is stable at 2-8°C for 12 hours.

5) p- Nitro phenol (standard) solution: Prepare this reagent to contain a concentration of 30mM%.

Sample :

Serum preserved by a few drops of 6M acetic acid. It should be free from haemolysis. Haemolysed serum contains red cell phosphatase.

Wave length : 405 nm (Violet filter).

Procedure :

1) Take into the labelled tubes as follows.

S.No. Reagent / Sample	Test	Blank
1) Working PNP solution	1 ml.	1 ml.
Keep at 37°C for 5 minutes.		
2) Serum	0.2 ml.	-

keep at 37°C for 30 minutes

3) Serum - 0.2 ml.

4) 0.1N NaOH 4.2 ml. 4.2 ml.

2) Mix thoroughly and wait for 5 minutes.

3) Read the O.D. of test against blank.

4) Calculate the concentration of total acid phosphatase by referring to a standard graph-prepared as follows.

a) Pipette into the labelled tubes as follows

S.No. Reagent	S ₁	S ₂	S ₃	S ₄	S ₅	Blank
1. W. PNP (in ml.)	0.95	0.90	0.85	0.8	0.7	1
2. Nitro phenol standard (in ml.)	0.05	0.1	0.15	0.2	0.3	0
3. Distilled water (in ml)	0.2	0.2	0.2	0.2	0.2	0.2
4. 0.1 N - NaOH (in ml.)	4	4	4	4	4	4
Conc. of ACP (in I.U.)	2.5	5	7.5	10	15	0

5. If the determined value is more than 15 I.U. dilute the serum five times and multiply the measured value with '5'.

Normal value : 0.9 - 12 I.U.

Determination of Prostatic acid phosphatase:

Name of the method : PNP method

Principle : Tartarate inhibits prostatic fraction. Prostatic acid phosphatase is measured by subtracting value of non prostatic form from total acid phosphatase.

Sample : Same as in total acid phosphatase determination.

Wavelength : Same as in total acid phosphatase determination.

Requirements and Reagents :

Same as in total acid phosphatase determination.

Additional reagent :

Tartarate-citrate buffer :

Composition :

Tartaric acid - 1.5 g.

Citrate buffer - 250 ml.

Method of preparation:

Dissolve 1.5 g. of tartaric acid in 250 ml. of citrate buffer. Adjust pH to 4.9.

Stability of reagent: This reagent is stable for 6 months on refrigeration.

Procedure :

1) Pipette into the labelled tubes as follows.

S.No. Reagent/Sample	Total test	Test	Blank
1) W.PNP solution in ml.	1	1	1
2) Trartarate - Citrate buffer in ml.	-	0.2	-
Mix & keep at 37 ⁰ C for 5 minutes.			
3) Serum (in ml)	0.2	0.2	-
Mix & keep at 37 ⁰ C for 30 minutes.			
4) Serum (in ml.)	-	-	0.2
5) 0.1 N NaOH (in ml.)	4.2	4	4.2

2) Mix thoroughly and wait for 5 minutes.

3) Read the O.D. of total test and test against blank.

4) Calculate the value of total test and test by referring to a standard graph (given in the experiment for total acid phosphatase).

5) Calculate the value of prostatic acid phosphatase by subtracting this value from the value of total acid phsophatase.

Normal values 0 - 4 I.U.

Clinical significance : 1) Serum acid phosphatase is elevated in male patients with prostatic cancer .Its elevation in these patients is inhibited by tartrate .

2) Its determination is also significant in forensic medicine to identify rape cases .This enzyme is elevated in semen in such cases .

6) ALKALINE PHOSPHATASE

It has three isoenzymes. For details , refer to isoenzymes topic .

Determination of Alkaline Phosphatase:

Name of the method : PNP method

Principle : Alkaline phosphatase enzyme acts on paranitrophenyl phosphate and breaks it down to p-Nitrophenol. p-Nitrophenol is colourless. In presence of alkaline medium, it turns yellow. Colour intensity is proportional to the concentration of enzyme in the specimen.

Requirements:

1) Test tubes

2) Serological pipettes

3) Thermostatic water bath

4) Photo electric colorimeter.

Preparation of reagents:

1) AMP buffer (pH - 10.3) :

Composition :

2- amino 2-methyl 1-propanol - 78.5 ml.

Conc. hydrochloric acid - 18 ml.

Distilled water upto - 1 litre

Method of preparation :

Mix 78.5 ml. of 2- amino - 2 methyl -1 propanol and 18 ml. of conc. hydrochloric acid. Make up this solution to 1 litre with distilled water.

Stability :

This reagent is stable at 2-8⁰C

2. Working PNP solution (w. substrate):

Composition:

p-Nitrophenyl phosphate powder - 425 mg.

Magnesium chloride (30 mg%) solution - 5ml.

Method of preparation:

Dissolve 525 mg. of p-Nitrophenyl phosphate in 5 ml. of Magnesium chloride solution.

Stability : This reagent is stable at 2-8⁰C for one day.

3. 0.25 N Sodium hydroxide solution:

Composition :

Sodium hydroxide - 1g.

Distilled water - upto 100 ml.

Method of Preparation:

Dissolve 1 g. of sodium hydroxide in 75 ml. of distilled water and dilute to 100 ml. with distilled water.

Stability:

This reagent is stable at room temperature.

4)p- Nitrophenol standard (30m M/dL) Prepare this reagent to contain concentration of 30 m M/dL.

Specimen : Serum

Wavelength : 405 nm (Violet filter)

Procedure:

1) Pipette into the labelled tubes as follows

S.No.	Reagent/Sample	Test	Blank
1)	AMP buffer (in ml.)	2.7	2.7
2)	Working PNP solution (in ml.)	0.2	0.2
Mix and keep at 37 ⁰ C for 5 min.			
3)	Serum (in ml.)	0.1	-
Mix and keep at 37 ⁰ C for 15 min.			
4)	Serum (in ml.)	-	0.1
5)	0.25 N NaOH (in ml.)	3	3

2) Mix and keep at room temperature for 5 minutes.

3) Read optical density of test against blank.

4) Calculate the concentration of enzyme in the specimen by referring to the standard graph prepared as follows.

a) Pipette into the labelled tubes as follows.

S.No.	Reagent (in ml)	S ₁	S ₂	S ₃	S ₄	Blank
1)	AMP buffer	2.7	2.6	2.4	2	2.8
2)	Nitro phenol standard	0.1	0.2	0.4	0.8	0
3)	Magnesium chloride solution	0.2	0.2	0.2	0.2	0.2
Mix thoroughly and keep at room temperature for 5 minutes.						
4)	0.25 N NaOH	3	3	3	3	3
Concentrations		20	40	80	160	0

b) Mix thoroughly and keep at room temperature for 5 minutes.

c) Read optical density values against blank.

d) Prepare graph by plotting concentrations on X-axis and O. D values on Y-axis.

5) If the values are more than 160 I.U., dilute serum ten times and repeat the determination.

6) Multiply the measured value in the repeated determination with dilution factor (10).

Normal values:

20-90 I.U. in adults

93-221 I.U. in children

Clinical significance : 1) Alkaline phosphatase activity is elevated in hepatobiliary diseases and bone diseases.

2) In bone cancer, values are very high.

3) Elevation is marked in obstructive jaundice and biliary cirrhosis.

4) Values are elevated moderately in Hodgkin's disease, congestive cardiac failure, infective hepatitis and abdominal problems.

SUMMARY

Enzymes are biocatalysts. They are chemically proteins. They are characterized by their specificity. Their specificity is explained by enzyme-substrate complex formation.

Enzyme inhibition is of two types. 1) Competitive inhibition 2) Non competitive inhibition. Enzymes are classified according to the type of reaction they bring about. They are named by suffixing -ase to the substrate/activity. Enzymes have significance in medicine and diagnosis. Enzymatic methods of determination are available for certain constituents like glucose, urea etc. Co-enzymes are organic cofactors. Cofactors are non protein components on which enzymes depend for their activity. Transaminases are enzymes which transfer α -amino group of an amino acid to the α -carbon atom of an α -keto acid. Examples of transaminases are SGOT, SGPT etc. Determination of SGOT is significant for diagnosis of myocardial infarctions. SGPT determination is significant in hepatic and post hepatic conditions. Cardiac enzymes are SGOT, CPK, LDH and SHBD.

Phosphatases are a group of enzymes catalysing hydrolysis of esters of different organic phosphates. Determination of serum acid phosphatase is significant in diagnosis of prostatic cancer with metastases in males. Determination of serum alkaline phosphatase is significant in diseases of liver and bones.

ESSAY QUESTIONS

- 1) Define enzymes. Describe enzyme specificity mechanism.
- 2) Discuss the factors influencing enzyme action.
- 3) What is enzyme inhibition? Write about types of enzyme inhibition.
- 4) Classify enzymes and write about their nomenclature of enzymes.
- 5) Discuss the importance of enzymes.
- 6) Write about coenzymes.
- 7) Write a short note on isoenzymes.
- 8) Define transaminases and give note on transaminases.
- 9) Write about determination of SGOT.
- 10) Write the principle and procedure of determination of SGPT.
- 11) Describe the determination of creatine phosphokinase.
- 12) Write about determination of LDH.
- 13) Explain the determination of α -hydroxy butyrate dehydrogenase.
- 14) Write about determination of serum acid phosphatase.
- 15) Discuss the determination of serum alkaline phosphatase.

SHORT ANSWER QUESTIONS

- 1) Define enzymes
- 2) What is enzyme specificity?
- 3) Mention the factors influencing enzyme action.
- 4) What is meant by enzyme inhibition? Name the types of enzyme inhibition.
- 5) Name the classes of enzymes.
- 6) What are enzymes of significance in diagnosis of pancreatic diseases?

- 7) What are the applications of determination of SGOT and SGPT?
- 8) Mention significance of determination of serum acid and alkaline phosphatase.
- 9) List out cardiac enzymes.
- 10) Define Katal.
- 11) What is a Co-enzyme?
- 12) Mention Co-enzyme forms of few vitamins.
- 13) Write the definition of isoenzymes .
- 14) Mention the isoenzyme forms of LDH .
- 15) What are the units of CPK ?
- 16) Name the isoenzyme forms of ALP .
- 17) Define transaminases.
- 18) Exemplify transaminases.
- 19) Write the transamination reaction caused by AST.
- 20) List out the amino acids undergoing first stage of catabolism by the process of transamination.
- 21) What is the principle of SGOT determination?
- 22) Give the composition of GOT substrate.
- 23) Write the composition of phosphate buffer.
- 24) Give the principle of SGPT determination.
- 25) Write the wavelengths for determination of transaminases.
- 26) Write the principle for determination of CPK enzyme.
- 27) Give the composition of Tris buffer.
- 28) Write the stability aspects of buffer-cysteine solution and phosphocreatine solution used in CPK determination.
- 29) What is the composition of p-chloromercuri benzoic acid solution?

- 30) How do you prepare α -Naphthol solution ?
- 31) Write the principle of determination of LDH by colorimetric method.
- 32) Write about preparation and storage of NAD solution used in LDH determination.
- 33) What is the principle of determination of SHBD?
- 34) How do you prepare NADH_2 solution for SHBD determination?
- 35) What are phosphatases?
- 36) Mention different phosphates.
- 37) Give the principle of determination of serum acid phosphatase by PNP method.
- 38) Write the principle of prostatic acid phosphatase determination.
- 39) What are the three iso enzymes of Alkaline phosphatase?
- 40) Write the principle of determination of Alkaline phosphatase by PNP method.
- 41) How do you prepare AMP buffer for SALP determination?
- 42) Write the normal values of SAP and SALP.
- 43) What are the normal values of SGOT and SGPT?

XII - BODY FLUIDS

1) OUTLINES OF FORMATIONS OF DIFFERENT BODY FLUIDS

Water in the body is distributed in two compartments . They are - 1. Intracellular fluid compartment 2. Extracellular fluid compartment . There is continuous exchange of water between these two compartments . Substances in solution also exchange continuously among the three compartments . This is caused by dynamic equilibrium between the three compartments . Volume of water in blood plasma is about 3 L. Plasma contains about 200 cc of proteins also . Extracellular fluid contains plasma and tissue fluids .

Different body fluids other than plasma are synovial fluid , pleural fluid , pericardial fluid , peritoneal fluid etc. Formation of these fluids occurs due to difference in hydrostatic pressure between capillaries and tissue fluids .

Synovial fluid (SF) : It is the fluid present in joints like knee joint , ankle joint , hip joint , elbow joint , wrist joint , shoulder joint etc. In normal conditions , about 1 ml of synovial fluid is present in each big joint . Synovial fluid normally contains viscosity of forming a string of 4cm from drop of SF from a syringe needle . Normally , 1ml of SF will form a compact large clot surrounded by clear solution on addition to 20 ml of 5% V/V acetic acid in a small beaker . Viscosity of SF and formation of clot are due to hyaluronate .

Pleural fluid (Pl. F.) : It is the fluid of pleural surface . Normally , about 1 - 10 ml of pleural fluid will be present around the lungs . It is derived by ultrafiltration of plasma . Normal quantity of proteins in this fluid is 1 - 2 g.% with no fibrinogen . Abnormal fluid accumulation is caused by increased capillary permeability , decreased plasma colloid osmotic pressure , increased hydrostatic pressure , decreased lymphatic drainage .

Pericardial fluid (PF) : It is the fluid of pericardial sac . Under normal conditions , 20 - 50 ml of clear straw coloured fluid will be present . It may be bloody , milky , or similar to gold paint .

Peritoneal fluid : It is straw coloured , clear fluid present in peritoneal cavity

. Its normal volume is less than 100 ml .

2) COMPOSITION AND ANALYSIS OF C.S.F. INCLUDING a) CSF SUGAR ESTIMATION b) CSF PROTEINS ESTIMATION c) CSF CHLORIDES ESTIMATION *including interpretation of results*

CEREBRO SPINAL FLUID (C.S.F.):

It is a modified tissue fluid present in sub-arachnoid space, cerebral ventricles and the central canal of the spinal cord. It is a clear, colourless, watery fluid formed by the blood vessels of choroid plexuses which are situated in the cerebral ventricles.

Physical properties (Normal):

- | | |
|--------------------------------------|---|
| 1) Colour : No colour | 2) Appearance : Clear, no formation of clot on standing |
| 3) Volume : 100 - 150 ml. in adults. | 4) pH - 7.3 - 7.4 |
| 5) Specific gravity - 1.003 - 1.008 | 6) Pressure : 70 to 150 mm of H ₂ O |

Composition (Normal) :

- Total solids : 0.87 - 1.7 g%.
- | | |
|-------------------------|-----------------------------|
| 1) Glucose : 40-80 mg% | |
| 2) Proteins : 15-45 mg% | |
| Albumin : 50-70% | |
| Globulin : 30-50% | Alpha-1 Globulin : 3 - 9 % |
| | Alpha-2 Globulin : 4 - 10 % |
| | Beta Globulin : 10 - 18 % |
| | Gamma Globulin : 3 - 9 % |
- Fibrinogen : Absent
- | | |
|-------------------------------|-------------------------------|
| 3) Chlorides : 700-750 mg% | 4) Sodium : 144 - 154 meq/l. |
| 5) Potassium : 2 - 3.5 meq/l. | 6) Creatinine : 0.5 - 1.2 mg% |

- 7) Urea : 6 - 16 g% 8) Uric acid : 0.5 - 4.5 mg%
 9) Cholesterol : 0.2 - 0.6 mg% 10) Thyroxine : 0.1 - 0.2 mg %
 11) Iron : 1 - 2 mg % 12) Glutamine : 6 - 11 mg %

Cells : 0 - 8 lymphocytes/mm³.

neutrophils - Nil

Clinical significance of CSF analysis : C.S.F. analysis is required mainly in meningitis. It is also conducted in encephalitis, spinal cord tumour, subarachnoid haemorrhage, multiple sclerosis, C.N.S. syphilis etc. It is also carried in treatments of elevation of C.S.F. pressure with benign intra cranial hypertension etc.

Collection and distribution of CSF specimen : Collection should be done in two tubes. Collection should be done by a trained person under the supervision of a surgeon. If the personnel employed for collecting CSF is not skilled, it will lead to complications. CSF can be withdrawn by lumbar puncture technique from the sub-arachnoid space which is at the level below the second lumbar vertebra. Lumbar puncture is usually done at L₃ - L₄ or lower. In small children, it should be performed at L₄ - L₅ or lower. Lumbar puncture should be done using a sterile lumbar puncture needle. After withdrawal of the stylet, fluid should be passed through needle.

Lumbar puncture needle : Lumbar puncture needle is a long needle with a stylette inside. It should be sterile. If it is not sterile, it can introduce infection. If stylette is not there, it can cause development of dermoid cyst. The needle should have a small bore. Needle without small bore can cause leakage of CSF. It will cause post puncture head ache.

Collection tubes : Collection should be done into two tubes - tube 1 and tube 2.

Tube 1 : Tube 1 should be sterile and volume of CSF to be taken into this tube should be a few drops. This tube is for bacteriological examination.

Tube 2 : Volume of CSF to be collected into this tube should be about 3 - 5 ml.

Centrifuge CSF.

Supernatant : Supernatant of CSF should be sent for biochemical qualitative and quantitative examination.

Sediment : Sediment of CSF is used for -

a) Preparation of smears b) Preparation of wet mount c) India ink preparation
 a) Preparation of smears : 3 smears can be prepared. Out of them, one is for Gram's staining, 2nd is for acid fast staining and 3rd is for differential leukocyte count.

b) Preparation of wet mount : For Trypanosoma

c) India ink preparation : For diagnosis of cryptococcus infection.

Precautions about the collected specimen :

1. Specimen on collection should be examined within one hour.
2. Part of specimen for bacterial culture should not be stored in refrigerator since cool temperature is lethal to nesseria mningitidis, which is commonly required.
3. Part of specimen for chemical examination can be stored at 2 - 8 0 C not exceeding three hours.
4. Examine the specimen carefully and economically since it is difficult to collect.
5. Handle the specimen carefully avoiding risk since the specimen may contain infective organisms.
6. Examine the specimen as early as possible to avoid lysis. Trypanosomes and cells are rapidly lysed after collection.
7. Needle for lumbar puncture should contain a stylette inside, be long, sterile and it should have a small bore.
8. Progression of paresis to paralysis may follow lumbar puncture in patients with spinal cord tumour.
9. Lumbar puncture should be performed in the morning rather than in late afternoon or evening.

Functions of CSF:

- 1) Bathing of the brain and spinal cord.
- 2) It acts as a shock absorber or buffer to the brain and the spinal cord.
- 3) It supplies nutrition and takes away the waste products from the brain and spinal cord.
- 4) It protects the brain and spinal cord against infection and acts as a buffer against shock.

Routine examination of CSF :

It consists of 1) Physical examination 2) Cell count 3) Qualitative and quantitative chemical examination

1) Physical examination of CSF :

- Requirements : 1) pH meter or pH papers
2) Hand refractometer etc.

Procedure : 1. Observe the specimen for colour, appearance, presence of blood, appearance of clot etc. and report in the part of report sheet for physical examination. Evaluate colour by holding the tube against a white paper along with a tube of distilled water for comparison.

2. Determine pH of specimen with pH paper.
3. Determine specific gravity of specimen if required.
4. Report the findings of physical examination in the part of report sheet for physical examination.

Interpretation with observed findings : Normal CSF is crystal clear. If a pale yellow or pink colour is observed, it should be centrifuged at high speed for 5 minutes and supernatant be observed. Pale pink to pale orange or yellow colouration of supernatant is called xanthochromia. Xanthochromia is graded from 1 to 4. Xanthochromia is caused by concentration of CSF protein more than 100 mg%, bilirubinaemia, intra cerebral or subarachnoid haemorrhage etc.

Turbidity is graded from '0 to 4+'. Zero grading for crystal clear appearance and grading of four plus for nonappearance of news print through the tube. Turbidity results from large number of leukocytes or bacteria. Slight opalescence is typical of tuberculous meningitis and grossly purulent appearance is indicative of some cases of pyogenic meningitis.

Clotting is seen in traumatic tap, marked elevation of CSF protein or moderate elevation of CSF protein associated with tuberculous meningitis.

2) Cell count :

Cell count of CSF is 2 types as that of leukocytes. They are -

- 1) Total count
- 2) Differential leukocyte count

- 1) Total count : Total count of blood cells is done in this.

Requirements : Neubaur counting chamber or Fuchs Rosenthal counting chamber, Diluting fluid, Microscope, RBC / WBC pipette etc

Procedure : 1. Dilute the specimen with diluting fluid using blood pipette. Dilution is done by taking Unna's polychrome methylene blue in RBC pipette to 1 mark and spinal fluid to 101 mark. This fluid colours red cells yellow and white cells blue. When specimen is turbid due to presence of many cells, WBC diluting fluid should be used for diluting in WBC pipette.

2. Charge the counting chamber. Neubauer's counting chamber or Fuch's Rosenthal chamber may be used for charging the fluid diluted in blood pipette.

3. Count the cells. If Fuch's Rosenthal chamber is used for charging, count the cells in the entire ruled area i.e. 16 squares having 16 square millimeters area and depth of 0.2 mm. If Neubauer's counting chamber is used, count the RBCs and WBCs in the 9 large squares.

4. Calculate the total count using the formula -

$$\text{Cells / mm}^3 = \text{Cells counted} \times 1.1 \text{ (For Neubauer)}$$

$$= \text{Cells counted} \times 0.33 \text{ (For Fuch's Rosenthal)}$$

2) Differential leukocyte count : Differential leukocyte count of CSF is done by taking a smear made from sediment of tube 2. DLC of CSF is done in the following way as DLC of blood specimen.

Requirements : 1. Microscope

2. Slides and glass spreader
3. Cedar wood oil for immersion
4. Leishman's or Wright's stain
5. Buffer solution (pH - 7)

Making a glass spreader : A glass spreader for making smear is prepared by marking a glass slide with smooth edges using glass cutter into 4 equal divisions

, each measuring about 19 mm and breaking at each division .4 spreaders are made .

Leishman's or Wright's Stain : Leishman 's stain , Wright 's stain and Giemsa's stain are polychromic staining solutions . They contain methylene blue and eosin .Eosin is acidic dye and methylene blue is basic dye .

Composition and method of preparation of Leishman's stain is given for example .

Composition of Leishman's stain :

Powder of Leishman's stain----- 0.15 g.

Methyl alcohol without acetone -- 1.33ml

Method of preparation of Leishman's stain : Powder the crystals of stain well in a mortar with a pestle .Add enough quantity of methanol to dissolve the stain and triturate . Add the remaining quantity of methanol .Transfer to a well stoppered bottle .

Buffer solution :

Composition of buffer solution :

Sodium dihydrogen phosphate ----- 3 . 76 g.

Potassium dihydrogen phosphate ----- 2 .10 g.

Distilled water ----- 1 L.

Keep this buffer at room temperature .

Procedure :

1. Cover the smear with 10 - 15 drops of staining solution .
2. Add equal quantity of buffer after 1 minute .
3. Wash the smear with tap water after 10 minutes .
4. Drain the slide .
5. Examine the film first under low power .
6. Now observe under oil immersion and note .

Clinical interpretation : 1. Large number of polymorphs are due to pyogenic meningitis caused by nesseria meningitidis , haemophilus influenzae , pneumococci , streptococci , staphylococci , coliforms , some times virus , rarely fungus etc .

2. Mixed reaction (neutrophils , lymphocytes and monocytes) is seen in subacute bacterial meningitis , tuberculous meningitis , mycotic meningitis and viral meningoencephalitis etc .

3. Monocytic and lymphocytic reaction is seen in viral meningitis , multiple sclerosis , tuberculous meningitis , fungal meningitis and syphilitic meningitis etc .

4. Lymphocytic reaction alone also is seen in the diseases as mentioned i in mixed reaction of lymphocytes and monocytes .

5. Malignant /benign cells may be seen in certain CNS neoplasms .

6. Blasts may be seen in leukaemic cell infiltrates in the meninges .

3)Qualitative and quantitative chemical examination :

Qualitative chemical examination for CSF sugar : Qualitative chemical examination for CSF sugar is done by Benedict's qualitative test for CSF sugar. Benedict's qualitative test for CSF sugar :

Principle :

Glucose sugar present in CSF reduces cupric ions present in the Benedict's qualitative reagent to cuprous ions in the presence of alkaline medium provided by sodium carbonate. Benedict's qualitative reagent which is originally blue colored gives yellow ppt with opalescent green supernatant when CSF sugar level is normal.

Requirements:- 1) Test tube

2) Benedict's qualitative reagent

3) Spirit lamp or water bath.

Composition of Benedict's reagent.

Sodium citrate – 173g.

Sodium carbonate anhydrous – 100g.

Copper sulphate - 17.3g

Distilled water - up to 1000ml.

Method of preparation of Benedict's reagent :

1. Dissolve 173 g. of sodium citrate and 100g of anhydrous sodium carbonate in about 750 ml. of distilled water and gently heat in 1 litre beaker or flask.
2. Dissolve 17.3g. of copper sulphate in 100ml of distilled water in another beaker .
3. Pour the copper sulphate solution into citrate-carbonate solution with constant stirring.
4. Transfer into 1 litre volumetric flask with washings.
5. Mix, cool and dilute to 1 litre with distilled water.
6. If it is not clear, filter and transfer into reagent bottle, label and store.

Procedure:

- 1) In a test tube, take 1 ml of CSF.
- 2) Add 1 ml of Benedict's qualitative reagent.
- 3) Boil over a spirit lamp for 2 minutes or heat over a boiling water bath for 5 minutes.
- 4) Observe the change and note.

Report:

<u>S.No.</u>	<u>Observation</u>	<u>Inference</u>
1	Slightly yellow precipitate with Opalescent green supernatant.	Normal
2	No change.	Sugar diminished
3	Orange or brick red ppt.	Sugar increased

Qualitative chemical examination for CSF Globulin : CSF globulin is qualitatively identified by following tests . 1. Pandy 's test 2. Ross jone's test

Pandy 's test for globulin : 2 ml of saturated solution of phenol is taken in a small test tube and 1 drop of CSF is added to it . Cloudiness is observed against a black back ground . It is graded from '0 to 4+' .

Ross jone's test for globulin : 0.5 ml of CSF is superimposed over 1 ml of

saturated solution of ammonium sulphate . Appearance of white ring at the junction of two liquids indicates presence of globulin . Thin white ring at the junction which disappears on mixing indicates 1+ . Heavy cloudiness persisting even after mixing indicates 4+ reaction .

These two tests are invalid if CSF contains blood .
Quantitative chemical examination : Quantitative determinations of glucose , proteins and chlorides are dealt here .

a. CSF SUGAR ESTIMATION :

Normal range - 40-80 mg% .

CSF glucose determination is significant in diagnosis of various clinical conditions.

<u>Clinical condition</u>	<u>Values</u>
1) Bacterial infection	0-40 mg%
2) Viral infection	slightly low or normal
3) Fungal infection	0-40 mg%
4) Acute purulent meningitis	0-40 mg%
5) Tuberculous meningitis	0-40 mg%
6) Brain tumour	0-40 mg%
7) Brain tumour	elevated
8) Acute syphilitic meningitis	0-40 mg%
9) Encephalitis lethargica	80-120 mg%
10) Cerebral haemorrhage	Variable

Quantitative determination of CSF sugar by Folin and Wu method :

Principle : When protein free filtrate is heated with an alkaline copper solution, a precipitate of cuprous oxide is produced by the reducing action of glucose. This in turn is dissolved by phosphomolybdic solution and reduces it to a blue colored compound. Colour intensity of blue colour formed is compared with the colour intensity developed in a similarly treated standard solution of glucose using colorimeter. Tungstic acid produced by reaction between sodium tungstate and sulphuric acid precipitates proteins and useful in preparation

of protein free filtrate. Removal of protein helps in removing the interference of protein with glucose in the colorimetric estimation. Sodium carbonate present in the copper tartrate solution provides alkaline medium for reduction of copper to take place.

Requirements :

1. 10 ml. test tube
2. Folin & Wu tubes
3. Water bath
4. Beakers
5. Cuvettes
6. Centrifuge
7. Colorimeter

Reagents :

1. 10% Sodium tungstate solution:

Composition : Sodium tungstate - 10g.

Distilled water - up to 100 ml.

Method of preparation : 10g. of sodium tungstate is accurately weighed, dissolved in approximately 75 ml. of water and diluted to 100 ml. with distilled water.

2. 2/3 N sulphuric acid:

Composition:

Conc. Sulphuric acid -2ml

Distilled water-upto-100ml

Method of preparation : Take 2 ml. of concentrated sulphuric acid, and dilute to 100 ml. with distilled water. Standardize the prepared solution against a standard solution of alkali and adjust the normality if necessary.

3. Alkaline copper tartrate solution:

Requirements :

Anhydrous sodium carbonate - 20g.

Tartaric acid - 3.75 g.

Copper sulphate - 2.25 g.

Distilled water upto- 500 ml.

Method of preparation :

1. Dissolve 20g. of anhydrous sodium carbonate in 200 ml. of distilled water.
2. Dissolve 3.75g. of tartaric acid in the solution prepared in step-1.
3. Dissolve 2.25g copper sulphate in the solution prepared in step-2.
4. Dilute the solution prepared in step 3. to 500 ml. with distilled water.

4. Phosphomolybdic reagent :

Requirements :

Molybdic acid - 17.5 g.

Sodium tungstate 2.5 g.

10% sodium hydroxide solution 100 ml.

Phosphoric acid - 62.5 ml.

Distilled water upto 250 ml.

Method of preparation :

1. Dissolve 17.5g of molybdic acid and 2.5g of sodium tungstate in 100 ml. of 10% sodium hydroxide solution.
2. Add 100 ml. distilled water and boil for 30 to 40 minutes. This will expel all the ammonia present in molybdic acid.
3. Cool and transfer to 250 ml. flask with washings.
4. Add 62.5 ml. of phosphoric acid.
5. Dilute to 250 ml with distilled water.

5. Glucose stock standard solution (1% w/v)

Composition : Glucose analar - 1g.

0.25% w/v Benzoic acid aqueous solution up to 100 ml.

Method of preparation :

Dissolve 1g. of glucose analar in about 75 ml. of 0.25% aqueous

benzoic acid solution and dilute to 100 ml. with 0.25% aqueous solution of benzoic acid solution.

6. Glucose Working Standard (0.1% w/v) :

Composition : Glucose stock standard - 1 ml.
Distilled water upto 10 ml.

Method of preparation :

Dilute 1 ml. of glucose stock standard solution to 10 ml. with distilled water. This will give glucose solution of 0.1% w/v (100 mg / 100 ml.) concentration.

Wave length : 440 milli microns. (Dark blue filter)

Specimen : C.S.F.

Preparation of protein free filtrate :

1. In a test tube, take 3.5 ml. of distilled water, 0.1 ml. of C.S.F, 0.2 ml. of 10% sodium tungstate solution and mix.
2. Add 0.2 ml. of 2/3N sulphuric acid and mix.
3. Filter or centrifuge after 5 minutes.
4. Collect the filtrate into another test tube.

Procedure :

1. Take 3 folin and Wu sugar tubes. Label them as test (T), standard (S) and blank (B).
2. Proceed as follows :

Reagent	T	S	B
1. Protein free filtrate	2 ml.	-	-
2. Working Standard	-	0.1 ml.	-
3. Distilled water	-	-	2 ml.

- | | | | |
|----------------------------|-------|-------|-------|
| 4. Alkaline copper Reagent | 2 ml. | 2 ml. | 2 ml. |
|----------------------------|-------|-------|-------|

Mix well, remove and cool in a beaker of cold water for 2-3 minutes.

- | | | | |
|----------------------------|-------|-------|-------|
| 5. Phosphomolybdic reagent | 2 ml. | 2 ml. | 2 ml. |
|----------------------------|-------|-------|-------|

Stand for few minutes until cuprous oxide has completely dissolved.

- | | | | |
|--------------------|---------------------------|----------------------------|----------------------------|
| 6. Distilled water | upto 12.5 ml. in F.W.tube | upto 12.5 ml. in F.W. tube | upto 12.5 ml. in F.W. tube |
|--------------------|---------------------------|----------------------------|----------------------------|

3. Set the colorimeter to 100% transmission at 440 milli microns wave length using blank.

4. Determine optical densities of T and S.

5. Determine the concentration with the formula

$$\text{concentration of C.S.F. glucose in mg\%} = \frac{\text{O.D. of test}}{\text{O.D. of standard}} \times \text{concentration of standard (100 mg\%)}$$

Determination of C.S.F. glucose by O-Toluidine method :

Principle : Glucose reacts with orthotoluidine in hot acidic medium to form a green coloured complex. Acidic medium favours the reaction between O-Toluidine and glucose. Acidic medium is provided by glacial acetic acid present in the O-Toluidine reagent. Intensity of the colour developed is proportional to the concentration of glucose in C.S.F.. Concentration of glucose in C.S.F. can be calculated by comparing with the intensity of colour developed in similarly treated standard.

Requirements :

1. Dispenser
2. Test tubes
3. Pipette
4. Water bath

5. Centrifuge
6. Colorimeter

Reagents :

1. O-Toluidine reagent :

Composition:

- O-Toluidine - 60 ml.
- Thiourea - 1.5 gms.
- Glacial acetic acid up to 1 litre.

Method of Preparation :

1. Take 60 ml. of O-Toluidine.
 2. Dilute approximately to 1L with glacial acetic acid.
 3. Dissolve 1.5 gms. of thiourea in it.
 4. Dilute upto 1 litre with glacial acetic acid.
2. Glucose stock standard solution (1% w/v) :

- Glucose A.R. - 1gm.
- 0.25% w/v aqueous solution of benzoic acid up to 100 ml.

Procedure :

Dissolve 1 gm. of glucose A.R. in approximately 75 ml. of 0.25% w/v aqueous solution of benzoic acid and dilute to 100 ml. with 0.25% w/v of aqueous solution of benzoic acid. It will give 1%w/v solution.

3. Glucose working standard solution (0.1% w/v) :

Composition :

- Glucose stock standard solution - 1 ml.
- Distilled water - upto 10 ml.

Procedure :

Dilute 1 ml. of glucose stock standard solution to 10 ml. with distilled

water. It will give 100 mg% solution of glucose standard.

Specimen : C.S.F.

Wave length : 640 milli microns (red filter).

Procedure :

1. Take 3 test tubes and label them as Test (T), Standard (S) and Blank

2. Proceed as follows:

Reagent	T	S	B
O-Toluidine reagent	5 ml.	5 ml.	5 ml.
C.S.F.	0.1 ml.	-	-
Working standard	-	0.1 ml.	-
Distilled water	-	-	0.1 ml.

3. Mix thoroughly and put all the tubes on boiling water bath for exactly 8 minutes.
4. Remove and cool in a cold water bath.
5. Set the colorimeter to 100% transmission with blank at 640 milli microns wave length.
6. Determine the optical densities of T and S.
7. Determine the concentration of C.S.F. glucose with the formula

concentration of C.S.F. glucose in mg% =

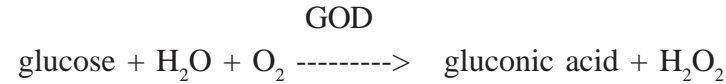
$$\frac{\text{O.D. of test}}{\text{O.D. of standard}} \times \text{concentration of standard (100 mg\%)}$$

Determination of C.S.F. glucose by GOD-POD (Enzymatic method) :

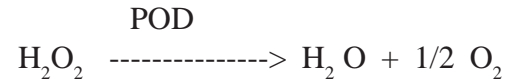
Principle :

Glucose undergoes oxidation by the action of the enzyme glucose oxidase

to give gluconic acid.



H₂O₂ formed in this reaction is cleaved into water and oxygen by the action of peroxidase enzyme.



Nascent oxygen formed in this reaction reacts with 4- amino phenazone to give pink coloured compound. Colour intensity of this compound is directly proportional to the concentration of glucose in C.S.F. Concentration of glucose in C.S.F. can be calculated by comparing with the intensity of colour developed with similarly treated standard.

Wave length : 530 nm

Specimen : C.S.F.

Reagents:

1) Buffer-Enzyme reagent.

Composition :

Glucose oxidase	- 650 units
Peroxidase	- 500 units
4-Aminophenazone	- 20 mg.
Sodium azide	- 30 mg.
Phosphate buffer (M/10)	- 100 ml.

Method of preparation : Dissolve GOD, POD, 4-Aminophenazone and Sodium azide in 100 ml. of M/10 Phosphate buffer.

2) Phenol reagent (100 mg.dL)

Composition:

Phenol - 1 g.

0.1 N HCl - upto 1 litre.

Method of Preparation :

Dissolve 1 g. phenol in about 750 ml. of 0.1 N HCl and dilute to 1 litre with 0.1 N HCl.

Procedure :

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

2) Pipette the reagents into the tubes as follows.

S.No.	Reagent	T	S	B
1.	Glucose reagent	3 ml.	3 ml.	3 ml.
2.	C.S.F.	0.02 ml.	-	-
3.	Glucose Standard	-	0.02 ml.	-
4.	Distilled water	-	-	0.02 ml.

3) Mix and keep at 37°C for 15 minutes or at lab temperature for 3 minutes.

4) Determine O.D.'s at 530 nm wave length.

5) Determine C.S.F. glucose concentration using the formula-

$$\text{C.S.F. glucose} = \frac{\text{O.D. of test}}{\text{O.D. of standard}} \times \text{concentration of standard (100 mg\%)}$$

b) CSF PROTEINS ESTIMATION

Normal range is 15-45 mg%. Albumin constitutes 50-70% and globulin constitutes 30-50%. Its determination is significant in diagnosis of various clinical conditions.

<u>Clinical condition</u>	<u>Values</u>
1) Bacterial infection	45-500 mg% (increase in globulin)
2) Viral infection	45-300 mg%
3) Fungal infection	Normal
4) Acute purulent meningitis	45-1000 mg%
5) Tuberculous meningitis	45-500 mg%
6) Brain tumour	Normal
7) Brain tumour	Normal (globulin elevated)
8) Acute syphilitic meningitis	Normal (globulin normal)
9) Encephalitis lethargica	Normal or increased
10) Cerebral haemorrhage	Increased (globulin normal)

Quantitative determination of CSF proteins by turbidimetric method :

Principle : Proteins are denatured by the action of acids. On denaturation, they change from soluble form to insoluble form and exhibit turbidity. Measure of turbidity gives concentration of proteins.

Requirements:

- 1) Test tubes.
- 2) Serological pipettes.
- 3) Volumetric pipettes .
- 4) Photometer.

Reagents :

1)3% W/V Sulfosalicylic acid reagent :

Composition :

Sulfosalicylic acid	- 3g.
Distilled water	- upto 100ml.

Method of preparation :

- 1) Dissolve 3g of Sulfosalicylic acid in approximately 75 ml of distilled water.
- 2) Dilute to 100ml with distilled water.

Stability: This reagent is stable at room temperature.

2) 0.9% Sodium chloride solution (Normal saline):

composition:

Sodium chloride AR.	-0.9g.
Distilled water	-up to 100ml.

Method of preparation:

- 1) Dissolve 0.9g of sodium chloride A.R. in approximately 75ml of distilled water.
- 2) Dilute to 100ml with distilled water .

Stability:- This reagent is stable at room temperature.

3) 6% W/V stock protein standard:

Composition:

Bovine albumin	- 6g.
Sodium azide	- 0.1g.
Normal saline up to	- 100ml.

Method of preparation :

- 1) Dissolve 6g of bovine albumin in approximately 75ml of normal saline.
- 2) Add 0.1g of sodium azide and mix.
- 3) Dilute to 100ml with normal saline.

Stability: This reagent is stable for one year on refrigeration.

4) Working protein standard (60mg%) :

Composition:

Stock protein standard	-0.1.
Normal saline up to	- 10ml

Method of preparation :

Dilute 0.1ml of stock protein standard to 10ml with normal saline.

Stability: It should be freshly prepared before use.

specimen: CSF

Wave length:- 640nm.

Procedure:-

- 1) Take 3 tubes and label them as Test, Std and Blank.

2) Pipette into them as follows.

S.No	Reagent / sample	Test	Std	Blank
1)	3% Sulfosalicylic acid	4ml	4ml	4ml
2)	CSF	1ml	-	-
3)	Working standard	-	1ml	-
4)	Distilled water	-	-	1ml

3) Mix thoroughly.

4) Keep at room temperature (25 ± 5°C) for 5 minutes.

5) Measure the optical density values of Test, Std against blank.

6) Calculate the Conc. of CSF proteins with the formula-

$$\text{CSF proteins} = \frac{\text{OD of test}}{\text{OD of Std.}} \times 60 \text{ mg\%}$$

If the values are higher than 200mg% dilute the sample 5 times and multiply the determined value with dilution factor (5).

c) CSF CHLORIDES ESTIMATION

Normal range of CSF chlorides is 700-750 mg%. Its determination is significant in diagnosis of various clinical conditions.

Clinical condition	Values
1) Bacterial infection	600-700 mg%
2) Viral infection	moderately decreased
3) Fungal infection	Normal or slightly decreased
4) Acute purulent meningitis	600-700 mg%
5) Tuberculous meningitis	500-600 mg%
6) Brain tumour	Normal or slightly decreased
7) Brain tumour	Normal
8) Acute syphilitic meningitis	Normal (globulin normal)
9) Encephalitis lethargica	Normal
10) Cerebral haemorrhage	Normal

CSF Chlorides determination can be done by:

1) Silver nitrate titration method.

2) Schales & Schales titration method.

Normal range of CSF chlorides is 700-760mg%.

Determination of CSF chlorides by silver nitrate titration method :

Principle: Silver nitrate reacts with chlorides in CSF and yields silver chloride and nitrate. Potassium chromate (5%) solution is used as indicator.

Requirements: 1) Burette.

2) Conical flask.

1) Silver nitrate (0.5814 mg%) solution :

Composition:

N/10 Silver nitrate solution – 17.1 ml.

Distilled water – up to 50ml.

Method of preparation:

Dilute 17.1 ml of N/10 silver nitrate solution to 50ml with distilled water.

Storage:- Store in brown coloured bottle in dark.

2) Potassium chromate 5% aqueous solution

composition : Potassium chromate – 5g

Distilled water – up to 100ml.

Method of preparation :

Dissolve 5g of Potassium chromate in approximately 75ml of water and dilute to 100ml with distilled water.

Stability: It is stable at room temperature.

Specimen: CSF

Procedure:

1) Take 2ml of CSF in a small conical flask.

2) Add 3ml of distilled water.

3) Add 2 drops of 5% potassium chromate indicator.

4) Titrate with silver nitrate solution from a burette till the last drop gives red colour of silver nitrate.

5) Note the volume.

6) Calculate with the formula -

CSF chlorides = Vol of AgNO₃ solution used in titration (in ml) X 100mg%.

Determination of CSF chlorides by schales and schales titration method:

Principle : Protein free Filtrate of CSF is titrated with Mercuric nitrate solution. Diphenyl carbazone is used as indicator. Chloride ions in CSF react with Mercuric ions. At the end point, mercuric ions in excess react with Diphenyl Carbazone indicator to form blue-violet coloured complex. Specimen with low protein content can be titrated directly with out removing proteins.

Requirements: 1) Test tubes.

2) Graduated pipettes.

Reagents: 1) Mercuric nitrate solution:

Composition:-

Mercuric nitrate - 3g.

2N Nitric acid - 20ml.

Distilled water up to - 1 litre.

Method of preparation:

Dissolve 3 g. of Mercuric nitrate in about 75ml of distilled water. Add 20ml of 2N nitric acid, dilute to 1 litre with distilled water.

Stability: This reagent is stable at room temperature. It is stored in amber coloured bottle.

2) 2N Nitric acid:

Composition:

Concentrated Nitric acid - 2ml.

Distilled water up to - 16ml.

Method of preparation:

Dilute 2ml of conc. Nitric acid to 16 ml. with distilled water and standardise.

Stability: This reagent is stable at room temperature.

3) Diphenyl carbazone indicator solution :

Composition :

Diphenyl carbazone - 100mg.

95% V/V ethanol - up to 100 ml.

Method of preparation :

Dissolve 100mg of diphenyl carbazone in 75 ml of 95% V/V ethanol and dilute to 100ml with 95% V/V ethanol.

Stability:- It is stable at 2-8⁰C .It is stored in amber coloured bottle.

4) 95% V/V ethanol:

Composition :

Absolute alcohol - 95ml.

Distilled water up to - 100ml.

Method of preparation:-

Dilute 95ml of absolute alcohol to 100ml with distilled water.

Stability: It is stable at room temperature.

5) Chloride standard (100 meq/l):

Sodium chloride A.R-5.85g.

Distilled water up to - 100ml.

Method of preparation:-

Dissolve 5.85g of sodium chloride A.R. in 75ml of distilled water and dilute to 100 ml. with distilled water.

Stability: It is stable at 2-8⁰C.

6) 2/3 N sulphuric acid :

Composition :

Concentrated sulphuric acid - 2ml.

Distilled water - up to 100ml.

Method of preparation:

Dilute 2ml of concentrated sulphuric acid to 100ml with distilled water and standardise.

Stability:- This reagent is stable at room temperature.

7) 10% W/V sodium tungstate solution:

Composition:-

Sodium tungstate - 10 g.

Distilled water upto - 100 ml.

Method of preparation:-

Dissolve 10g of Sodium tungstate in 75ml of distilled water and dilute to 100ml with distilled water.

Stability:- This reagent is stable at room temperature.

Specimen:- CSF.

Procedure:

- 1) To 4ml of distilled water, add 0.5ml of CSF, 0.25ml of 2/3 N H₂SO₄ and 0.25 ml of 10% W/V sodium tungstate solution.
- 2) Mix thoroughly, centrifugate at 3000 RPM and pipette 2ml of protein free filtrate into a test tube.
- 3) Add one drop of indicator.
- 4) Titrate against mercuric nitrate solution.
- 5) Note the volume of mercuric nitrate solution at the end point when colourless solution turns to violet blue.
- 6) Dilute the chloride standard solution 10times and titrate against mercuric nitrate solution.
- 7) Note the volume at the end point.
- 8) Calculate with the formula.

Concentration of CSF chlorides =

$$\frac{\text{Volume of Mercuric nitrate solution against CSF}}{\text{Volume of Mercuric nitrate solution against chloride standard.}} \times 100 \text{ meq/L.}$$

Procedure for specimen of low protein content:-

- 1) To 1.8ml of distilled water taken in a test tube add 0.2ml of undiluted CSF.
- 2) Add 1 drop of diphenyl carbazone indicator.
- 3) Titrate against mercuric nitrate solution and note the volume of mercuric nitrate solution used up against CSF chlorides.
- 4) To 1.8ml of distilled water taken in a test tube, add 0.2ml of chloride standard.

- 5) Add 1 drop of indicator and titrate against mercuric nitrate solution.
- 6) Note the volume of mercuric nitrate solution used up against chloride standard and calculate with formula-

CSF chlorides =

$$\frac{\text{Volume of mercuric nitrate against CSF}}{\text{Volume of mercuric nitrate against chloride Standard.}} \times 100 \text{ meq/L.}$$

Summary

CSF is a modified tissue fluid present in sub-arachnoid space, cerebral ventricles and the central canal of the spinal cord. C.S.F. analysis is required mainly in meningitis. CSF can be withdrawn by lumbar puncture technique. Supernatant of CSF should be sent for biochemical qualitative and quantitative examination. Sediment of CSF is used for -Preparation of smears, Preparation of wet mount and India ink preparation. Sugar in CSF can be qualitatively identified by Benedict's qualitative test. CSF globulin can be determined qualitatively by Pandy's test and Ross jone's test. CSF glucose can be quantitatively determined by Folin and Wu, O-Toluidine and GOD-POD methods. CSF proteins can be quantitatively determined by turbidimetric method. CSF chlorides can be quantitatively determined by Silver nitrate titration method and Schales and Schales titration method.

Essay questions

- 1) Write the physical properties and composition of CSF.
- 2) Explain collection and distribution of CSF.
- 3) Write about physical examination of CSF.
- 4) Explain cell count of CSF.
- 5) Write about Benedict's qualitative test for CSF sugar.
- 6) Write the qualitative tests for globulin.
- 7) How do you determine CSF sugars by Folin and Wu's method?
- 8) Explain how you determine CSF sugars by o-Toluidine method.
- 9) Write about determination of CSF proteins.

- 10) Describe the method of determination of CSF chlorides by titration method.
- 11) Explain the process of determining CSF chlorides by Schales and Schales titration method.

Short Answer Questions

- 1) Write the principle of Benedict's qualitative test for CSF sugars.
- 2) How do you report Benedict's qualitative test for CSF sugars?
- 3) Give the principle of determination of CSF sugar by Folin and Wu method.
- 4) Write down the principle of O-toluidine method of determining CSF sugar.
- 5) Write about preparation of 3% sulphosalicylic acid reagent.
- 6) What is the principle of determining CSF proteins by turbidimetric procedure?
- 7) Give the composition of 6% stock protein standard.
- 8) Write the principle of CSF chlorides determination by silver nitrate method.
- 9) What is the principle of Schales and Schales method of determining CSF chlorides?

XIII - AUTOMATION AND USAGE OF COMPUTERS IN BIOCHEMICAL ANALYSIS

AUTOMATION

With changing scenario in the field of medicine, Diagnostics is given much importance than ever before. Trend of the patients in this country is getting oriented towards super speciality checkup. With this tendency alterations towards keeping the health, need arose towards accuracy, reliability and speed in diagnostics. View in a biochemistry laboratory is in the process of undergoing drastic changes these days to include automation in place of tedious, time consuming, labour requiring, costly, less accurate and less reliable procedures.

Automation is a self regulating process and results are displayed on digital units with facility of getting a printout also. With advent of automation, multi step processes are replaced by methods involving different grades of automation.

Advantages of Automation :

- 1) Time factor : Lot of time is saved. Speed of analysis is increased in automation. Doctor can take decision earlier and delay in therapy is avoided.

ex : 1½ to 2 hours time requiring determination of SGOT enzyme is reduced to a few minutes after automation.

- 2) Accuracy: Results obtained with analysers are more accurate than with other methods.

It is due to:

- 1) avoided manual errors
- 2) effective thermostating
- 3) automated wavelength adjustment etc.

Self monitoring of the process also adds to enhanced accuracy which leads to reliability.

- 3) Cost factor : Though investment cost is high, working cost is greatly reduced due to less volumes of reagents required in these methods. Time saving gives chance to perform several tests and it adds to increased income.
- 4) Spectrum of tests : With a photoelectric colorimeter only a range of wavelength is available and hence determinations of that range can only be done. On autoanalysers, available wavelength range is high and wide range of determinations can be performed with it. UV-Kinetic and end point determinations can be done with analysers.
- 5) Ease of operation : It is very easy to learn to operate it. Number of technicians can be reduced. It also adds to reduced cost factor.

Classification of Analysers: Depending on the degree of automation, analysers are classified into-

- 1) Semi autoanalysers
 - 2) Batch analysers
 - 3) Random access auto analysers
- 1) Semi auto analysers: Analysers requiring manual performance as well as mechanised performance are called as semi auto analysers. Sample-Reagent/Standard-Reagent mixing and feeding to the analyser are manually performed. Evaluation and display of the result are mechanically performed.
 - 2) Batch analysers: Analyzers which can be used to contain reagents to determine concentration of one substance (for ex: glucose) at a time are called as batch analysers. In this type of analysers, Sample-Reagent / Standard-Reagent mixing and feeding operation are also automatic.
 - 3) Random access auto analysers: They are also called as stat analysers. Analysers which can be used to contain reagents to determine concentrations of selected substances at a time are called as Random-access auto analysers. Print out of result can also be got. Fully automated

analysers are popular in developed countries where as in developing countries semiautomated analysers are widely used.

Examples of Semi automated analyzers :

<u>S.NO</u>	<u>Name of analyser</u>
1)	RA-chemistry analyser
2)	BTR-820
3)	ERBA chem-5
4)	SEAC
5)	Clinicon 4010
6)	Hitachi 4020
7)	Semi autopacer



Fig. 13-1
SEAC

Examples of Batch analysers :

- 1) Erbachem-10
- 2) Clinicon corona
- 3) Autopacer



Fig.13-2
CORONA

Examples of Random access auto analysers :

- 1) Hitachi - 704 and 705
- 2) BT - 2245
- 3) ERBA Stallion :200
- 4) Abbott spectrum
- 5) Centrifugal fast analysers

6) Dry chemistry analysers

7) 550 - Express

ERBA Chem-5 : ERBA Chem-5 is a semi automatic analyser supplied by Transasia Biomedicals Pvt. Ltd.



Fig. 13-3
Erbachem-5

Features of ERBA Chem-5 :

- 1) Analytical options available with this instrument are end point, kinetic and fixed time. Modes - mono chromatic and bichromatic.
- 2) It can display, print and memorise graphs of linear and non linear reactions.
- 3) It has open and economic reagent system.
- 4) It has 128 test memory which can be directly selected through key board.
- 5) It is simple in operation and maintenance is safe.
- 6) User-friendly software present in the instrument helps the operator step by step.
- 7) This instrument has got 256 characters liquid crystal video display.
- 8) It has got dual cuvette system.

Construction and technical specifications:

- 1) It is equipped with 8 filters for determinations of various components in body fluids and drugs. Wavelengths of light provided by 8 filters are 340, 380, 405, 492, 510, 540, 578 and 630 nm.
- 2) Printer is of full graphics thermal printer. It prints graphs and curves.
- 3) Cuvette volume is 33 mL.
- 4) It can be interfaced to an IBM-Compatible computer.
- 5) Light source is quartz halogen lamp (12V, 20VA)
- 6) Temperature control is achieved by peltier effect with electronic flow cell.

It is programmable for 25^o, 30^o and 37^oC.

- 7) It has good photometric precision.
- 8) Keyboard is 24 key membrane panelled. Photometric range is 0-2.5 O.D.
- 9) Zero setting and calibration against standard are automatic.
- 10) Computer has latest micro processor ≥ 80 . It has battery backup for permanent storage of test parameters and calibration curves for 128 tests.

Applications : It is useful for determinations of several components in biological fluids and drugs.

Profile 16-ERBA Random access analyser : It has state of art features.

Features :

- 1) It has got truly walk away random access system.
- 2) It has 48 user programmable determinations.
- 3) On board refrigeration and reagent storage at specified temperature facilities are there.
- 4) Quality control features are present.
- 5) There is facility for computation of dilution ratio for repeating pathological and hyper active sera.
- 6) There is stat facility for urgent samples.
- 7) It has facility for printing of patient reports.
- 8) Patient report storage facility on Hard disk or floppy disk.
- 9) There is facility for automatic flagging of out of range samples.
- 10) It has user-friendly key board for entering patient data which helps through help screens.

Examples of determinations on Auto analysers :

<u>Name of the determination</u>	<u>Method</u>
1) Serum Total Bilirubin	Jendrassic & Grof
2) Serum Direct Bilirubin	Jendrassic & Grof
3) Serum Indirect Bilirubin	Jendrassic & Grof
4) SGPT	UV Kinetic
5) Serum Alkaline phosphatase	Visible kinetic

6) Gamma G.T.	End point reaction
7) SGOT	UV - Kinetic
8) SBDH	UV - Kinetic
9) LDH	UV - Kinetic
10) Serum inorganic Phosphorous	Direct U.V.
11) Blood glucose	End point, UV - Kinetic

Example of Determination of auto analyser :

Determination of Total Bilirubin on autoanalyser by Jendrassic & Grof method

Refer to LIVER FUNCTION TESTS.

Automation of Flame photometry : Autoanalyser flamephotometer is available for determination of sodium and potassium .Both one channel module and two channel modules are available . One channel module determines either sodium or potassium at a time and to switch from one to other, filter is reversed.. Two channel module determines both sodium and potassium at the same time. Results are recorded on either two one-pen recorders or simultaneously on a two-pen recorder .

Automation of blood gas analysis : Blood gas analysis is also automated . Automatic blood gas analysers are in use for about more than two decades.

Blood gas analysers have warm up time of about half an hour . There are also models in which warmup time is reduced . In AVL model , warmup time is only five minutes . Figure of AVL blood gas analyser is given in blood gas analysis .

USAGE OF COMPUTERS IN BIOCHEMICAL ANALYSIS

Computing has developed drastically from abacus of olden days to the present day computer . Computer was first invented by Charles Babbage . The present day computer is its development . Now a days computers find applications in every walk of life . With the advent of computerisation of every field , medical field also has utilised its application in several aspects including clinical data storage, processing , medical records maintenance , diagnostics , pharma care etc. Computer aided diagnostics has its own importance in modernisation of medical & health services . In a medical lab , it finds application in reception , registration

,printing the reports & automation of several instrumental techniques in different specialisations of medical lab technology including biochemistry , microbiology , clinical pathology , histopathology , blood bank etc . It is already in use by specialists of medical community in endoscopy and other latest techniques related to diagnostics , therapeutics and surgery.

Computer finds application in biochemistry in automated analysis , quality assurance , research and development , storage of analysed data etc . In view of the above , a lab technician involved in biochemical analysis as well as other divisions is required to be well versed in computer fundamentals . Auto analysers run the quantitative determinations automatically aided by computerised programming . Auto analysers have key board facility to operate programmable determinations , display , print out patient reports, storage facilities on hard disk. Help screens assist the technician to perform automated biochemical analysis .

What is a computer ?

A computer is an electronic machine to input , memorise , process and output data .A computer consists of input unit , memory unit , process unit and output unit .Main characteristics of a computer are its speed , accuracy and versatility to perform a variety of tasks , automatic programme executability without human intervention and capacity to carry out repetitive tasks routinely any number of times .Commonly used input units are punched card reader , floppy disk reader , video terminal etc . The form in which data is memorised in computers are characters and numbers . Characters include letters and special symbols .Other type of data are decimal numbers such as 1234 , 485 etc .A processing unit in a computer interprets instructions in a programme and carries them out. The most common output units are line printers and character printers.

Fig.13-4 Diagram of a computer and accessories

Software of a computer : List of instructions to be executed by a computer are called software of a computer . Computer software can be conveniently classified into application software and system software . Application software is set of programmes necessary to carry out operations necessary for a specified application .System software are general programmes written for the system which provide the environment to facilitate the writing of application software .

Programming language : Sequence of machine instructions is called a machine language programme . Computer programming languages are developed with the primary objective of facilitating a large number of people to use computers without knowing the internal structure of a computer . Most commonly used high level languages are FORTRAN , COBOL , BASIC , PASCAL , SNOBOL and LISP etc .

Hardware of a computer : Electronic circuits used in building the computer that executes software is called hardware of a computer .

What is bioinformatics ?

Bioinformatics is used to describe the application of information science and technology in life science . It was first used in mid 1980s . It can be defined as use of computers to retrieve , process , analyse and simulate biological information . Even narrower definition of it is “application of information technology to the management of biological data”.A more appropriate definition of bioinformatics is “It is the science of how information is generated , transmitted, received and interpreted in biological systems ”.

What is internet ?

Internet is interconnection of one computer with other .Network can link people .Exchange of information can be done through E - mail , Voice mail etc

Things of need for internet accessibility :

- * a computer
- * account with service provider
- * a modem

Website : Each place which is visited on web is called website .

Buzz words : Each website has its own unique URL (Uniform resource locator). Example - www . wherever . com

Uses of network :

- * Centralised storage of information
- * Sharing of data
- * Remote access to data
- * Communication and access to information

Types of network :

Different types of network are -

1. Local area network which is limited to interconnecting 20 - 30 computers . It is a network through cable . Range is 1 - 20 KM.
2. Metropolitan area network which is interconnection of computers in an organisation .
3. Wide area network which provides countrywide or worldwide connectivity.

E - Mail : For doing e-mail some kind of e-mail programme is needed .ex - Microsoft outlook , Microsoft outlook express etc .

Things of need for e-mail :

- * e-mail address of the addressee
- * incoming mail server
- * outgoing mail server
- * e-mail user name of the sender
- * e-mail password of the sender

Chatting : Online chit-chat is called chatting

Video conferencing : Coming into communication online using web cameras and switching to audio and video is called videoconferencing .

All these channels of communication can be applied to get access to the latest developments in medical technology and to connect to specialists in diagnostics .

Summary

Automation is a self regulating process and results are displayed on digital units. Autoanalysers are classified into 1) semi-auto analysers, 2) Batch analysers and 3) Random access auto-analysers. Auto analyser methods are available for several constituents of body fluids. Automated methods are advantageous over older manual methods.

Computer is used in diagnostics to store , process & print clinical data ,reports and to aid in automated performance of medical lab processes . Computer aided channels of communication help in updating to the latest developments and also to getting connected to specialists in the field .

Essay Questions

- 1) Write about advantages of automation in Biochemistry lab.
- 2) Classify analysers.
- 3) Give examples of semi automatic, batch and random access auto analysers supplied by manufacturers.
- 4) Write about ERBA Chem-5
- 5) Write about profile 16-ERBA Random access analyser.
- 6) List out determinations on auto analysers
- 7) Write about computer applications in biochemistry .

Short Questions

- 1) What is automation?
- 2) Define semi auto analysers.
- 3) What are batch analysers?
- 4) What is a random access auto analyser?
- 5) What is a computer ?
- 6) Mention some uses of computer in biochemical analysis .
- 7) What is internet ?
- 8) Explain e-mail .
- 9) What is chatting ?

XIV - QUALITY ASSURANCE IN BIOCHEMICAL LABORATORY

a) INTRODUCTION AND IMPORTANCE OF QUALITY ASSURANCE , GENERAL PRINCIPLE

Quality control of analytical methods used in diagnostic testing procedures will help in achieving accuracy. Quality control is possible by well organized, adequately staffed and accurately performed processes. Assurance of accuracy in results is needed for accurate diagnosis and hence for deciding the right treatment at right time. Quality control system must be established in Diagnostic centers. Certain basic operational rules have to be established to make quality assurance system function effectively. Quality assurance is an excellent means of improving laboratory efficiency. Concept of quality control refers to the process of striving to achieve perfection without errors. Primary responsibility of maintaining quality lies with personnel involved in diagnosis. Removal of responsibility from personnel involved in diagnosis can result in imperfect quality control. Quality assurance personnel must establish control points to monitor quality. These begin with pure chemicals / reagents used in diagnosis, instruments, skilled and trained personnel involved in diagnostic analysis.

Parameters of quality analysis :

Parameters of quality analysis are -

- 1) *Specificity*
- 2) *Sensitivity*
- 3) *Linearity*
- 4) *Precision*
- 5) *Accuracy*
- 6) *Ruggedness:*
- 7) *System suitability*

1) *Specificity:* It is an important quality criterion. Analysis of a component of a mixture can interfere with other components of that mixture. If interference takes place, method is non-specific. If interference does not occur, method is

specific.

2) *Sensitivity:* Sensitivity is the limit of detection. When a sample contains a component in very low concentration, signal from the instrument will also be small. In such a case it is uncertain whether the signal is due to noise of the instrument or due to component.

3) *Linearity:* Linearity of a method gives characteristic trend of parameters such as absorbance, peak heights, peak area or response ratio as a function of concentration of component to be measured. At least five different concentrations should be employed between 80-120% or 50-150%. By plotting concentration versus response, linearity of observed data points can be visualised.

4) *Precision:* Precision indicates how close are two measured values to each other. Precision does not imply accuracy. Accuracy expresses correctness of a measurement whereas precision expresses reproducibility. Precision always accompanies accuracy. High degree of precision does not imply accuracy. Precision of a method is usually established during development stage by the multiple analyses of the sample judged to be typical of the material to be analyzed. These analyses usually do not account for any additional sources of variation such as day to day variation, lab to lab variation, small modification in the technique, varying skill of technicians, undetected instrumental factors, and other unexpected system errors.

Mean deviation or relative mean deviation is a measure of precision.

5) *Accuracy:* Accuracy is defined as nearness of a measured value to its true value. It normally refers to the difference between mean of the set of results and value accepted as true or correct value for the quantity measured. There are two ways of determining accuracy. They are - a .Absolute method

- b .Comparative method

a .Absolute method : Synthetic sample is made which contains known amounts of constituents. Primary standards have to be used, which are commercially available or prepared by the analyst and they should be purified rigorously. As a rule, results of analysis of unknown are compared with results of analysis of

standards. Analyst should prepare six samples of standard in 80% to 120% or often 50% to 150% of the expected content and assaying each of those prepared samples. Acceptance criterion in the accuracy test is expressed in terms of standard deviation of the method as determined in precision test. It is also required to know the effect of presence of foreign substances. It is necessary to know the influence of as many substances as feasible.

b. Comparative method : When it is not possible to prepare synthetic sample containing constituents in desired composition, standard samples of material, whose content of constituents has been determined by possibly accurate methods of analysis is useful. Though this method involving secondary standards is theoretically not satisfactory, it is useful in applied analysis.

6) *Ruggedness*: Ruggedness tests describe influence of small but reasonable changes in the procedures of quality of analysis. Ex:- Source and age of reagents, heating rate, humidity, voltage fluctuations, thermometer errors, column temperature, variations of column to column, technician to technician and instrument to instrument etc. Eight measurements are enough to investigate seven variables when appropriate experimental design is employed. Various types of inter laboratory checks should also be carried out. It is necessary to check the length of shelf life of prepared solutions or reagents.

7) *System suitability*: These studies study the reliability of performance of a given analytical system on a given day. System in this context means all components such as technicians, hardware, solvents, electronics considered together. System suitability tests are composed of system's measurement precision and system's power of resolution.

To select appropriate method, analyst should have thorough knowledge of physico chemical properties of the substance being determined, its degradation products, degradation mechanisms and degradation reaction rates.

Evaluation of some of the analytical methods:

Some of the analytical methods are -

- 1) Electrometric methods
- 2) Solvent extraction methods
- 3) Spectrophotometric methods
- 4) Chromatographic methods

- 1) *Electrometric methods*: Titrimetric methods can be used for precise analysis of substance being determined. Often they do not offer desired specificity for analysis of component being determined. Titrimetry may be used when degradation products do not interfere with titration. When suitable procedures for eliminating the interferences are available, one can use titrimetry for analysis with component specificity.
- 2) *Solvent extraction methods* : It is possible to extract acidic, neutral or basic compounds from organic solvents selectively on the basis of partition behavior of their ionised and unionised species. Double extraction procedure provides some degree of specificity.
- 3) *Spectrophotometric methods* : Direct spectrophotometric methods such as spectrophotometry in visible range and U.V. range usually lack selectivity. Selectivity or specificity can be improved through separation of appropriate group. IR analysis is primarily for quantitative determination of degradation products of the components to be determined. Nuclear magnetic resonance (NMR) spectroscopy offers specificity along with simplicity of operation. But it too lacks sensitivity and precision.
- 4) *Chromatographic methods* : Some of the chromatographic methods used in analysis are paper, thin layer, column, gas, liquid, ion exchange and electro chromatographies. GLC and HPLC prove most useful for quantitative determinations.

Automated systems of analysis :

Automation usually enhances quality, quantity and efficiency of an operation. Introduction of automation into clinical analysis has dramatic change in the traditional look, capability, precision and acceptability. Use of automation in instrumental

methods of clinical analysis, data handling, and data storage is on the raise. Automated chemical, microbiological, enzymatic and other procedures evolved. Automated continuous testing has potential application in routine testing. Automated procedures enhance reliability of data, provide immediate feed back on process control and save time.

In a clinical laboratory, where sufficiently large number of similar specimens must be subjected to similar types of examination routinely, automated methods of analysis provide more efficient and precise testing than manual methods. Many laboratories have found it convenient to utilize automated methods as alternative to the traditional methods.

Maintenance, storage and retrieval of records:

Proper control of records in analytical operation are useful for quality analysis.

Comparision of results : There are three common methods for comparision of results. They are - a. Student's t - test b. Variance ratio test (or F - test)
c. Chi square distribution

Student's t - test is useful for small samples. Its purose is to compare the mean from a sample with some standard value. Variation ratio test is used to compare the precision of two sets of data. Chi square test is used to determine whether or not a set of data has difference significantly from a theoretical or defined distribution.

Errors:

Errors in quantitative analysis can be classified in to-

- I. Determinate or constant errors.
- II. Indeterminate or random errors.

I. Determinate errors : They are also called as constant errors. A determinate error is one which can be determined or approximately calculated and measurements can be corrected accordingly.

Types of determinate errors: 1) Instrumental errors 2) Reagent errors
3) Personal errors 4) Operational errors 5) Additive errors

6) Proportionate errors 7) Errors of method

1) Instrumental errors : They are caused due to errors in the instruments and glass ware used. For ex: a) Weighing errors caused by arms of unequal length of balance, Improperly calibrated weights b) Incorrectly graduated burettes cause errors in measuring liquids and enchance errors in quantitative determinations by titrimetry.

2) Reagent errors : Errors arising due to impurities in the reagents are called as reagent errors. If pure reagents are not available, degree of impurity must be measured and correction must be made accordingly. Attack of reagents upon glassware, porcelain etc. result in introduction of foreign substances.

3) Personal errors : Constitutional inability of some individuals cause personal errors. Constitutionally some individuals cannot make certain observations accurately. Personal errors are also caused by personal carelessness and employing of improper techniques by the personnel .

Ex:- a) Errors in reading a burette.

b) Errors in judging end point in visual titrations sharply.

b) Loss of material in various steps of an analysis.

c) Improper washing of precipitate.

d) Use of reagents containing impurities when pure chemicals are available.

4) Operational errors : These are errors arising in operation. These are due to factors for which individual is responsible. They are mostly physical in nature and occur when sound analytical technique is not followed. Ex - loss of materials in various steps.

5) Additive errors : An additive error is one which is not dependent on the quantity of the component being determined. It can be revealed by taking samples of different weights.

6) Proportionate errors : It is one which is proportional to the quantity of components being determined. A proportional error may arise from an impurity in a standard substance. Ex - ignition of various weights at appreciably lower temperature.

7) Errors of method : These are errors originating from incorrect sampling and also due to non completion of a reaction. In quantitative analysis, errors can occur owing to failure of reactions to proceed to completion, occurring of induced and side reactions, reaction of substances other than the substance being determined and difference between observed end point and stoichiometric end point.

II. Indeterminate errors : They are also called as random errors. They are accidental and the technician has little or no control. They are difficult to define. But their existence is revealed by measurement of a quantity.

Indeterminate errors are two types.

- 1) Errors due to variation within determinate errors : For ex: errors due to worn out knife edge of a balance .
- 2) Erratic errors: It is difficult to pin point such errors. For ex: fluctuations in balance room can cause erratic errors in weighing.

If a large number of observations are made, it shows that -

- a) Small errors occur more frequently than the large.
- b) Large errors occur infrequently.
- c) Positive and negative errors of same numerical magnitude are likely to occur equally.

b) INTERNAL AND EXTERNAL QUALITY CONTROL

There are two steps in quality control in a clinical laboratory.

- 1) Internal quality control.
- 2) External quality control.

1) Internal quality control: Control of quality within internal sources is called as internal quality control. Minimisation of errors is very important in internal means of quality assurance. Internal quality control can be maintained by -

1) Use of standardised glassware, reagents and instruments : All instruments used in analysis should be calibrated. Glassware like flasks, burettes, pipettes should be calibrated. Weights used must be calibrated. Reagents used must be standardised. This will minimise instrumental and reagent errors.

2) Employing conscientious and well trained staff : Staff employed must be well trained and qualified . They should have analytical aptitude and they should have constitutional ability of analysing. They should be alert and should not be careless while conducting analysis .

3) Use of quality reagents : Reagents used must be of good quality. Standard solutions used in quantitative methods of analysis must be prepared by 'Analar' grade or 'G.R.' chemicals. Where AR & GR quality is not needed and when purity within certain limits is required, GPR quality is used. Reagents used in quantitative analysis have to be standardised prior to use. L.R. quality can be used for preparation of qualitative reagents such as Fehling's solution, Selvinoff's reagent, Benedict's reagent, Fouchet's reagent etc.

4) Employing appropriate methods of analysis : If appropriate methods of analysis are not used it will cause method errors. Minimisation of method errors is important in internal quality control.

5) Employing independent methods of analysis : In some cases, accuracy can be established by carrying out analysis using a different method and results have to be tallied. Results obtained by two different methods should be concordant. Values can be treated as correct within small limits of deviation.

6) Running of parallel determinations : Parallel determinations can be run for the purpose of serving as check on result. They indicate only the precision of analysis. Variations should be too small. If variations are found to be large, determinations have to be repeated until satisfactory agreement arrives.

Phases in internal quality control : There are two phases in internal quality control. They are - a) Preventive phase b) Retrospective phase.

a) Preventive phase : In preventive phase, precautions are taken to prevent

errors in specimen collection, serum / plasma separation, specimen analysis, photometric readings, calculation of test values etc.

b) Retrospective phase : Retrospective phase includes comparison between optimum conditions variance (OCV), routine condition variance (RCV). O.C.V. refers to results obtained under optimum conditions by using freshly prepared reagents and by using standardized 'A' grade glassware. R.C.V. refers to results obtained by using routine reagents and regularly used glass ware. Difference between OCV and RCV should not be more than 3%.

2) External quality control : Quality control from external sources is called as external quality control. Some of the ways are by-

1) Counter checking with other labs : Quality can also be checked by sending a specimen analysed in our laboratory to other well recognised laboratory , tallying the results got in our laboratory with those got in the other laboratory . There should be concordance of results . If there is any variation, it should be very little. A record of counterchecking should be maintained.

2) Analysis of specimen brought from other well recognised labs and comparative study : Specimens brought from other well recognised labs can be tested in our laboratory and results have to be studied from comparative aspect. Standard substances and standard solutions brought from other labs have to be tested in our laboratory and comparative study with purity testings of our standard substances and standard solutions has to be undertaken. A record also should be maintained for this activity also.

3) Occasional exchange of primary standards with well recognised labs : There should be occasional exchange of primary standards with other well recognised labs. A record has to be maintained for this data also. This will also enable steps of confidence enhancement and act as one of the quality control measures from external means.

Summary

Quality control of analytical methods help in achieving accuracy. Parameters of quality analysis are specificity, sensitivity, linearity, precision, accuracy, ruggedness, system suitability. Some of the analytical methods are electrometric methods, solvent extraction methods, spectrophotometric methods, chromatographic methods. Automation enhances quality. Proper control of records are useful for quality analysis. Errors can be classified into determinate or constant errors and indeterminate errors. Determinate errors are instrumental errors, reagent errors, personal errors, additive errors, proportionate errors etc. Indeterminate errors are errors due to variation within determinate errors, erratic errors etc. Quality control in a clinical laboratory is two types. Internal quality control and external quality control.

Essay Questions

- 1) What are different parameters of quality analysis? Explain.
- 2) Write about evaluation of some of the analytical methods.
- 3) What are different errors? Explain.
- 4) Write about quality control in a clinical laboratory.

Short Answer Questions

- 1) Write about specificity.
- 2) What is meant by precision?
- 3) Mention some analytical methods.
- 4) What are major classes of errors?
- 5) What are different types of quality control?
- 6) What is meant by internal quality control?
- 7) Mention the phases in Internal quality control.
- 8) Explain OCV and RCV.
- 9) Mention the ways of external quality control.

XV - DIAGNOSTIC TESTS

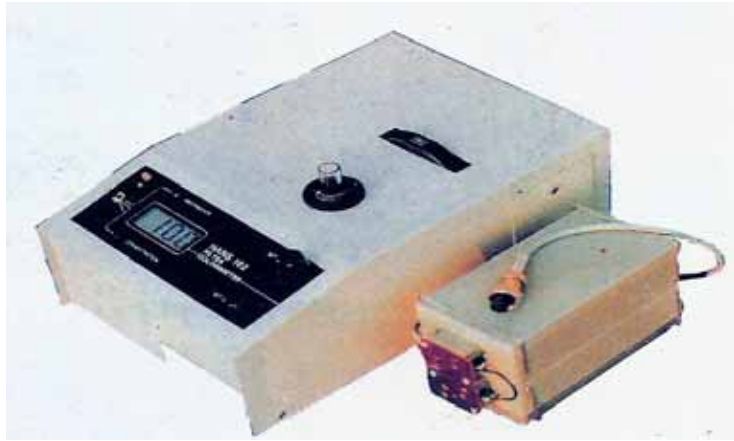


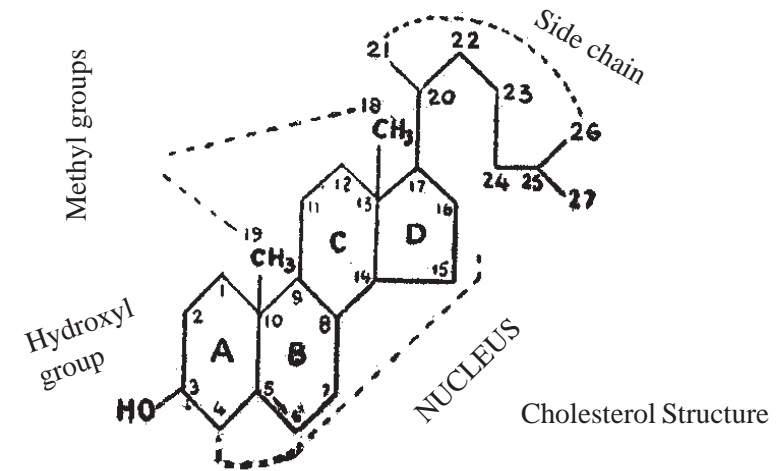
Fig.15-1 Digital photo electric photometer

a) LIPID PROFILE :SERUM CHOLESTEROL,HDL CHOLESTEROL

Lipid profile : lipid profile is profile of determinations significant in diagnosis of primary and secondary dyslipoproteinaemias . Important lipid profile tests are -

- 1) Total lipids
- 2) Serum total cholesterol
- 3) Serum HDL cholesterol
- 4) Total cholesterol/HDL cholesterol ratio
- 5) Serum triglycerides
- 6) Serum phospholipids
- 7) Electrophoretic fractionation of lipoproteins to determine
 - a) Chylomicrons
 - b)L.D.L.(Low density lipoprotein)
 - c)V.L.D.L.(Very low density lipoprotein)
 - d)H.D.L.(High density lipoprotein)

Cholesterol: Cholesterol is the most abundant steroid in animal tissues. Steroids are derivatives of perhydro cyclopentanophenanthrene. Perhydrocyclopentano phenanthrene is saturated tetracyclic hydrocarbon.



Structure of cholesterol : Cholesterol is a steroidal alcohol containing hydroxyl group at carbon-3 of ring A, branched aliphatic chain of 8 carbons at carbon 17 of ring 'D', methyl groups attached to carbon-10 and carbon-13 and double bond between carbon-5 and carbon-6. Cholesterol is present in plasma membranes of animal cells and in lipoproteins of plasma. Its melting point is 150°C. It is water insoluble. It can be extracted from animal tissues by using organic solvents such as Benzene, Chloroform, Ether or hot alcohol etc. Cholesterol is the precursor of many other steroids in animal tissues - bile acids, androgens, estrogens, progesterone and adrenocortical hormones.

Determination of Total cholesterol :

Clinical Significance: Determination of serum cholesterol is significant from diagnostic point of view.

Cholesterol levels are increased in-

- 1) Cardiovascular diseases and atherosclerosis
- 2) Type II familial hyper cholesterolaemia
- 3) Obstructive jaundice
- 4) Diabetes miletus
- 5) Hypothyroidism
- 6) Obesity

- 7) Nephrosis
- 8) Xanthomatosis etc.

Cholesterol levels are lowered in-

1) *Conditions like :*

- a) Liver disease
 - b) Hyperthyroidism
 - c) Anaemia
 - d) Stress
 - e) Sepsis
 - f) Antibiotic therapy in which there is impairment of absorption from G.I.T.
- 2) Pernicious anaemia
 - 3) Haemolytic jaundice
 - 4) Hyperthyroidism.
 - 5) Final stages of cancer
 - 6) Hypolipo proteinaemias
 - 7) Severe infection

Instructions to the patients :

- 1) 12 hours fasting before test.
- 2) Normal dieting for 7 days prior to testing.
- 3) Non consumption of alcohol for 24 hours prior to testing.
- 4) Avoiding lipid lowering drugs as Estrogen, Oral contraceptive pills, Salicylates etc.

Note: Water is allowed.

Name of the method : Watson method

Principle : Cholesterol on reaction with acetic anhydride in presence of glacial acetic acid and conc. Sulphuric acid gives green coloured complex. Colour intensity is proportional to the concentration of cholesterol in serum.

Requirements :

- 1) Test tubes.
- 2) Serological pipettes.

- 3) Dispensers.
- 4) Photo electric colorimeter.
- 5) Push button pipette.

Reagents:

- 1) Dimethyl benzene sulfonic acid reagent :

Composition :

- | | |
|--|----------|
| 2,5 - dimethyl benzene sulfonic acid-5.6g. | |
| Glacial acetic acid | - 200ml. |
| Acetic anhydride | - 300ml. |

Method of preparation :

Mix 5.6g of 2, 5 dimethyl benzene sulfonic acid with 200ml of glacial acetic acid and 300ml of acetic anhydride.

Storage & Stability: This reagent is stored in amber coloured bottle. It is stable when stored in amber coloured bottle at room temperature for one year.

- 2) Concentrated Sulphuric acid.
- 3) Cholesterol stock standard (1% W/V):

Composition: Cholesterol-1g.

Glacial acetic acid- upto 100ml.

Preparation: It is prepared to contain a concentration of 0.1/W/V with glacial acetic acid as solvent.

Stability: This reagent is stable at 2-8°C.

- 4) Cholesterol working standard(200mg%).

Composition :

- | | |
|----------------------------------|--|
| Cholesterol stock standard-10ml. | |
| Glacial acetic acid-up to 50ml. | |

Method of preparation: Dilute 10ml of cholesterol stock standard to 50ml with glacial acetic acid. It will have a concentration of 200mg%.

Stability: This reagent is stable at 2-8°C.

Specimen: Serum.

Note: Hemolysed and Icteric sera shall not be used in this test.

Wave length: 575nm (green-yellow filter)

Procedure :

- 1) Take three tubes and label them as T, S and B.
- 2) Pipette in to them as follows.

S.NO	Reagent/sample	Test	Std.	Blank
1)	DBSA reagent	2.5ml	2.5ml	2.5ml
2)	Serum	0.1ml	-	-
3)	Cholesterol working std	-	0.1ml	-
4)	Distilled water	-	-	0.1ml

- 3) Mix well and keep at room temperature for 10minutes.
- 4) Read the O.D. values of test and standard against blank.
- 5) Calculate the concentration of cholesterol with the formula-

$$\text{Serum total cholesterol} = \frac{\text{OD of Test}}{\text{OD of Standard}} \times 200 \text{ mg\%}$$

Normal range: 150-250mg%

ENZYMATIC METHOD OF DETERMINATION OF SERUM TOTAL CHOLESTEROL (auto analyser method):

Principle : Cholesterylesters are hydrolysed by the action of enzyme chlesterylester hydrolase to give cholesterol and fatty acids. Free cholesterol and cholesterol obtained by enzymatic hydrolysis are oxidised to cholestone 4-en 3-one by the action of the enzyme cholosterol oxidase. H₂O₂ is also produced due to enzymatic oxidative action on cholesterol and is subjectrd to enzymatic action of peroxidase. Nacent oxygen liberated in this reaction reacts with 4-amino phenazone and phenol to form a coloured compound. Colour intensity is proportional to the concentration of total cholesterol.

Reagents:

- 1) Buffered enzyme-chromogen reagent (PH-7,Conc-M/10)

Composition :

Cholesterylester hydrolase	-	10units.
Cholesterol oxidise	-	15units.
Peroxidase	-	3500units.
4-Aminophenazone	-	50mg.
Sodium azide	-	30mg.
Phasphate buffer	-	upto 100ml.

Stability: This reagent is stable at 2-8⁰C.

- 2) Phenol reagent (30mg/dl) :

Composition :

Phenol	-	30mg.
Distilled water	-	upto 100ml.

Stability: This reagent is stable at 2-8⁰C.

- 3) Cholesterol stock standard : (1%W/V)

Refer to Watson method of detemination of total cholesterol.

- 4) Cholesterol working standard (200mg%) :

Refer to Watson method of determination of total cholesterol.

- 5) Working Reagent :

Composition :

Buffered enzyme-chromogen reagent-2 parts.

Phenol reagent-1 part.

Method of preparation :

Mix 2 parts of buffered enzyme-chromogen reagent and 1 part of phenol reagent.

Stability : This reagent is stable at 2-8⁰C for 12hours. Store it in amber colored bottle.

Sample : Serum.

System parameters:

Reaction type :	End point.
Wave length :	510 nm.
Flow cell temperature :	25 ⁰ C.
Incubation temperature :	37 ⁰ C.
Incubation time:	20 minutes.
Sample volume:	0.01 ml.
Concentration of Std:	200 mg%.
Zero setting:	With reagent blank.

Procedure :

- 1) Take three tubes and label them as 'T', 'S' and 'B'.
- 2) Pipette in to them as follows

S.No.	Reagent/Sample	Test	Std	Blank
1	Working reagent	1ml	1ml	1ml
2	Serum	0.01ml	-	-
3	Working Std	-	0.01	-
4	Distilled water	-	-	0.01ml

- 3) Mix well and keep at 37⁰C for 20 minutes.
- 4) Read the O.D.values of test and standard against blank.
- 5) Calculate the conc. of serum total cholesterol with the formula-

$$\text{Serum total cholesterol} = \frac{\text{OD of Test}}{\text{OD of Std.}} \times 200 \text{ mg\%}$$

- 6) If the values are above 500mg% dilute the serum specimen and multiply the determined value with 10.

Enzymatic method of determination of serum total cholesterol using colorimeters/spectrophotometers:

Principle, Reagents, and sample:- Same as in Enzymatic method on auto analysers.

Wave length: 510 nm.

Procedure: use the following volumes instead of volumes used in method on

autoanalysers.

Working reagent	-	1ml.
Serum	-	0.02ml.
W.standard	-	0.02ml.
Distilled water	-	2ml.

Determination of HDL cholesterol :

Clinical Significance: Determination of HDL cholesterol is clinically significant in diagnosis of coronary heart disease. Lower levels of HDL cholesterol indicate increased risk of coronary heart disease. Determination of serum total cholesterol and HDL cholesterol help in indicating the risk of coronary heart disease.

Watson method of determination of HDL Cholesterol :

Principle : Phosphotungstic acid and magnesium chloride precipitate LDL, VLDL & chylomicrons. HDL cholesterol left in the supernatant can be determined usually.

Requirements: 1 to 5 same as in Watson's method of determination of serum total cholesterol.

6) Centrifuge.

7) Centrifuge tubes.

Reagents : 1 to 3 : Same as in Watson's method of determination of serum total cholesterol.

4)Phosphotungstic acid reagent :

Composition:

Phosphotungstic acid	-	2.25g.
IN Sodium hydroxide	-	8ml.
Distilled water	-	42ml.

Method of preparation:

Dissolve 2.25g of phosphotungstic acid in 8ml of IN Sodium hydroxide solution and 42ml of distilled water.

Stability : This reagent is stable at room temperature for several months.

5) Magnesium chloride solution:-

Composition:

Magnesium chloride	–	40.68g.
Distilled water	–	up to 100ml.

Method of preparation:

Dissolve 40.68g of magnesium chloride in 75ml of distilled water and dilute to 100ml with distilled water.

Stability: This reagent is stable at room temperature.

6) Cholesterol stock standard :

Refer to Watson method of determination of total cholesterol.

Stability: This reagent is stable at 2-8°C.

7) Cholesterol working standard solution(100mg%) :

Composition :

Cholesterol stock standard	-	5ml.
Glacial acetic acid	-	upto 50ml.

Method of preparation:

Dilute 5 ml. of stock standard to 50ml with glacial acetic acid.

Stability: This reagent is stable at 2-8°C.

Wave Length: 575 nm (Yellow-Green filter).

Specimen: Serum.

Procedure:

- 1) In a centrifuge tube, take 0.5ml of serum, 0.05ml of phosphotungstic acid reagent and 0.02ml of MgCl₂ reagent.
- 2) Mixwell and centrifuge at 3000 RPM for 20 minutes and collect supernatant.
- 3) Take three tubes and label them as T, S and B.
- 4) Pipette into these tubes as follows.

<u>S.No.</u>	<u>Reagent / Sample</u>	<u>Test</u>	<u>Std</u>	<u>Blank</u>
1)	DBSA reagent	2.5ml	2.5ml	2.5ml
2)	Supernatant	0.1ml	–	–
3)	Cholesterol working std.	–	0.1ml	–

4) Distilled water – – 0.1ml.

- 5) Mix well and keep in water bath at room temperature for 5minutes.
- 6) Add 0.5ml of conc. H₂SO₄ to each tube.
- 7) Mix thoroughly and cool to room temperature by keeping in water bath at room temperature.
- 8) Read O.D. values of Test and Standard against Blank.
- 9) Calculate the concentration of HDL cholesterol with the formula.

$$\text{Serum HDL cholesterol} = \frac{\text{OD of test}}{\text{OD of std.}} \times 114 \text{ mg\%}$$

Normal values: Men – 30-60 mg%.
Women – 40-70 mg%.

b) GLYCOSYLATED HAEMOGLOBIN

Clinical significance: Determination of glycosylated haemoglobin is clinically significant because its level is elevated in diabetic patients. Red cells of normal human being contains three genetical species of haemoglobin. They are HbA, HbA₂ and HbF. Other variants are HbA₁ a, HbA₁ b & HbA₁ c. These are collectively measured as HbA₁. It reflects average blood sugar concentration over an extended period of time. Its determination helps in preventing diabetic complications.

Colorimetric method of determination of glycosylated haemoglobin:

Principle : Red cells are washed with normal saline 4-6 times to remove interfering substances. Carbon tetrachloride is used to prepare haemolysate. Hexoses bound to haemoglobin are hydrolysed by heating haemolysate. Haemolysate is heated at 100°C in presence of oxalic acid. Resulting chromogen is measured colorimetrically.

Reagents :

- 1) 0.9%.W/V. Sodium chloride solution (Normal saline) :

Composition :

Sodium chloride : 0.9g.

Distilled water : up to 100ml.

Method of preparation : Dissolve 0.9 g. of sodium chloride in 75ml of distilled water and dilute to 100ml with distilled water.

Stability: This reagent is stable at room temperature.

2) Carbon tetrachloride .

3) Drabkin's solution.

Composition :

Potassium ferri cyanide - 400mg.

Potassium dihydrogen phosphate - 280mg.

Potassium cyanide - 100mg.

Nonidet - 1ml.

Distilled water - upto 1 litre.

Stability : This reagent is stable at 2-8°C in a polyethylene container.

4) 0.3 N oxalic acid:

Oxalic acid solution is prepared to contain concentration of 3N.

Stability: This reagent is stable at room temperature.

5) 40% W/V Trichloro acetic acid:

Stability: This reagent is stable at room temperature.

6) 0.1% W/V Thiobarbituric acid.

Stability: This reagent is stable at room temperature.

Sample: EDTA or Heparin added blood.

Wave length - 443 nm.

Procedure :

1) Prepare Test as follows.

a) Centrifugate blood specimen.

b) Aspirate plasma and buffycoat.

c) Wash packed cells with normal saline for six times atleast.

d) Add one quarter of distilled water to packed cells and one quarter of carbon tetrachloride.

e) Centrifugate for 20 min. at 3000 RPM.

f) Aspirate haemolysate and determine Hb content.

g) Adjust Hb conc. to 10g% with Normal saline using the formula $C1V1=C2V2$.

h) To 1.36 ml. of haemolysate, add 0.64ml of normal saline.

i) To 20 ml. of haemolysate, add 1ml of oxalic acid, mix well, keep in boiling water-bath for one hour.

j) Cool the tubes to room temperature.

k) Add 1 ml. of trichloro acetic acid reagent and mix well.

l) Centrifugate at 3000 RPM and collect supernatant.

m) Add 0.5ml of thiobarbituric acid to 2ml of supernatant and mix well.

n) Keep at 37°C for 40 minutes.

2) Prepare blank by adding 0.5ml of thiobarbituric acid to 2ml of distilled water.

3) Read the optical density of test against blank.

4) Calculate glycosylated heemoglobin with formula.

$$\text{Glycosylated heemoglobin} = \frac{\text{OD of Test}}{0.029} \times 1$$

Normal values : 4-7%

Determination of Glycosylated haemoglobin by Ion exchange-colorimetry method :

Principle: Haemolysate is first prepared and subjected to Ion exchange with cation exchange resin. Nonglycosylated haemoglobin left in the supernatant is determined colorimetrically.

Requirements:

1) Plastic tube

2) Vortex mixer

3) Ion exchange resin

4) Photo electric colorimeter

5) Cuvette

Reagents: 1) EDTA.

2) Lysing reagent.

3) Lyophilised control.

Storage: 2 & 3 Reagents should be stored in refrigerator.

Procedure:

- 1) Prepare Haemolysate by mixing 0.5ml of lysing reagent to 0.1ml of EDTA added blood.
- 2) Take 3ml of Ion exchange resin into plastic tube by pipetting.
- 3) Add 0.1ml of haemolysate to Ion exchange resin taken in plastic tube.
- 4) Mix the contents in the plastic tube well on a vortex mixer for about 5 minutes.
- 5) Separate the resin.
- 6) Pour supernatant into a burette and read the O.D at 415 nm against distilled water taken as blank. This is O.D. of GHb.
- 7) Take 0.02 ml of haemolysate prepared in step-1 into a cuvette containing 5ml of distilled water.
- 8) Read the O.D. against distilled water taken as blank at 415nm. This is O.D. of THb.
- 9) Calculate with the formula-

$$\text{GHb} = \frac{\text{OD of GHb}}{\text{OD of THb}} \times 10 \times \text{Temperature factor (T}_f\text{)}.$$

$$T_f = 1 \text{ at } 23 \pm 2^\circ\text{C}.$$

$$T_f = 0.7 \text{ at } 30^\circ\text{C}.$$

c) SERUM CALCIUM

Clinical Significance : Calcium is the major mineral element of body. Mainly, it is present in bones and teeth. It is present in body fluids in small concentrations. Normal range of total serum calcium ranges from 9-11 mg/dL. Factors influencing

elevation of serum calcium levels are hyperparathyroidism, hypervitaminosis D etc. Factors influencing lowering of serum calcium levels are improper absorption of calcium from intestines, hypoparathyroidism, vitamin D deficiency etc.

Hypercalcaemia : Condition of elevation of serum calcium above normal range is called hypercalcaemia. It can occur in hyperparathyroidism, hypervitaminosis D, multiple myeloma etc.

Hypo Calcaemia : Condition of lowering of serum calcium below normal range is called hypocalcaemia. Hypocalcaemia occurs in hypoparathyroidism, rickets, osteoporosis etc.

Titrimetric method of determination of serum calcium :

Principle: This determination is a titrimetric procedure. It depends on reaction between 1) 'Calcium' present in serum and EDTA.

2) 'Calcium' present in Ca-standard and EDTA.

Reagents:

1) ***2N Sodium hydroxide solution :***

Composition :

Sodium hydroxide-40g.

Distilled water up to – 500ml.

Method of preparation:

Dissolve 40g of sodium hydroxide in 350ml of water and dilute to 500ml with distilled water and standardise.

Stability : This reagent is stable at room temperature.

2) ***Indicator solution :*** Alcoholic solution of calcon is used as indicator. This gives purple colour when calcium is in the solution. It changes blue at the end point of the reaction.

3) ***Stock solution of EDTA (0.05M) :***

Composition: EDTA-14.8g.

Distilled water: up to 1 litre.

Method of preparation :

Dissolve 14.8g of EDTA in about 750ml of water and dilute to 1 litre with

water in a 1litre volumetric flask.

4) *Working solution of EDTA (0.001M) :*

Composition:

Stock solution of EDTA-5ml.

Distilled water up to – 250ml.

Method of preparation :

Dilute 5ml of stock solution of EDTA to 250ml with distilled water.

Standardise with calcium standard solution.

5) Stock solution of calcium standard(40mg%)(0.1% W/V Calcium carbonate solution):

Composition :

Anhydrous calcium carbonate -0.1001mg

IN .HCL - 2ml.

Distilled water -up to 100ml.

Method of preparation:

- Weigh 0.1001g of anhydrous calcium carbonate.
- Keep at 110⁰C in a hot air oven over night.
- Cool in a dessicator.
- Take 15ml of distilled water in a hundred ml. volumetric flask.
- Add 2ml of IN. HCL to the flask.
- Transfer anhydrous calcium carbonate into the volumetric flask.
- Heat to 60⁰C to solubilise salt.
- Rinse down the walls of the volumetric flask with distilled water and cool to room temperature.
- Dilute to 100ml with distilled water.

6) Working solution of Calcium standard(10mg%):

Composition:

Stock solution of calicum standard - 10ml.

Distilled water - up to 40ml.

Method of preparation:

Dilute 10ml of stock solution of calcium standard to 40ml with distilled water.

Procedure:

- Take working solution of EDTA in a burette.
- Take 2 Erlene meyer flasks of 100ml capacity and label them as ‘T’ and ‘S’.
- Take reagents/sample in to the two flasks as follows.

<u>S.NO</u>	<u>Reagent/sample</u>	<u>Test</u>	<u>Standard</u>
1.	Serum	1ml	–
2.	Working std. soln. of calcium	–	1ml
3.	2N. NaOH soln.	4ml	4ml
4.	Distilled water	25ml	25ml
5.	Indicator solution	2drops	2drops

4) Titrate the contents of T and S flasks with working EDTA solution from the burette until purple changes to light blue.

5) Note the volumes of Titrant required for titrating Test and Standard.

6) Calculate the concentration of calicum in serum with formula-

$$\text{Serum calcium} = \frac{\text{Volume of working EDTA required in 'T'}}{\text{Volume of working EDTA required in S}} \times 10 \text{ mg\%}$$

Colorimetric method of determination of Serum calcium :

Principle :Calcium in the specimen on reaction with saturated solution of sodium chloranillate forms precipitate of calcium chloranillate. Excess of chloranillic acid in the precipitate is removed by washing with Isopropyl alcohol. This precipitate on reaction with EDTA forms a chelate of EDTA and calcium and releases chloranillic acid. It gives purple colour, colour intensity of which is proportional to the concentration of serum calcium.

Requirements:

- Test tubes
- Thermostatic water bath
- Centrifuge
- Filter paper

- 5) Vortex mixer
- 6) Photoelectric colorimeter

Reagents :

1) Saturated solution of sodium chloranillate :

Composition :

Sodium chloranillate - 6.13g
Deionised water - 500ml.

Method of preparation :

Take about 6.13g of sodium chloranillate in 500ml of deionised water. Shake well, filter out to separate undissolved Sodium chloranillate.

2) 50% V/V Iso propyl alcohol:

This solution will contain 50 ml. of Isopropyl alcohol in 100 ml. solution.

3) EDTA Solution:

Composition :

EDTA -25g.
Distilled water - up to 500ml.

Method of preparation:

Dissolve 25g of EDTA in about 350ml of distilled water and dilute to 500ml with distilled water.

4) Stock solution of calcium standard (100mg%):

Composition :

Pure calcium carbonate(A.R) - 2.5g.
Conc. HCl - 8ml.
Distilled water up to - 1 litre.

Method of preparation :

a) Dissolve 2.5g of calcium carbonate A.R. in 100ml of distilled water and 8ml of concentrated HCl. Use gentle heat to facilitate dissolution.

b) Transfer into 1litre volumetric flask after dissolution takes place.

c) Dilute to mark with distilled water.

5) Working solution of calcium standard(10 mg% = 2.5mmol/L):

Composition :

Stock solution of calcium standard - 10ml.

Distilled water - up to 100ml.

Method of preparation:

Dilute 10ml of stock solution of calcium standard to 100ml with distilled water.

Specimen: Heparinised plasma or serum

Wave length- 520 nm.

Procedure:- 1) Take two test tubes and label them as 'T' and 'S'.

2) Pipette in to them as follows.

<u>S.No</u>	<u>Reagent/Sample</u>	<u>Test</u>	<u>Std</u>
1)	Serum	2ml	-
2)	Working solution of calcium standard	-	2ml
3)	Saturated sodium chloranillate (Add forcefully)	1ml	1ml

3) Mix well by swirling and place in water bath at 37°C for 3 hours.

4) Centrifuge for 10 minutes at 2000 RPM.

5) Decant the supernatant immediately.

6) Drain the tubes for 2minutes by inverting over a filter paper.

7) Wipe the mouth of tube with filter paper to remove excess of chloranillate.

8) Add 1drop of Isopropyl alcohol to each tube.

9) Break up the precipitate with help of a vortex mixer or by tapping against finger.

10) Wash the ppt with 6 to 7 ml of 50% Isopropyl alcohol.

11) Centrifuge and drain the tubes on a filter paper.

12) Dry the mouths of tubes by wiping with filter paper.

13) Add 1drop of EDTA solution to the precipitate and break up the precipitate with help of vortex mixer or by tapping against finger.

14) Allow the tubes to stand for 10 minutes.

15) Read the O.D. values against EDTA solution as blank.

16) Determine the concentration of calcium in serum using the formula-

$$\text{Serum calcium} = \frac{\text{OD of test}}{\text{OD of std}} \times 10 \text{ mg\% (or } 2.5 \text{ m mol./L)}$$

Cresolphthalein complexone method (cpc method) of determination of serum calcium using colorimeter :

Principle : Calcium in the specimen reacts with cresolphthalein complexone.

Interference of magnesium is eliminated by 8-hydroxy quinoline.

Requirements :

- 1) Test tubes
- 2) Measuring cylinder
- 3) Beaker
- 4) Serological pipettes
- 5) Photo electric colorimeter

Reagents :

1) Cresolphthalein complexone reagent (cpc reagent):

Composition :

Cresolphthalein complexone	-	20mg
Conc. Hydrochloric acid	-	0.5ml
8-hydroxy quinoline	-	1.25ml
Dimethyl sulfoxide	-	50ml
Glass distilled water	-	upto 500ml

Method of preparation: Mix 20mg of cresolphthalein complexone in 0.5 ml. of conc. hydrochloric acid, followed by 1.25g. of 8- Hydroxy quinoline and 50 ml. of dimethyl sulfoxide. Dilute to ½ litre with glass distilled water.

Stability: This reagent is stable at room temperature for 3 months.

2) Potassium cyanide-diethyl amine reagent :

Composition :

Potassium cyanide	_	250mg
Diethylamine	_	20ml
Distilled water	up to _	500ml

Method of preparation :

Mix 250mg of potassium cyanide and 20ml of diethylamine in 350ml of distilled water and dilute to 500 ml. with distilled water.

Stability : This reagent is stable at room temperature for 3 months.

3) Stock solution of calcium standard(100mg%): Refer to colorimetric method of determination of serum calcium.

4) Working solution of calcium standard(10mg%): Refer to colorimetric method of determination of serum calcium.

5) EDTA 4% W/V solution:

Composition:

EDTA - 4g.

Distilled water - up to 100ml.

Method of preparation:

Dissolve 4g of EDTA in 75ml of distilled water and dilute to 100ml with distilled water.

Stability : This reagent is stable at room temperature for several months.

6) Working reagent :

Composition :

Cresol phthalein complexone – 1part.

Potassium cyanide – diethylamine reagent – 1part.

Method of preparation :

Mix equal parts of cresolphthalein complexone reagent and potassium cyanide - diethylamine reagent.

Stability : This reagent is stable at room temperature(25±5°C) only for 24hours.

Specimen: Serum or heparinised plasma

Wave length : 575 nm.

Procedure :

- 1) Take two test tubes and label as 'T' and 'S'.
- 2) Take 6ml of working reagent in each of the two tubes.
- 3) Add 0.05ml of serum or heparinised plasma to 'T' and 0.05ml of working standard to 'S'.

- 4) Take another tube and label them as 'B.' Take 6ml of working reagent and 0.05ml of distilled water in this tube.
- 5) Mix thoroughly and keep at room temperature for exactly 10 minutes.
- 6) Read the O.D values of T and S against blank.
- 7) Determine conc. of serum calcium with formula –

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

d) INORGANIC PHOSPHATE

Clinical significance : Phosphorous is essential along with calcium for the formation of bones and teeth .It is one of the principal mineral of the body.Its daily requirement is 240 -1200 mg (Adults 800 mg).Normal value of seum inorganic phosphorous is between 2.5 -4.5 mg/dL in adults.It is between 4 -6 mg in children.

Conditions causing elevation of serum inorganic phosphorous are -

a)Chronic nephritis b)Hypo parathyroidism c) Hypper vitaminosis D etc.

Conditions causing lowering of serum inorganic phosphorous are -

a)Hyper parathyroidism b)Rickets c)Steatorrhoea d) Fanconi's syndrome
e) Osteomalacia etc.

Excretion of inorganic phosphorous in urine in 24 hours is about 1 gm. on an average diet.Urinary excretion is increased in hyperpara thyroidism and it is lowerwd in hypo parathyroidism.It is also reduced in rickets due to impairment in absorption of phosphorous .

Determination of Serum inorganic Phosphorous :

Name of the method :Gomorri's method

Principle :Acid molybdate reacts with Inorganic phosphate forming phosphomolybdic acid .Metol (colour reagent) reduces phosphomolybdic acid

to a blue coloured complex which is estimated colorimetrically .

Specimen : Serum separated from 3 - 5 ml of blood within 30 minutes of collection.

It need not be a fasting specimen .

- Requirements :
- 1) Test tubes
 - 2) Centrifuge tubes
 - 3)1 ml & 5ml graduated pipettes
 - 4)Stop watch
 - 5)Photo electric colorimeter

Reagents : 1) 10 % w/v Trichloro Acetic acid

Composition :

T.C.A.	-	10 g.
Distilled water	-	upto 100 ml.

Method of preparation : It is prepared by dissolving T.C.A. in quantity of distilled water sufficient to dissolve T.C.A. and then making up the volume to 100 ml.with distilled water.

Stability :This reagent is stable at room temperature .

2) Molybdate reagent

Composition :

Ammonium molybdate	-	5 g.
10 N. Sulphuric acid	-	100 ml
Distilled water	-	300 ml

Method of preparation : It is prepared by dissolving Ammonium molybdate in

100 ml of 10 N Sulphuric acid and 300 ml distilled water.

Stability : This reagent is stable at room temperature.

3) Colour reagent :

Composition :

Metol - 1 g.
3% Sodium metabisulphite solution - 100 ml

Method of preparation : It is prepared by dissolving metol in quantity of distilled water sufficient to dissolve metol and then making up the volume to 100 ml. with distilled water.

Stability : This reagent is stable at room temperature.

4) Phosphorous standard (5 mg %) :

Composition :

Potassium dihydrogen phosphate - 0.2197 g.
Glass distilled water - 1L

Method of preparation : It is prepared by dissolving potassium di hydrogen phosphate in glass distilled water .

Stability : This reagent is stable at 2 - 8 0 C.

Procedure : 1) Take 2 centrifuge tubes and label them as test(T) and diluted standard (D.S.).

2) Pipette into them as follows.

S.No.Reagent/Sample	Test	Diluted Std
---------------------	------	-------------

1) T.C.A. reagent in ml	4.5	4.5
2) Serum in ml	0.5	-
3) Standard in ml	-	0.5 ml

3) Mix, centrifuge and obtain clear filtrate.

4) Take 3 test tubes and label them as Test (T), Standard (S) and Blank (B).

5) Pipette into them as follows .

S.No.Reagent/Sample	Test	Std	Blank
1) Filtrate in ml	2.5	-	-
2) Diluted std. in ml	-	2.5	-
3) Distilled water in ml	-	-	2.5
4) Molybdate reagent in ml	0.5	0.5	0.5
5) Colour reagent in ml	0.5	0.5	0.5

5. Mix thoroughly and keep in dark for 10 minutes .

6. Read optical densities at 660 nm (red filter) wave length .

7. Calculate using the formula

$$\text{Serum inorganic Phosphorous} = \frac{\text{O.D. of test}}{\text{O.D. of std}} \times 5 \text{ mg \%}$$

Determination of urine inorganic phosphorous :

Principle , name of the method , requirements and reagents : Same as in serum inorganic phosphorous .

Specimen : 24 hours urine preserved by thymol crystals .

Procedure :

- 1) Dilute the urine specimen 10 times with 10% TCA. If urine is turbid after adding TCA, centrifuge and separate supernatant.
- 2) Dilute the standard (5mg %) ten times with TCA reagent
- 3) Take 3 test tubes and label them as T, S and B.
- 4) Take 2.5 ml. dilute urine, 0.5ml. molybdate reagent and 0.5 ml. colour reagent in the tube labelled as T.
- 5) Take 2.5 ml. of dilute standard, 0.5 ml. molybdate reagent and 0.5 ml. colour reagent in the tube labelled as "S".
- 6) Take 2.5 ml. of distilled water, 0.5 ml. of molybdate reagent and 0.5 ml. of colour reagent in the tube labelled as "B".

Calculation :

$$\text{Concentration of inorganic phosphorous in urine (mg\%)} = \frac{\text{O.D. of T}}{\text{O.D. of S}} \times 50$$

Quantity of inorganic phosphorous preserved over 24 hours in mg. =

Volume of 24hrs. urine in ml. X Concentration of inorganic phosphorous

100

Determination of urine inorganic phosphorous :

Determination of Urine inorganic phosphorous :

Method : Gomori's method :

Principle : Acid molybdate reagent reacts with inorganic phosphate to form phosphomolybdic acid. Colour reagent metol reduces phosphomolybdic acid to give blue coloured compound. Intensity of blue colour is proportional to the concentration of inorganic phosphorous.

Requirements :

- 1) Test tubes.
- 2) Centrifuge tubes.
- 3) 1 ml. and 5 ml. pipettes with graduations.
- 4) Photo electric colorimeter.

Reagents :

- 1) 10% Trichloro acetic acid.
- 2) Acid molybdate reagent.
- 3) Colour reagent.
- 4) Phosphorous standard (5 mg%) reagent.

- 1) 10% trichloro acetic acid :

Composition : Trichloro acetic acid - 10 gms.
Distilled water upto 100 ml.

Method of preparation : Dissolve 10 gms. of trichloro acetic acid in 75 ml. of distilled water and diluted to 100 ml. with distilled water.

- 2) Acid molybdate reagent :

Composition : Ammonium molybdate - 5 g.
10N Sulphuric acid - 100 ml.
Distilled water - 300 ml.

Method of preparation :

- 1) Dissolve ammonium molybdate in 10N Sulphuric acid .
- 2) Add the solution prepared in step-1 to 300 ml. of distilled water gradually with constant stirring and cool.

- 3) Colour reagent :

Composition : Metol - 1 g.
3% w/v sodium metabisulphite solution up to 100 ml.

Method of preparation : Dissolve 1 g. of metol in 75 ml. of 3% w/v sodium

metabisulphite solution and dilute to 100 ml. with 3% w/v sodium metabisulphite solution.

4) Phosphorous standard (5 mg%) reagent :

Composition : Potassium dihydrogen phosphate - 0.2197 g.

Glass distilled water - 1 litre

Method of preparation : Dissolve 0.2197 g. of Potassium dihydrogen phosphate in 1 litre of glass distilled water.

Storage : 1) Store the reagents 1,2 and 3 at room temperature. 2) Store the Phosphorous reagent at 2-8°C.

Specimen : 24 hours urine specimen preserved by thymol crystals.

Procedure :

- 1) Dilute the urine specimen 10 times with 10% TCA. If urine is turbid after adding TCA, centrifuge and separate supernatant.
- 2) Dilute the standard (5mg %) ten times with TCA reagent
- 3) Take 3 test tubes and label them as T, S and B.
- 4) Take 2.5 ml. dilute urine, 0.5ml. molybdate reagent and 0.5 ml. colour reagent in the tube labelled as T.
- 5) Take 2.5 ml. of dilute standard, 0.5 ml. molybdate reagent and 0.5 ml. colour reagent in the tube labelled as "S".
- 6) Take 2.5 ml. of distilled water, 0.5 ml. of molybdate reagent and 0.5 ml. of colour reagent in the tube labelled as "B".

Calculation :

$$\text{Concentration of inorganic phosphorous in urine (mg\%)} = \frac{\text{O.D. of T}}{\text{O.D. of S}} \times 50$$

Quantity of inorganic phosphorous preserved over 24 hours in mg. =
Volume of 24hrs. urine in ml. X Concentration of inorganic phosphorous

100

e) ANALYSIS OF HORMONE METABOLITES

Analysis of hormone metabolites helps in assessment of hormonal function in body . Determination of some of metabolites of adrenal medullary and cortical hormones is given .

Vanillyl mandelic acid

This investigation is needed for assessment of adrenal medullary function . Vanillyl mandelic acid is metabolite of adrenal medullary hormones in body . Adrenal medullary hormones are adrenaline and noradrenaline. They are also called catecholamines. Adrenaline and noradrenaline are also called epinephrine and norepinephrine . Metanephrine and 3 , 4 - dihydroxy mandelic acid are intermediate products in the metabolism of adrenaline . Normetanephrine and 3 , 4 - dihydroxy mandelic acid are intermediate products in the metabolism of noradrenaline . Vanillyl mandelic acid is chemically 4- hydroxy - 3 - methoxy mandelic acid .

Clinical significance : Normal value of VMA is upto 9mg. /24 hours . High levels of VMA are observed in pheochromocytoma . Mild to moderate elevation is observed in neuroblastomas , ganglioneuromas

and ganglioblastomas etc. Normal value of adrenaline is 100 - 230 mug/24 hours . Normal value of norepinephrine is 100 - 230 mug. / 24 hours .

Normal value of metanephrine is 24 - 96 mug/ 24 hours . Normal value of normetanephrine is 12 - 288 mug./24 hours .

Determination of Vanillyl mandelic acid :

- Reagents :
1. 6N Hydrochloric acid
 2. Sodium chloride
 3. Ethyl acetate

4. 1 M potassium carbonate
5. 2 % Aqueous solution of sodium meta periodate
6. 10 % Aqueous solution of sodium metabisulphite
7. 5N Acetic acid
8. 1 M Phosphate buffer pH 7.4
9. Toluene
- 10 Vanillin standard 100 mug./ ml in water

- Procedure :
- 1 . Collect 24 hours urine sample using 6N HCl as preservative .
 - 2 . Take 2 % of total volume of 24 hours urine collected .
 - 3 . Dilute the collected sample of urine to 5.5 ml and add 0.5 ml of 6N HCl .
 - 4 . Mix thoroughly and add 3 g. of finely powdered sodium chloride and 30 ml of ethyl acetate .
 - 5 . Shake vigorously for 4 - 5 minutes to get a saturated solution .
 - 6 . Allow to stand and transfer 25 ml of ethyl acetate layer into a second stoppered test tube .
 - 7 . Add 1.5 ml of 1 M potassium carbonate solution and shake for half an hour .
 8. Centrifuge at 2000 RPM speed and take 1 ml of carbonate layer into a 3rd tube .
 9. Mix 0.1 ml of periodate solution and incubate at 50⁰ C for 30 minutes .

- 10 . Allow to cool .
- 11 . Add 0.1 ml of sodium meta bisulphite solution and mix well .
- 12 . Add 0.6 ml of phosphate buffer and adjust pH between 5 - 7 by adding 5 N acetic acid .
- 13 . Mix 20 ml of toluene and shake for 3 minutes ,
- 14 . Allow to stand for few minutes and take 15 ml of toluene layer into another glass stoppered tube .
- 15 . Add 4 ml of 1 M potassium carbonate .
- 16 . Shake vigotously and wait for few minutes .
- 17 . Take the reading against blank (prepared with water in place of urine)
- 18 . Add 3.9 ml of 1 M potassium carbonate solution to 0.1 ml of vanillin standard and read against blank .
- 19 . Calculate using the formula -

Reading of un known

$$\text{VMA} = \frac{\text{Reading of un known}}{\text{Reading of standard}} \times 16 . 5 \text{ mg / 24 hours}$$

Reading of standard

17 Ketosteroids , 17 ketogenic steroids (17 KGS) and 17 hodroxy corticosteroids(17 OHCS)

17 Ketosteroids (17 KS) , 17 ketogenic steroids (17 KGS) and 17 hodroxy corticosteroids(17 OHCS) are metabolites of adrenal cortical and testicular hormones and thus their determination is needed in disturbances of adrenal cortex and testes .

Clinical significance : Normal value of 17 ketosteroids in 24 hours urine in males is about 8 - 18 mg. and it is 5 - 15 mg. in females . Decreased levels are seen in

males with hypogonadism and in females with Addison's disease. Elevation is seen in patients with testicular tumour, adrenal hyperplasia, adrenal hyperplasia and Cushing's syndrome.

Determination of 17 Ketosteroids (17 KS) :

Reagents :

1. Concentrated HCl .
2. 1 N NaOH
3. Anhydrous sodium sulphate
4. Chloroform
5. 1 % Solution of m - dinitro benzene in ethanol
6. Aldehyde free ethanol
7. Tetramethyl ammonium hydroxide
8. 30 % v/v aqueous solution of ethanol
9. Ether
10. Standard solution of dehydroepiandrosterone (10 mg %)

Procedure :

1. Collect 24 hours urine sample .
2. Measure the volume of collected urine specimen .
3. Prepare Zimmerman's reagent by adding equal volumes of 1 % Solution of m - dinitro benzene in ethanol and tetramethyl ammonium hydroxide solution
4. Pipette out 4 ml of urine into a glass stoppered tube containing 0.5 ml of concentrated HCl .
5. Boil over water bath for 20 minutes to cause release of metabolites from bound form .
6. Cool and add 8 ml of chloroform .
7. Shake vigorously .

- 8 . Allow to stand for some time to cause separation of layers .
9. Pipette out aqueous layer .
10. Add 3 ml of 1 N NaOH to chloroform layer .
11. Shake for some time and discard aqueous layer .
12. Repeat twice each time with 3 ml of distilled water .
13. Add a small quantity of anhydrous sodium sulphate to chloroform extract .
14. Take 4 ml of chloroform extract into a test tube and allow to dry over water bath .
- 15 . Take 0.2 ml of standard in another test tube and allow to dry .
- 16 . Add 0.25 ml of freshly prepared Zimmerman reagent to each tube .
- 17 . Prepare blank .
18. Allow the tubes to stand in dark at 250C for 1 hour .
- 19 . Add 3 ml of ethanol to each tube and read absorbance at 520 nm. against blank .
- 20 . Calculate using the same formula -

$$\text{Ketosteroid} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 0.01 \text{ mg}$$

Where V = volume of urine in 24 hours .

f) BLOOD GAS ANALYSIS



Fig.15-2 Blood gas analyser

Blood gases are oxygen and carbon dioxide .Their determination is needed for

1. Assessment of adequacy of oxygenation 2. Assessment of adequacy of ventilation 3. Assessment of acid - base status .For determination of blood gases , arterial blood is needed rather than venous blood due to several reasons.

Reasons for use of arterial blood rather than venous blood are -

1. Arterial blood is a good mixture of blood from all parts of body . Hence it is good to sample arterial blood rather than venous blood which represents that extremity from where it has been collected.
2. Arterial blood gives information of how well oxygenation has taken place in the lungs .

Collection of arterial blood specimen : 1. Instruct the patient to take supine or sitting or position .

2. Raise the wrist with a pillow and instruct the patient to extend the fingers downwards .
3. Palpate the artery .
4. Rotate the patient 's hand back and forth until a good pulse is felt .
5. Clean the site of collection with antiseptic agent like betadine .
6. Anaesthetise the site of collection with small quantity of 1% xylocaine .
7. Puncture the artery with 20 or 21 gauge sterile needle .
8. Attach pre heparinised syringe of 12 ml capacity .
9. Draw blood by pulling back the plunger taking care not to pull the needle out of artery .
10. Collect 3 - 6 ml of sample .
11. Withdraw needle .
12. Keep sufficient absorbant bandage over the site of puncture and apply pressure for about 2 minutes .
13. Expel any air bubbles in the sample as soon as possible .
14. Cap the syringe and rotate gently to cause proper mixing of blood with heparin .
15. Preserve by storing in the refrigerator if it is not tested within 15 - 25 minutes .

Precautions : 1. Apply enough pressure at the puncture site and watch for bleeding.

2. Collection of arterial blood should be done without trauma .
3. Protect from room air .
4. Be aware of air bubbles in the syringe as they will cause change of concentration of gases .

Determinations related to blood gases :

They are analyses of oxygen and carbondioxide .

Blood oxygen analysis

Blood gas analyses conducted are 1. Determination of Oxygen saturation

2. Determination of oxygen capacity

3. Determination of oxygen content 4. Measurement of P_{O_2} .

Clinical significance : Oxygen content of arterial blood in males is 16 - 23 per 100 ml . Average value is 20 ml .In venous blood , it is 11 - 16 ml. and average value is 14 - 15 ml .Thus , oxygen utilisation by tissues is about 25 % . These values in females are 10 - 15 % less than the values in males . Oxygen saturation of arterial blood is about 95 % . It is 70 - 75 % for venous blood. P_{O_2} in inspired air is about 150 mm of Hg. Its value in alveolar air is nearly 100 mm of Hg. Value of alveolar arterial blood is also same. Cyanosis occurs when saturation is less than 70 - 75 % i.e. pressure falling below 50 mm of Hg. Tissue damage begins between 40 - 30 mm of Hg and saturation is below 50 % . Death occurs when saturation is below 20 % . Decreased arterial blood O₂ is seen in chronic obstructive lung disease , patients with post operative respiratory complications , Flail chest , kyphosclerosis , neuromuscular impirment and obesity hypoventilation etc .

Hypoxaemia : Reduced P_{O_2} is termed hypoxaemia .

Hypoxia : When hypoxaemia is severe enough to damage tissues it is termed hypoxia. It is four types - 1. Hypoxic 2. Anaemic hypoxia 3. Stagnant hypoxia 4. Histotoxic hypoxia .In hypoxic type , oxygen capacity is normal .Saturation of arterial blood is below normal .It is due to shortage of oxygen at high altitudes

and also in diseases like pneumonia in which there is rapid shallow breathing .In anaemic hypoxia , oxygen saturation is normal . Oxygen capacity of arterial blood is reduced due to conversion of some of haemoglobin into nonfunctional form . In stagnant hypoxia , there is some circulatory deficiency . Arterial oxygen capacity may be normal .

Oxygen saturation of arterial blood is normal . Because of reduced circulation , an increased amount of oxygen is removed by tissues so that saturation of venous blood is below normal . In histotoxic hypoxia , tissue cells cannot utilise oxygen normally in conditions of poisoning by alcohol , potassium cyanide etc .

PO₂ is increased in polycythaemia , hyperventilation in arterial blood specimen . Decreased levels of PO₂ are associated with anaemia , cardiac decompensation , insufficient atmospheric O₂ , intracardiac shunts , chronic obstructive disease , restrictive pulmonary disease , hypoventilation due to neuromuscular disease etc . Decreased arterial PO₂ with normal or decreased arterial blood PCO₂ tension is associated with diffuse interstitial pulmonary infiltration , pulmonary oedema , pulmonary embolism , post operative extra corporeal circulation etc .

Determination of Oxygen saturation : One method of determining oxygen saturation is using Kipp haemoreflector . Another method is spectrophotometric technique . Method of determining oxygen saturation using Kipp haemoreflector is given here .

Determination of Oxygen saturation using Kipp haemoreflector :

Name of the method : Reflection oximetry

Construction of haemoreflector : Haemoreflector consists of a light source , filter , revolving head and galvanometer . Revolving head mounted on the reflector contains three cells . One cell is specimen cell . Second cell is indian ink cell . Third cell is sensitivity adjustment cell containing red dye stuff .

Principle : Intensity of light reflected by a layer of blood is determined . Light is more reflected by oxyhaemoglobin than haemoglobin . Logarithm of reflected quantity of light is linear function of oxygen saturation .

- Requirements :
1. Haemoreflector
 2. Dithionate
 3. Heparin
 4. 2% Sodium chloride
 5. 0.3 % Sodium salicylate
 6. 0.05% Sodium cyanide

Wave length : 600 - 680 nm.

Specimen : Blood

Procedure : 1. Adjust the galvanometer reading to zero by placing the compensating cell in the light path .

2. Adjust the galvanometer reading to value given in the calibration certificate by placing dyestuff cell in the light path .

3. Place the specimen cell in the light path .

4. Read the value of oxygen saturation from calibration curve supplied .

Heparin is used as anticoagulant . 2% Sodium chloride is used to dilute the blood specimen . 0.3 % Sodium salicylate is used to prevent agglutination and sedimentation . Sodium cyanide solution is used to convert any methaemoglobin to cyanmethaemoglobin .

Determination of oxygen capacity and oxygen content : Determination of oxygen capacity and oxygen content can be done using Van Slyke apparatus .

Principle : Saponin in oxygen reagent is used to lyse the cells . Potassium ferri cyanide is used to liberate oxygen . Caprylic acid is used to prevent frothing . Sodium hydroxide is used to absorb carbon dioxide .

Reagents : 1. Oxygen reagent

2. 2.2% Sodium hydroxide solution

1. Oxygen reagent :

Composition : Potassium ferricyanide ----- 3g.

Saponin ----- 3g.

Caprylic alcohol ----- 3 ml

Distilled water ----- upto 1 L.

Method of preparation : 1. Dissolve 3 g. of potassium ferricyanide and 3g. of saponin in water .

2. Add 3 ml of caprylic alcohol and make upto volume with distilled water .

2. Sodium hydroxide 2% solution :

Composition : Sodium hydroxide ----- 2 g.

Distilled water ----- upto 100 ml.

Method of preparation : 1. Dissolve 2 g. of sodium hydroxide in enough quantity of water .

2. Make the volume with distilled water .

Procedure : 1. Mix oxygen reagent properly and measure 7.5 ml into cup C of Van Slyke apparatus .

2. Transfer it into pipette by lowering mercury reservoir .

3. Deaerate by producing vacuum .

For measuring oxygen capacity , take blood into a separating funnel of about 300 ml capacity . Take care to distribute the cells evenly and completely throughout the specimen . Allow blood to form a layer over surface of funnel and rotate to ensure complete aeration of haemoglobin . For measurement of oxygen content , take blood under paraffin . Mix thoroughly before doing the estimation .

For both of the estimations proceed as follows .

1. Force 6 ml of deaerated oxygen reagent into the cup.
2. Pipette 2 ml of blood by means of ostwald pipete .
3. Place the tip of pipette at the bottom of cup under the liquid .
4. Run blood and all the reagent except 1 ml into the pipette of the apparatus.
5. Seal off the tap by placing a little amount of mercury in the bottom of the cup.
6. Lower mercury reservoir to produce a partial vacuum and shake until a constant volume of gas is obtained in the calibrated part of pipete .Five to

ten minutes is usually required .

7. Run the liquid into side tube F .
8. Wash out cup C with distilled water and place it in a few ml of 2 % sodium hydroxide .
9. By suitably placing mercury reservoir , run in slowly 0.5 ml of this solution deaerated previously .
10. Introduce some mercury into cup C and run a fine stream down . This would take hydroxide with it , otherwise it would remain at the upper end of the pipette .
11. Mix and run as much of liquid as possible into F . Again , read the volume of gas . Level of mercury in the reservoir should be adjusted to be higher than that of mercury in the pipette by 1/13 th of height of any liquid still in the pipette . This gas is mixture of oxygen of haemoglobin and of oxygen and nitrogen in physical solution .
12. Ascertain temperature and barometric pressure .
13. Calculate the corrected volume of oxygen using the formula -

$$\text{Volume of oxygen corrected to } 0^{\circ}\text{C} = \frac{V (B - W)}{760 (1 + 0.00367 t)}$$

where V = volume of oxygen + nitrogen in ml

B = barometric pressure in mm of Hg .

w = vapour tension of water

t = temperature in 0 °C

Oxygen capacity in ml per 100 ml of blood =

$$\frac{V (B - W)}{760 (1 + 0.00367 t)} \times \frac{100}{2} = 1.9$$

Measurement of P_{O_2} : Partial pressure reflects amount of oxygen passing from pulmonary alveoli into blood . It is influenced by amount of oxygen inhaled . This test measures the pressure exerted by O_2 dissolved in plasma . It gives the effectiveness of lungs to oxygenate blood . partial pressure of oxygen can indicate the severity of impairment of lungs to diffuse oxygen across alveolar membrane into circulating blood .

$$P_{O_2} = \frac{(\text{Barometric pressure} - \text{water vapour pressure}) \times \% O_2}{100}$$

It is measured by collecting arterial blood sample . Oxygen electrode is available for attachment with Astrup instrument . Partial pressure of oxygen can be measured quickly using this method .

Blood CO_2 analysis

Analyses of CO_2 are - 1. Determination of total carbondioxide (TCO_2)

2. Determination of partial pressure of CO_2 (PCO_2).

Total carbondioxide (TCO_2) test is a measure of alkalinity or acidity of venous , arterial or capillary blood . This test measures CO_2 from dissolved CO_2 , total H_2CO_3 , HCO_3^- , carbamino carbondioxide . 95% of this CO_2 is derived from bicarbonate , which is regulated by kidneys . Remaining 5 % is derived from other sources . Total CO_2 content thus gives only little information about the functioning of lungs . HCO_3^- in the extracellular space first exists in the form of CO_2 , then as H_2CO_3 and finally most of it is converted to sodium bicarbonate ($NaHCO_3$) by the action of buffers of red cells and plasma .

Partial pressure of CO_2 is a measure of pressure or tension exerted by CO_2 in blood . It is proportional to partial pressure of CO_2 in alveolar air . This test is used to detect respiratory abnormality and also to determine acidity or alkalinity . It is indicative of alveolar ventilation . PCO_2 in arterial sample reflects efficiency of gaseous exchange with blood in lungs .

Clinical significance : CO_2 level is elevated in severe vomiting , emphysema , aldosteronism , use of mercurial diuretics etc . It is decreased in severe diarrhoea , starvation , acute renal failure , salicylate toxicity , diabetic acidosis , use of chlorothiazide diuretics etc . PCO_2 is elevated in hypoventilation . It is decreased in hypoxia , nervousness , anxiety , pulmonary emboli , pregnancy , other causatives of hyperventilation etc . Causes of elevated PCO_2 are obstructive lung diseases such as chronic bronchitis , emphysema etc . Reduced function of respiratory center as in overreaction , head trauma , anaesthesia and other rare causes of hypoventilation also cause elevation of PCO_2 . Elevated PCO_2 can also occur even with normal lungs if respiratory center is depressed .

Determination of total carbondioxide (TCO_2) : Venous or arterial blood collection is done for this purpose anaerobically . 6ml of blood is collected in a heparinised syringe . If blood cannot be analysed immediately it should be stored in an iced container . Plasma is separated by centrifuging . TCO_2 is measured using blood gas analyser . It can also be determined using titration method . In this method excess of acid is added and back titrated to know the value of TCO_2 .

Determination of PCO_2 : PCO_2 can be measured using the formula

$$PCO_2 = \frac{(\text{Barometric pressure} - \text{water vapour pressure}) \times \% CO_2}{100}$$

Arterial blood sample is collected under liquid paraffin for this purpose .

A small quantity of blood is introduced into blood gas analyser without exposing to air . Measure of CO_2 tension can be done with a silver - silver chloride electrode .

Summary

Lipid profile tests are important in diagnosis of primary and secondary dyslipoproteinaemias . Cholesterol is a steroidal alcohol . Some of the methods

of determination of total cholesterol are 1) Watson method 2) Enzymatic method. One of the methods of determination of HDL cholesterol is Watson method.

Glycosylated haemoglobin determination is clinically significant in diagnosis of diabetes mellitus. It can be determined by 1) Colorimetric method 2) Ion exchange – Colorimetry method.

Determination of serum calcium is significant in diagnosis of diseases in which serum calcium levels are elevated and lowered. It is determined by 1) Titrimetric method 2) Colorimetric method 3) Cresol phthalein complexone method.

Serum inorganic phosphate can be determined using Gomori method. Conditions causing elevation of serum inorganic phosphorus are -Chronic nephritis, Hypoparathyroidism, hypervitaminosis D etc.

Conditions causing lowering of serum inorganic phosphorus are - Hyperparathyroidism, rickets, steatorrhea, Fanconi's syndrome, Osteomalacia etc.

Blood gas analysis consists of blood oxygen analysis and blood CO₂ analysis.

Blood oxygen analysis includes oxygen content and capacity. It also includes determination of partial pressure. Blood CO₂ analysis includes total CO₂ and partial pressure.

Essay Questions

- 1) What are important lipid profile tests? Write about cholesterol.
- 2) Write about determination of serum total cholesterol by Watson method.
- 3) Describe the enzymatic method of determination of serum total cholesterol.
- 4) Write about determination of HDL cholesterol.
- 5) Explain the method of determination of glycosylated haemoglobin by colorimetric method.
- 6) Discuss the method of determination of glycosylated haemoglobin by Ion

exchange – Colorimetry method.

- 7) What are different methods of determining serum calcium? How do you determine serum calcium by titrimetric method?
- 8) How do you determine serum calcium by colorimetric method?
- 9) Write cresolphthalein complexone method of serum calcium determination.
- 10) Write about determination of serum inorganic phosphate.
- 11) Discuss the clinical significance of blood oxygen analysis.
- 12) How do you determine blood oxygen content and capacity?
- 13) Write about blood carbon dioxide analysis.

Short answer questions

- 1) Mention different lipid profile tests.
- 2) Name the clinical conditions requiring lipid profile tests for their diagnosis.
- 3) What is the source of cholesterol?
- 4) What is the chemical nature of cholesterol?
- 5) Name the biosynthetic products of cholesterol in animal tissues for which cholesterol is precursor.
- 6) Mention a few conditions in which serum total cholesterol is elevated.
- 7) Name a few conditions in which serum total cholesterol lowers.
- 8) What are the instructions given to the patient undergoing serum total cholesterol determination?
- 9) Write the composition of DBSA reagent used in serum cholesterol determination by Watson method.
- 10) What are the stability aspects of cholesterol stock standard and DBSA reagent?
- 11) Mention the wavelengths for 1) Determination of serum cholesterol 2) Glycosylated haemoglobin.

- 12) Give the principle of enzymatic method of determination of serum total cholesterol.
- 13) Mention the system parameters of serum total cholesterol determination using auto analyser.
- 14) Write the composition of buffered enzyme-chromogen reagent.
- 15) Write the clinical significance of determination of serum total cholesterol along with HDL cholesterol.
- 16) How do you separate HDL cholesterol from other fractions of lipoproteins in HDL cholesterol determination?
- 17) Mention the normal values of-
 - a) Serum total cholesterol
 - b) Serum HDL cholesterol
- 19) What are different genetical species of haemoglobin?
- 20) What do you mean by glycosylated haemoglobin?
- 21) Write down the principle for determining glycosylated haemoglobin by colorimetry-ion exchange method.
- 22) List out the different methods of serum calcium determination.
- 23) How do you prepare 100ml of 2N NaOH solution?
- 24) Prepare 100ml of working solution of calcium standard (10mg%) from stock solution of calcium standard(40mg%)?
- 25) Write the formula to determine serum calcium by titrimetric method.
- 26) Name the reagents used in serum calcium determination by colorimetric method.
- 27) Give the principle of flame photometric method of serum calcium determination .
- 28) What is the principle of inorganic phosphate determination ?

- 29) Mention the normal values of serum inorganic phosphate .
- 30) Write the clinical significance of serum inorganic phosphate .
- 31) What are the blood gases ?
- 32) Mention the analyses of blood oxygen .
- 33) Write the analyses of blood CO₂ .
- 34) Define PCO₂
- 35) Write the definition of PO₂
- 36) Write the formula of PCO₂
- 37) Give the formula of PO₂

GLOSSARY

Acute thyroiditis	= Inflammation of thyroid gland caused by bacterial infection
Addison's disease	= Inadequate secretion of corticosteroid hormones by adrenal glands
Amenorrhoea	= Cessation of menstruation
Aminoaciduria	= Appearance of amino acids in urine
Anions	= Negatively charged ions
Aqueous solution	= Solution with water as solvent
Ascending development	= Method of development in which solvent front moves upward
Azospermia	= Complete absence of sperms in semen
Basal metabolic rate	= Heat production per unit body surface area per day
Bile duct carcinoma	= Cancer of bile duct
Buffer	= Solution which keeps pH constant within specified range
Carcinogenicity	= Ability to cause cancer
Cardiac tonic	= Agent which strengthens cardiac muscle
Cations	= Positively charged ions
Chronic thyroiditis	= Inflammation of thyroid gland caused by abnormal immune response
Colitis	= Infection of colon
Corrosive	= Causing Corrosion
Cushing's syndrome	= Excessive secretion of corticosteroid hormones
Cyst	= An abnormal sac or closed cavity filled with liquid or semisolid matter
Descending development	= Method of development in which solvent front moves downwards
Elution	= Development
Exophthalmos	= Protrusion of Eye balls
Fistula	= Abnormal communication between two hollow organs or between hollow organ with exterior
Gastric Carcinoma	= Cancer of stomach
Haemolytic Jaundice	= Jaundice caused due to excessive destruction of RBC
Heparin	= An anticoagulant synthesised in the body

Hepatotoxic	= Causing harm to liver
Hypercholesterolaemia	= Condition of elevation of cholesterol in blood
Hyperglycaemia	= Condition of elevation of blood sugar above normal
Hypoglycaemia	= Condition of blood sugar lowered below normal
Hypolipoproteinaemia	= Condition of lowering of lipoproteins in blood
Hypophysectomy	= Removal of pituitary by cutting
Hyospermia	= Lowered sperm count
Lethargy	= Mental and physical sluggishness
Lymphoma	= Malignant tumour of lymph nodes
Malignancy	= Tumour which invades and destroys tissue of origination and spreading to other parts of body through blood stream
Metabolism	= Chemical changes taking place in the body
Micropipette	= Pipette used for pipetting small quantities of liquids
Neonate	= Newborn
Nephrosis	= Degeneration of tubular epithelium of kidneys
Normal saline	= 0.9% sodium chloride aqueous solution.
obesity	= Condition of excessive accumulation of fat in the body
Occult	= Hidden
Pancreatic carcinoma	= Cancer of pancreas
Pancreatitis	= Inflammation of pancreas
Perfusion	= Passage of fluid through a tissue
pH	= Negative logarithm of Hydrogen ion concentration
Prothrombin	= A Plasma protein participating in blood clotting
Radiotherapy	= Treatment of a clinical condition using radio active isotopes
Renal glycosuria	= Condition of temporary glycosuria due to some abnormality in tubular reabsorption of glucose.
Sepsis	= Destruction of tissues by putrefaction caused by micro organisms or their toxins
Steatorrhoea	= Abnormal increase of fat in faeces
Sterilisation	= Process of killing or removing living micro organisms
Sterility	= Infertility
Thyroid Carcinoma	= Thyroid cancer
Xanthomatosis	= Multiple small fatty tumours

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Dr .Raghu Rama Rao & Late Dr .Ravi Shankar.*