HIGH INSTITUTE OF MEDICAL TECHNOLOGY

LABORATORY MEDICINE DEPARTMENT

BIOCHEMISTRY UNIT

PRACTICAL SHEETS FOR III YEAR

LABORATORY SAFETY

Laboratory rules

- ✓ Always wear a laboratory white coat, gloves and shoes with closed toes & heels.
- ✓ Don't eat, drink or smoke in the laboratory and never store the food or drink in the refrigerator.
- ✓ Don't apply cosmetic or contact lenses in the laboratory. Dangling jewelry, long hair, bread may be risky.
- ✓ Don't draw reagents or specimens through a pipettes directly by mouth.
- ✓ Put needles & sharps in puncture – resistant containers.
- ✓ Don't throw any solid into the sink. If you have to pour strong acids or alkalis make sure that you let a lot of tap water rinse it away.
- ✓ Don't waste reagents.
- ✓ Report to the instructor, if there is any accident of any type.

Chemical safety

- o Bottles of chemicals and solutions should be handled carefully, and a cart should be used to transport a heavy or a multiple number of containers from one area to another.
- o Glass containers with chemicals should be transported in rubber or plastic containers that protect them from breakage.
- o A bottle should never be held by its neck, but instead firmly around its body with one or both hands.
- o When working with acid or alkali solutions, safety goggles should be worn & acids must be diluted by slowly adding them to water, while mixing; water should never be added to concentrated acid.
- o Acids, caustic materials, and strong oxidizing agents should be mixed in the sink. This provides water for cooling.

- All bottles containing reagents must be properly labeled before adding the reagent.
- The label should bear the **name** and **concentration** of the reagent, the initials of the **person** who made up the reagent, the **date** on which the reagent was prepared, the **expiration** date & storage and potential hazards instructions [corrosive, toxic, irritants, flammable, explosive, reactive].
- Organic solvents represent a potential fire hazard and hazards to health from inhalation of toxic vapors or skin contact. Their use should be carried out using a fume hood. Solvents should be stored in a metal storage cabinet.
- Disposal of flammable solvents in sanitary sewers is not allowed.
- Separate safety cans should be used for ether and for chlorinated solvents; all other solvents may be combined in a third can.

Electrical hazards

- Worn wires on all electrical equipment should be replaced immediately; all equipment should be grounded using three-prong plugs.
- an extension cord may have to be used temporarily.
- If several outlets are needed in an area, a strip with its own fuse or circuit breaker may be installed at least 3 in. above bench-top level.
- Electrical equipment and connections should not be handled with wet hands, nor should electrical equipment be used after liquid has been spilled on it.
- The equipment must be turned off immediately and dried thoroughly; a fan or hair dryer will speed up the drying process.
- In case of a wet or malfunctioning electrical instrument that is used by several people, the plug should be pulled.

Fire safety

- Fire sources are flammable liquids, electrical and trash fires. Fire extinguishing by water, CO_2, foam, dry chemicals, or by fire extinguishers.
- Gas cylinders must be stored separately away from fire sources.
- Fire blankets for smothering fire on clothing should be available in an easily accessible wall-mounted case.
- An extinguisher should be provided near every laboratory door & should be tested by qualified personnel at intervals specified by the manufacturer.

Biological hazards

- Exposure to infectious pathogens can result from:
    1- Accidental puncture with hypodermic needles.
    2- Spraying of infectious materials by a syringe or spilling and splattering of these materials on benchtops or floors.
    3- Centrifuge accidents.
    4- Cuts or scratches from contaminated glassware.
- Never perform mouth pipetting and never blow out pipets that contain potentially infectious material.
- Barrier protection, such as gloves, masks, and protective eyewear and gowns, must be used when drawing blood from a patient, when handling all patient specimens & during removal of stoppers from tubes.
- Phlebotomists should change gloves and dispose of them between patients.
- Wash hands whenever gloves are changed. Encourage frequent hand washing in the laboratory& whenever leave the laboratory.
- Facial barrier protection should be used if there is a significant potential for the spattering of blood or body fluids.
- Dispose of all sharps appropriately in rigid containers without handling them.

- Wear protective clothing, which serves as an effective barrier against potentially infective materials. When leaving the laboratory, the protective clothing should be removed.
- Make a habit of keeping your hands away from your mouth, nose, eyes, and any other mucous membranes (reduce the possibility of self-inoculation).
- Decontaminate all surfaces and reusable devices after use with disinfectants.
- Before centrifuging tubes, inspect them for cracks. Inspect the inside of the trunnion cup for signs of erosion or adhering matter.
- Periodically, clean out freezer to remove broken ampules and tubes of biological specimens using rubber gloves and respiratory protection.
- All samples should be considered as dangerous samples therefore a special care should be followed during handling or processing of the samples.

Safety equipment

- Two entrances
- Showers
- Fire extinguishers
- Fire blankets
- Fire alarm
- Fume hoods
- First aid kits
- Respirators
- Safety goggles
- Masks
- Gloves
- Fluid resistant coats and plastic or rubber aprons.

BIOCHEMISTRY LABORATORY

Types of samples: a variety of specimens are used in biochemical analysis:

1. Body fluids such as
   - i- blood,

a. Serum: is obtained from coagulated blood without anticoagulant then a wait of 15 to 30 min for completion of coagulation then centrifuge the sample.

b. Plasma: anticoagulant is added to the specimen and centrifugation can be done immediately after collection.

   - ii- Urine,
   - iii- Spinal fluid,
   - iv- Amniotic fluid,
   - v- Peritoneal fluid,
   - vi- Sputum & saliva,
   - vii- Synovial (joint fluid).

2. Feces,
3. Tissue and cells
4. Calculi (stones) and other materials.

Types of Anticoagulants

- ✓ **Ethylenediaminetetraacctic Acid (EDTA)**:

The chelating agent EDTA is particularly useful for hematological examinations because it preserves the cellular components of blood. It is used as the disodium or dipotassium salt. EDTA prevents coagulation by binding calcium, which is essential to the clotting mechanism.

- ✓ **Sodium Fluoride**:

Sodium fluoride is usually considered a preservative for blood glucose; however, it also acts as a weak anticoagulant. It exerts its preservative action by inhibiting the enzyme systems involved in glycolysis.

- ✓ **Citrate**:

Sodium citrate solution is widely used for coagulation studies, because its effect is easily reversible by addition of Ca^{+2}. It appears to preserve labile procoagulants, but it has little application in clinical chemistry.

- ✓ **Heparin**:

Heparin causes the least interference with tests. It is a mucoitin polysulfuric acid and is available as sodium, potassium, lithium, and ammonium salts. This anticoagulant accelerates the action of antithrombin III, which neutralizes thrombin and thus prevents the formation of fibrin from fibrinogen.

- ✓ **Oxalates**:

Sodium, potassium, ammonium, and lithium oxalates inhibit blood coagulation by forming rather insoluble complexes with calcium ions.

- ✓ **Iodoacetate**:

Sodium iodoacetate at a concentration of 2 g/L is an effective antiglycolytic agent and a substitute for sodium fluoride. Because it has no effect on urease, it can be used when glucose and urea assays are performed on a single specimen. It inhibits creatine kinase but appears to have no other significant effects.

Types of tubes:

General laboratory supplies

- ✓ **Glassware**:

Glass is a complex silicate. The thermal properties of glass can be significantly changed by addition of Boron oxide – borosilicate.

1. **Pipettes**:

They are used to measure and transfer liquids. They are of two types transfer & measuring pipettes.

- Transfer pipettes are designed to deliver a fixed volume of liquids, e.g. volumetric & Ostwald – Folin pipettes.
- Measuring (graduated) pipettes are designed to allow measurement of differing volumes of liquids, e.g. serological & Mohr pipettes.

Pasteur pipettes: they are used to add small, unmeasured quantities of liquids to containers and they require a bulb for use.

2. **Burettes**:

They are used for titration purposes.

3. **Beakers**:

Griffin beaker is used for preparation and transfer of solutions.

4. **Flasks**:

They are used for measuring and transferring volumes of liquids.

- Erlenmeyer flask
- Florence flask
- Volumetric flask

5. **Measuring cylinder**:

It's used to measure a specific volume of a solution or a liquid.

Micropipette:

Pipettes that are made to dispense less than 1 ml are called micropipettes. Their volumes are expressed in micro liters (µl); 1 ml = 1000 µl. Micropipettes are available in sizes ranging from 1 – 1000 µl.

Cleaning of glassware:

Most commonly glasswares are rinsed, soaked in weak detergent solution e.g. soap then washed many times by water then by distilled water and dried. For lipids, proteins, stains, and other greasy materials used in borosilicate glasswares must be cleaned as follows:

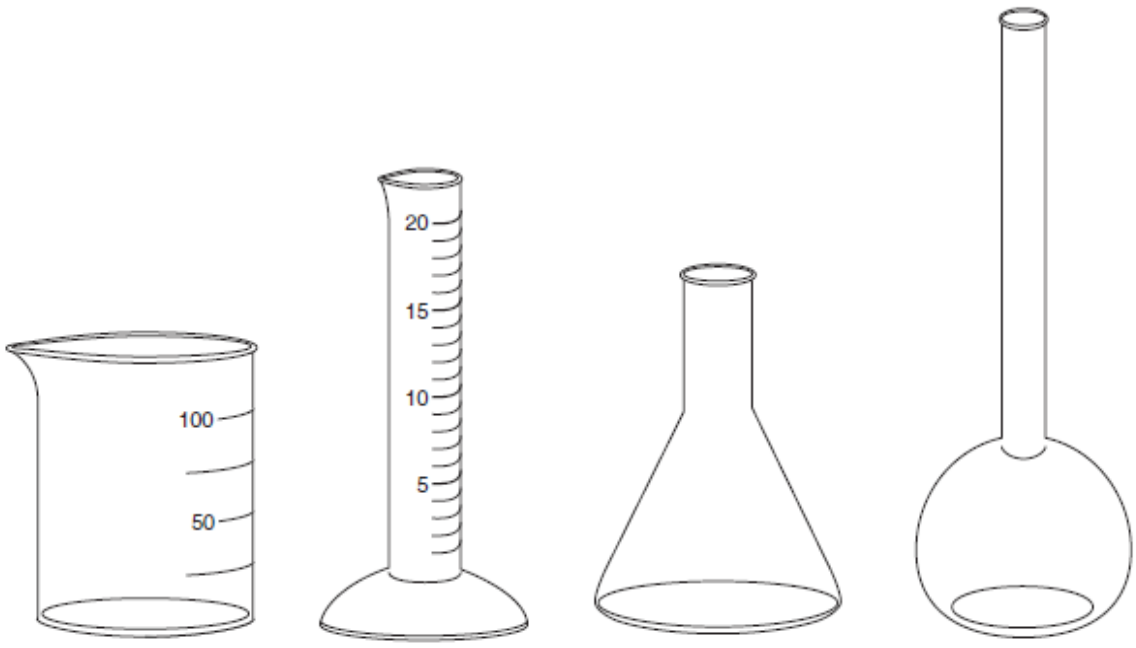
Chromic acid (Na-dichromate + concentrated H_2SO_4) ➔ diluted HCl ➔ T.W. ➔ D.W. ➔ drying.

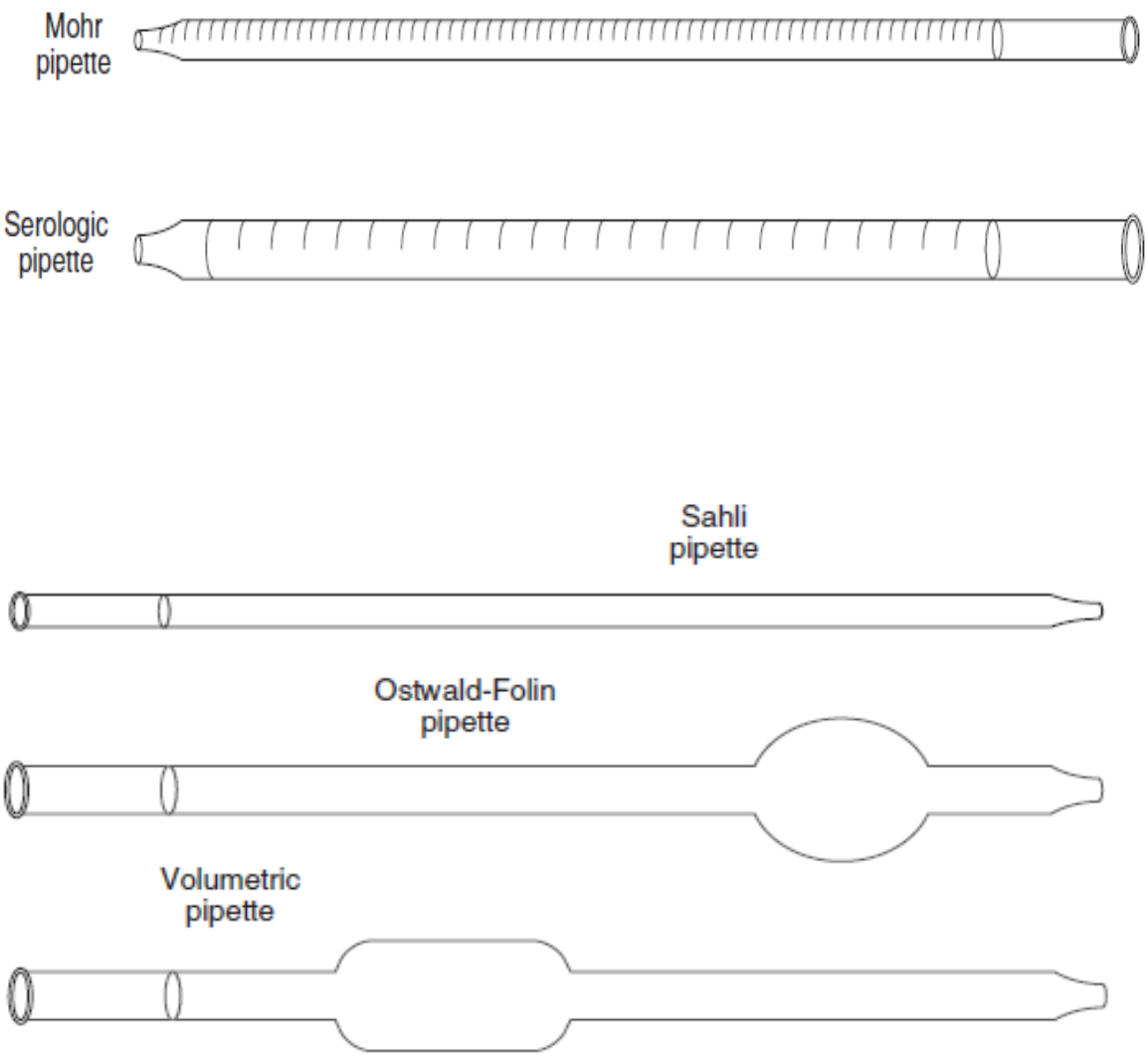
For pipettes, beakers, flasks & cylinders cleaning is as follows:

Chromic acid (Na-dichromate + concentrated H_2SO_4) ➔ T.W. ➔ D.W. ➔ drying.

Instructions for preparation of laboratory reports

Day	:	Date	:
Practical	:	Experiment	:
Test, title or aim	:		
Principle	:		
Materials	:		
Method or procedure	:		
Results or observations	:	Results in quantitative assay & observations in qualitative assay	





Volumetric, Ostwald-Folin, and Sahli transfer pipettes.

CENTRIFUGE

A centrifuge is a device that accelerates gravitational separation of substances that differ significantly in their masses. In the clinical laboratory, centrifugation is used:

1- To separate particles from a solution in which they are suspended. Examples of this application are:
   i. Removing cellular elements from blood to provide cell-free plasma or serum for analysis.
   ii. Concentration of cellular elements and other components of biological fluids for microscopic examination or chemical analysis.
   iii. Elimination of chemically precipitated protein from an analytical specimen.
   iv. Separating protein-bound or antibody-bound ligand from free ligand in immunochemical and other assays.
2- To separate two liquid phases of different densities, for example:
   i. Extracting solutes in biological fluids from aqueous to organic solvents.
   ii. Separating lipid components such as chylomi-crons from other components of plasma or serum, and lipoproteins from each other.

PRINCIPLE OF CENTRIFUGATION

The correct term is relative centrifugal force (RCF), also called relative centrifugal field has been used for determination of speed of centrifuge. Units are expressed as number of times greater than gravity (e.g., 500 X *g).*

Relative centrifugal force is calculated as follows:

$$\mathbf{RCF} = 1.118 \text{ X } l0^{-5} \text{X r X } n^2$$

Where $1.118X\ 10^{-5}$ is an empirical factor, r is the horizontal distance (i.e., the radius in centimeters) from the center of rotation to the bottom of the tube in

the rotor cavity or bucket during centrifugation, and n is the speed of rotation of the rotor in revolutions per minute (rpm).

$$n\,(\text{rpm}) = 1000\ X \sqrt{\frac{RCF}{11.18\ X\ r\,(\text{cm})}}$$

COMPONENTS OF A CENTRIFUGE

i. A rotor or centrifuge head.
ii. A drive shaft and a motor.
iii. A centrifuge chamber
iv. power switch,
v. Timer, speed control, tachometer, and brake.
vi. Protective shield to minimize aerosol production if a tube should break, or a refrigerator to reduce the temperature within the chamber.
vii. Centrifuges may also include audible or visible alarms to indicate malfunctions such as imbalance of the rotor.

TYPES OF CENTRIFUGES

Centrifuges may be classified generally into three types:

i. Horizontal-head or swinging-bucket,
ii. Fixed-angle or angle-head, and
iii. Ultracentrifuge.

There are many different models of the fixed-angle and angle-head centrifuges, including bench-top and floor models, as well as refrigerated and non-refrigerated versions.

N.B.: balance between tubes is very important to prevent tubes cracking.

SPECTROPHOTOMETER

- ✓ It's an instrument used to measure light that is either absorbed or transmitted through a solution in cuvette.
- ✓ Transmitted light is internally mathematical converted into absorbance unit to determine [light absorbing substance] present in the cuvette.
- ✓ All spectrophotometers have basically the same components including light source, monochromator, sample holder, detector and read out device.

Light source:

A suitable light source must meet the following requirements:

- It must produce a beam with sufficient power,
- It must provide a continuum of wavelength over the region of interest,
- It must stable.

✓A tungsten lamp provides a continuum of wavelength, and it's not used for measurements below 350 nm.

✓Hydrogen and deuterium discharge lamp give a continuous emission spectrum in the ultraviolet (UV) region, which ranges from 195 – 380 nm.

✓Mercury, xenon, and other types of lamps are available.

Entrance & exit slits:

Monochromator:

It's used for isolation of required range of wavelength; this can be done by using of filters, prisms, and differentiation gratings & are of 2 types:

- Absorption filters; produce a wide range of wavelengths.
- Interference filters; are made at fixed wavelengths.

Sample holder:

✓ It's also known as cuvette or cell; made of glass, quartz or plastic.

✓ Glass and transparent plastic cuvettes are used in the visible region.

✓ Quartz or silica cell are necessary for UV radiation.

✓ Cuvettes are of two types – macrocuvettes with a capacity of 3 ml only and microcuvettes with a capacity of 1 ml only.

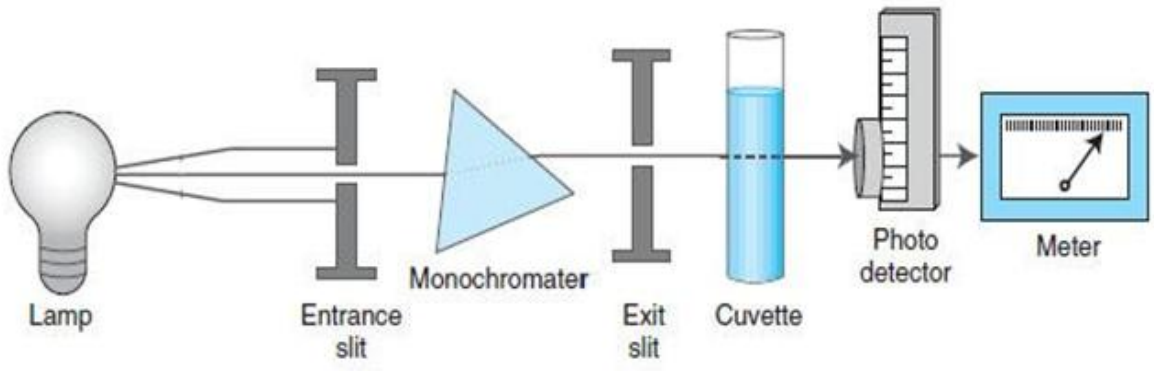
Detector:

It converts the electromagnetic radiation transmitted by a solution into an electrical signal. Four types of detectors can be used to measure the transmitted light:

- Barrier layer cell
- Silicon photodiode
- Phototube
- Photomultiplier

Readout device:

The magnitude of the electrical current from a detector can be displayed on a meter; digital readout device or recorder.

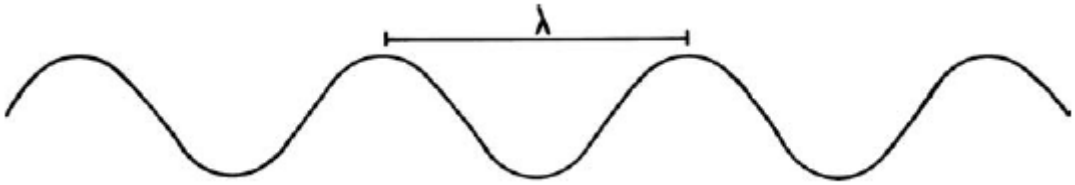


Components of a spectrophotometer.

Characteristics of wavelength:

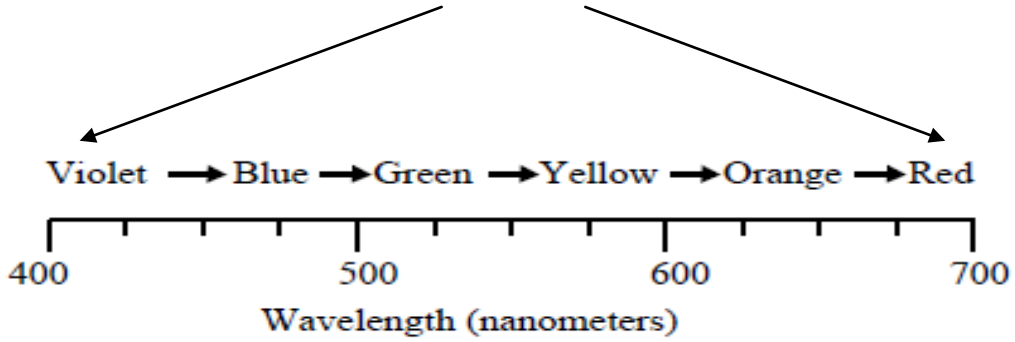
✓ Light and all electromagnetic radiation consist of photons or packets of energy traveling in waves.

✓ The electromagnetic spectrum is described in term of wavelength (λ) which is the distance between successive wave peaks and its unit is nanometer (nm) or A.



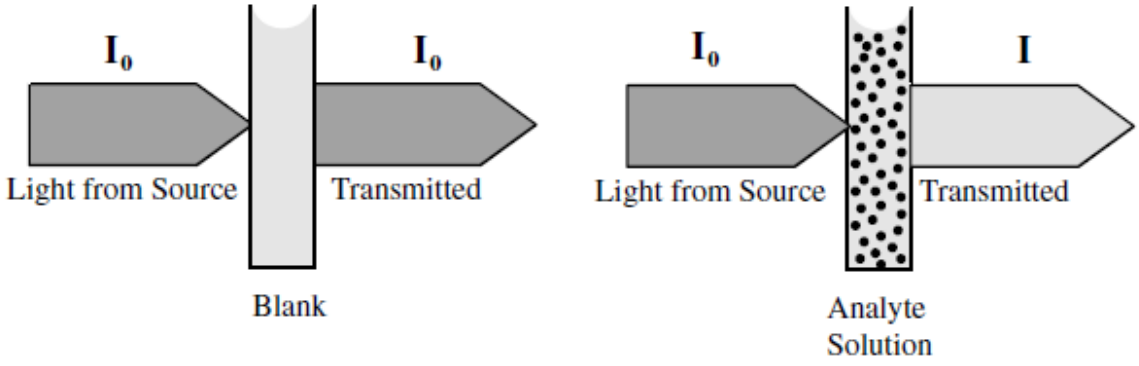
The Electromagnetic Spectrum:

Cosmic rays	γ rays	X ray	UV	Visible	IR	Microwaves (TV, radio, radar)



< 380 nm UV invisible light	380 – 440 nm violet visible light
440 – 500 nm blue visible light	500 – 580 nm green visible light
580 – 600 nm yellow visible light	600 – 620 nm orange visible light
620 – 750 nm red visible light	750 – 2000 nm IR invisible light

Absorbance, transmittance, and Beer's law:



$$\% T = \frac{I}{I_o} X 100$$

Beer-Lambert law

The concentration of a substance is directly proportional to the amount of light absorbed or inversely proportional to the logarithm of the transmitted light.

$$\text{Conc. } \alpha \text{ A}$$

$$\text{Conc. } \alpha \log \frac{1}{T} \rightarrow \text{A } \alpha \log \frac{1}{T}$$

Absorbance can't be measured directly but mathematically derived from % T. According to Beer-Lambert law

$$A = -\log T = -\log \frac{I}{I_o}$$

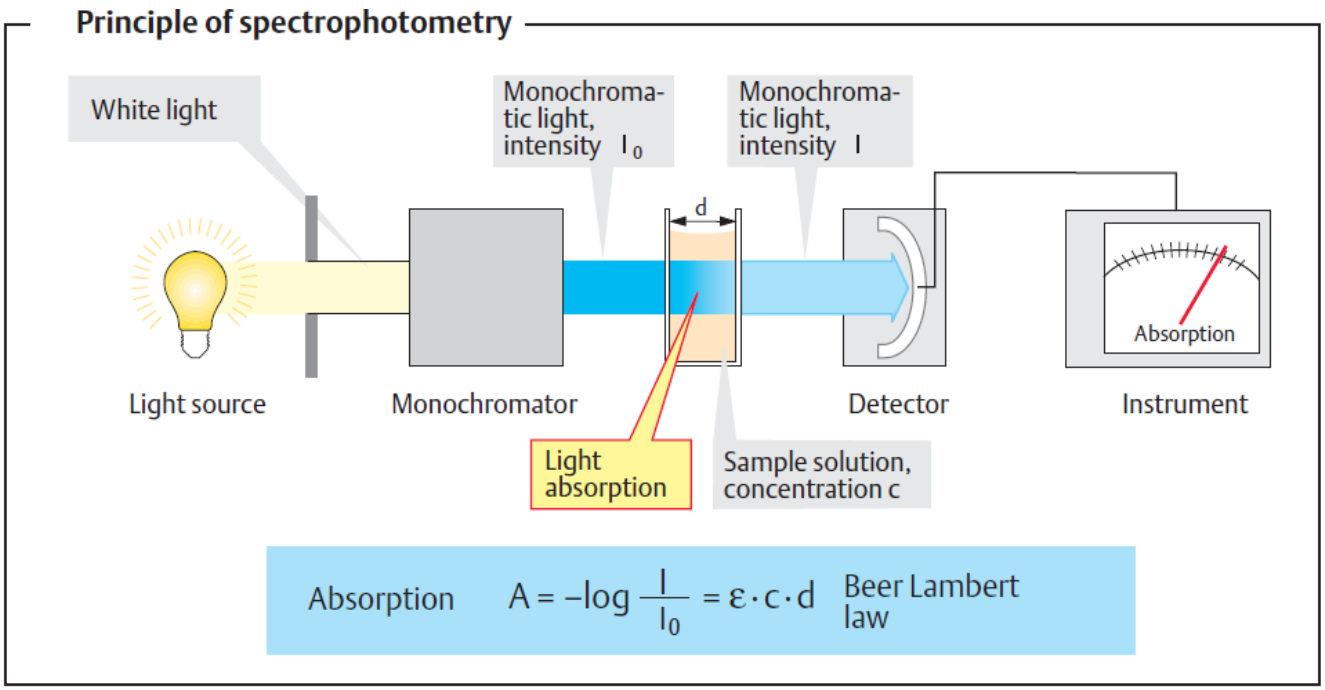
$$\mathbf{A = \log (100\ \%) - \log \%\ T = 2 - \log \%\ T = abc}$$

Where,

a: absorptivity constant for the substance,

b: length of the light path through the substance,

c: concentration of the substance



BIOCHEMICAL CALCULATIONS

SI units:

The metric system was introduced in 1901 to be used in chemistry and physics. Since then this system has been gradually expanded, and in 1960 it was given the name International System of Units (SI) and were introduced into medicine thereafter.

Basic SI units:

Quantity	Unit Name	Symbol
Length	metre	m
Mass	kilogram	kg
Time	second	s
Electric current	ampere	A
Thermodynamic temperature	kelvin	K
Amount of substance	mole	mol
Luminous intensity	candela	cd

Conversion factors:

Factor	Prefix
10^{-1}	deci
10^{-2}	centi
10^{-3}	mili
10^{-6}	micro
10^{-9}	nano
10^{-12}	pico
10^{-15}	femto

Units of weight and volume:

○ Units of weight	○ Units of volume
1 gm = 1000 mg = 10^3 mg	1 liter = 10 dl = 1000 ml
1 mg = 1000 µg = 10^3 µg (micro)	1 dl = 100 ml
1 gm = 10^6 µg	1 ml = 1000 µl
1 gm = 10^9 ng (nano)	0.5 ml = 500 µl
1 gm = 10^{12} pg (pico)	0.1 ml = 100 µl
	0.05 ml = 50 µl
	0.01 ml = 10 µl

Concentration units:

Concentration is the amount of solute dissolved in a given amount of solvent. There are many ways to express the concentration units, the most familiar units are:

- Percent

i- Mass: it's also called weight percent and is given by the formula:

% mass of solute in g =

[Mass of solute / (mass of solute + mass of solvent)] X 100

ii- W / V

iii-W / W or V / V

- Number of moles & mole fraction

Number of moles = mass (g) / molecular weight (g / mol)

Mole fraction =

Moles of a part of a compound / sum of moles of all parts of a compound

- Molality

It's the number of moles of solute dissolved in one kilogram of solvent;

Molality = moles of solute / kilograms of solvent = mol / kg.

- Formality
- Normality (N)

It's the number of equivalent weight of a solute in gram dissolved in one liter of solvent;

- Molarity (M)

It's the number of moles of solute dissolved in one liter of solution; i.e. it's the number of molecular weight of a solute in gram dissolved in one liter of solvent.

Molarity = moles of solute / liters of solution = mol / L or M

Q: Prepare 1 normal solution of NaOH (equivalent weight = 40)

S: weigh 40 g of NaOH and dissolve in 1 liter of D.W., shake well. The solution is said to be 1 N NaOH.

1 N = 40 g / 1000 ml, If 0.1 N is required ➔ N / 10 = 4 g / 1000 ml.

Q: Prepare 1 molar solution of oxalic acid (molecualr weight = 60)?

S: weigh 60 g of oxalic acid and dissolve in 1000 ml of D.W., shake well. The solution is said to be 1 M oxalic acid.

Q: How to prepare 250 ml of N/10 oxalic acid solution?

S: For normal solution 63 g / 1000 ml

For N /10 solution 6.3 g / 1000 ml

For N /10 solution [6.3 /4]/ 250 ml

For N /10 solution in 250 ml solution = 1.575 g /250 ml

Weigh 1.575 g of oxalic acid and dissolve in 250 ml of D.W., shake well.

How to prepare m.mol solution?

Dissolve a weight equivalent to molecular weight of a solute in milligram (mg) dissolved in one liter of solvent.

Example: prepare 1 m.mol & m.mol / 15 of urea in 1000 ml, 500 ml, 250 ml, and 100 ml (molecular weight of urea = 60)

Volumes (ml)	m.mol	m.mol / 15
1000	Weigh 60 mg	Weigh 4 mg
500	Weigh 30 mg	Weigh 2 mg
250	Weigh 15 mg	Weigh 1 mg
100	Weigh 6 mg	Weigh 0.4 mg

Conversion of traditional unit to SI unit and vice versa:

Normal values	Traditional unit mg / dl	SI unit m.mol / l
Fasting blood sugar	60 - 90	3.33 – 5
Blood urea	15 – 40	2.5 – 6.41
Serum cholesterol	150 – 200	3.8 – 5.17
Serum sodium		135 – 148
Serum potassium		3.5 – 5.5
Serum calcium	9 – 11	2.12 – 2.62
Plasma protein	6.8 – 8.4	
Serum creatinine	0 – 1.5	0.053 – 0.106
Serum iron	59.75 – 150	10.7 – 26.9
Serum triglyceride	0 – 160	

m.mol/l X molecular weight = mg/dl X 10

Molecular weight of come compounds

Compounds	Molecular weight	Compounds	Molecular weight
Glucose	180	Sodium	23
Urea	60	Potassium	39
Cholesterol	386.4	Calcium	40.1
Creatinine	113.13	Iron	56
Triglyceride	882		

Example: a patient his blood sugar level is 100 mg / dl, what is his blood sugar level in m.mol /l ?

Solution: m.mol / l = [mg / dl X 10] / molecular weight

m.mol / l = [100 X 10] / 180 = 5.5

Example: assume that a patient's blood urea level is 3 m.mol / l, what does it equal in mg /dl?

Solution: mg / dl = [m.mol / l X molecular weight] / 10

mg / dl = [3 X 60] / 10 = 18

SPECIMEN COLLECTION

Storage of blood specimens

1) Plasma or serum should be separated from cells as soon as possible and certainly within 2 hrs to minimize the metabolism of cells.
2) If it's impossible to centrifuge a blood specimen within 2 hrs the specimen should be kept at room temperature rather than at 4 °C to minimize hemolysis.
3) If the specimen can't be analyzed at once, the separated serum should be generally stored in capped tubes at 4 °C until analysis both to:
   i- Maintain stability of the specimen; and
   ii- Reduce evaporation.
4) If a specimen for a particular test is sufficiently unstable at 4 °C, the serum specimen should be held at – 20 °C in a freezer capable of maintaining this temperature.
5) Specimens for bilirubin or carotene must be protected from both daylight and fluorescent light to avoid photodegradation.
6) Specimen tubes should be centrifuged with stoppers in place. Closure lowers evaporation and prevents aerosolization of infectious particles.
7) Removal of the stopper before centrifugation allow loss if CO_2 and increase in blood PH.

Changes in blood metabolites on keeping

1. Glucose is converted to lactate, as a result of glycolysis occurring in blood cells specially RBCs. It's inhibited by fluoride but fluoride interferes with glucose oxidase and urease methods.
2. Glycolysis decreases serum glucose by approximately 5 – 7 % in 1 hr. (5 – 10 mg /dl) in uncentrifuged coagulated blood at room temperature.
3. The rate of glycolysis is higher in the presence of leukocytosis or

bacterial contamination.

4. Several substances pass through RBCs membrane e.g. K^+, LDH.
5. Loss of CO_2, since the PCO_2 of blood is much higher than air leading to fall in plasma total CO_2.
6. Phosphate increases due to hydrolysis of organic ester phosphate compounds of erythrocytes.
7. Enzymes activities are lost on long keeping
8. Formation of ammonia from nitrogenous substances.

Deterioration of urine samples on keeping

Urine samples tend to deteriorate unless preservative is added from the start of collection, or the specimen is refrigerated throughout the collection period. The changes in chemical composition that are liable to occur include:

1. Bacterial fermentation of glucose if present.
2. Fall in PH & Urea is converted to ammonia by bacteria.
3. Sometimes phosphate precipitates.
4. Urobilinigen is oxidized to urobilin and porphobilinogen changes to porphyrins.

SERIAL DILUTIONS

A dilution involves two entities, the solute, which is the material being diluted, and the diluent, the medium making up the rest of the solution. When a solution is diluted with water, its volume is increased and its concentration is decreased, but the total amount of solute remains unchanged. A simple formula can be used only if the concentration of the original solution is known:

C 1 X V 1 = C 2 X V 2, where

C 1: the original concentration of the solution to be diluted

V 1: the unknown volume to be taken from the undiluted solution

C 2: the needed dilution concentration

V 2: the needed volume of diluted solution (total volume)

V 2 = V 1 + volume of diluent

This formula can be used to determine the volume of a concentrated solution that is required to make a known volume of a solution of a desired lesser concentration.

The relationship between solute and diluent is expressed as a fraction. For example, if a 1:20 dilution is called for, this implies 1 part of solute and 19 parts of diluent. The number on the bottom of the fraction is the total volume, reached by adding the volumes of the solute and diluent together.

$$\frac{1}{\text{Dilution}} = \frac{\text{Amount of solute}}{\text{Total volume}}$$

To create a certain volume of a specified dilution, it is helpful to know how to manipulate this relationship. An algebraic equation can be set up to find either the total volume, the amount of solute, or the amount of diluent needed to make a dilution. Consider the following example:

2 ml of a 1:20 dilution is needed to run a specific test. How much serum and how much diluent are needed to make this dilution?

The equation is set up using the fraction for the dilution, indicating the relationship between the total volume and the solute, or amount of serum needed:

$$\frac{1}{20} = \frac{\chi}{2\,\text{ml}}$$

Note that the 20 represents the total number of parts in the solution, and that 2 ml is the total volume desired. Solving this equation for *x* gives 0.1 ml for the amount of serum needed to make this dilution. The amount of diluent is obtained by subtracting 0.1 ml from 2.0 ml to give 1.9 ml of diluent. To check the answer, simply set up a proportion between the amount of solute over the total volume. This should equal the dilution desired.

$$\frac{0.1\,\text{ml}}{2.0\,\text{ml}} = \frac{1}{20}$$

Thus the correct answer has been obtained. If, on the other hand, the amount of serum that is to be used is known, a problem can be set up in the following manner:

A 1:5 dilution of patient serum is necessary to run a test. There is 0.1 ml of serum that can be used. What amount of diluent is necessary to make this dilution using all of the serum? A slightly different formula can be used to solve this problem.

$$\frac{1}{\text{Dilution} - 1} = \frac{\text{Amount of solute}}{\text{Amount of diluent}}$$

$$\frac{1}{4} = \frac{0.1\,\text{ml}}{\chi}\text{, } x = 0.4 \text{ ml of diluent}$$

Note that the final volume is obtained by adding 0.1 ml of solute to the 0.4 ml of diluent. Dividing the volume of the solute by the total volume of 0.5 ml yields the desired 1:5 ratio. Depending on the unknown being solved for, either of these formulas can he used. To calculate the total volume, the total dilution factor must be used. If, however, the amount of diluent is to be

calculated, the formula using dilution – 1 can be used. The previous examples represent simple dilutions.

Occasionally in the laboratory it is necessary to make a very large dilution, and it is more accurate and less costly to do this in several steps rather than all at once. Such a process is known as a compound dilution. The same approach is used but the dilution occurs in several stages. For example, if a 1:500 dilution is necessary, it would take 49.9 ml. of diluent to accomplish this in one step with 0.1 ml of scrum. If only a small amount of solution is needed to run the test, this is wasteful; furthermore inaccuracy may occur if the solution is not properly mixed. Therefore, it is helpful to make several smaller dilutions. To use the example above, a 1:500 dilution can be achieved by making a 1:5 dilution of the original serum, a 1:10 dilution from the first dilution, and another 1:10 dilution. This can be shown as follows:

Serum ➔		
1:5 dilution →	1:10 dilution →	1:10 dilution
0.1 ml serum	0.1 ml of 1:5 dilution	0.1 ml of 1:10 dilution
0.4 ml diluent	0.9 ml diluent	0.9 ml diluent

Multiplying 5 X 10 X 10 equals 500, or the total dilution. Each of the simple dilutions is calculated individually by doing mental arithmetic, or by using the formula given for simple dilutions. In this example, the 1:500 dilution was made using very little diluent in a series of test tubes, rather than having to use a larger volume in a flask. The volumes were kept small enough so that mixing could take place easily, and the final volume of 1.0 ml is all that is necessary to perform a test.

STANDARD CURVE CONSTRUCTION
(CALIBRATION CURVE)

The preparation of a standard curve is necessary to check whether the method of assaying a particular substance follows Beer's law, that is whether the absorbance of the substance increases in a linear way with its concentration.

- Prepare standard solution (e.g. glucose 1 g/dl = 1 % = 1000 mg/100 ml).
- Apply the calculations made in the following table to make different dilutions of standard solution.
- The general formula for obtaining different concentrations of a solution by dilution with diluent (e.g. distilled water) is:

C 1 X V 1 = C 2 X V 2, where

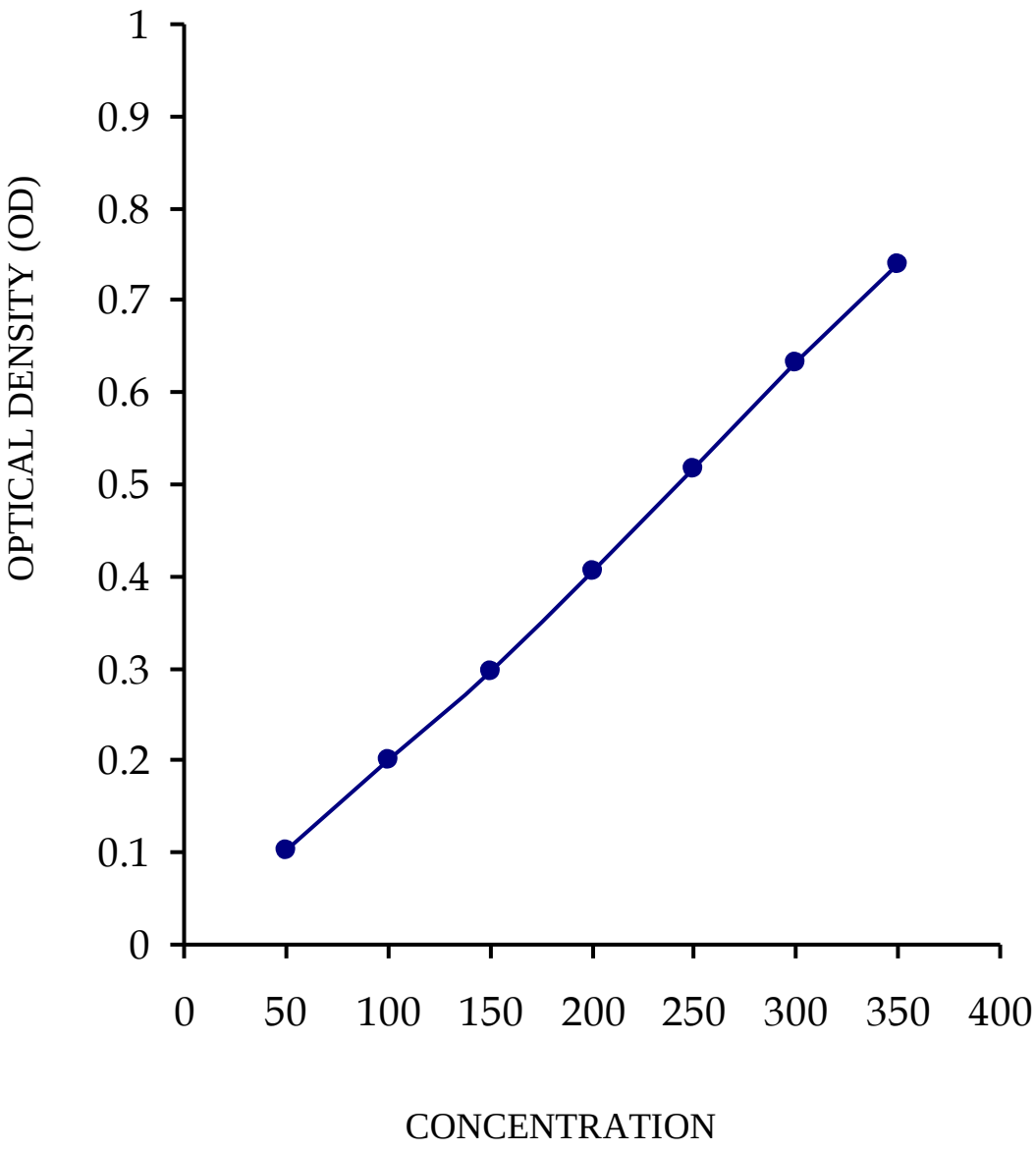
TV = V 2 = V 1 + volume of diluent

Tubes	Conc. (mg %)	Amount of mls needed (ml) C1X V1 = C2 X V2	DW (ml)	Total volume (ml)
1	50	1000 X V1 = 50 X 2 ➔ V1 = 0.1	1.9	2
2	100	1000 X V1 = 100 X 2 ➔ V1 = 0.2	1.8	2
3	150	1000 X V1 = 150 X 2 ➔ V1 = 0.3	1.7	2
4	200	1000 X V1 = 200 X 2 ➔ V1 = 0.4	1.6	2
5	250	1000 X V1 = 250 X 2 ➔ V1 = 0.5	1.5	2
6	300	1000 X V1 = 300 X 2 ➔ V1 = 0.6	1.4	2
7	350	1000 X V1 = 350 X 2 ➔ V1 = 0.7	1.3	2
8	400	1000 X V1 = 400 X 2 ➔ V1 = 0.8	1.2	2

Pour into cuvettes the intended volumes of working reagent and dilution series according to the estimation procedure of interest.

The standard curve is constructed by plotting a vertical axis (y – axis, ordinate) for the optical densities (absorbance) and a horizontal axis (x – axis, abscissa) the concentrations of the standard solutions.

The glucose concentration in the sample (test or unknown) can be then measured from the graph (standard curve).



BLOOD SUGAR ESTIMATION

Principle:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconate} + H_2O_2$$

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

Color intensity (red-violet) is directly proportional to [glucose] in the specimen.

Sample: Serum, plasma.

Stability: 1 day ➔ 2 – 8 ºC

Conditions:

Wavelength	:	546 nm
Cuvette	:	1 cm light path
Temperature	:	25 ºC/ 30 ºC/ 37 ºC
Measurement	:	Against reagent blank
Method	:	Enzymatic colorimetric

Procedure:

	Blank (B)	Standard (S)	Test (T)
Standard	-	10 µl	-
Sample	-	-	10 µl
Working reagent	1 ml	1 ml	1 ml

Mix well and incubate at RT for 10 minutes.

Calculation

$$[\text{Glucose mg/dl}] = \frac{\text{OD sample}}{\text{OD standard}} \text{ X } [\text{Standard}]$$

Where, OD (optical density) = A (absorbance)

[S] = 100 mg/dl

Normal value:

75 – 115 mg/dl

Clinical interpretation:

Blood glucose level increases in case of

1. DM, CNS disorders, Hypothalamic disease
2. Brain tumor or hemorrhage, liver disease, obesity, feeding after starvation.
3. Disturbances of metabolism associated with burns, infection, fractures, myocardial infarction, and uremia.
4. Certain drugs e.g. thaizides, corticosteroids, adrenocorticotropic hormone, birth control pills.

ORAL GLUCOSE TOLERANCE TEST (OGTT)

The ability of the body to manage its blood glucose level within the normal range is known as glucose tolerance. One of the biochemical tests that is employed in the diagnosis and in assessment of severity of diabetes is the glucose tolerance test. The response of plasma glucose levels to specific tolerance of glucose orally conforming to a period of 2 to 3 hrs without giving rise to glucosuria, constitute what is known as normal glucose tolerance. This is a measure of efficiency of carbohydrate metabolism in the person.

Standards for OGTT:

- Prior diet of > 150 gm of carbohydrate daily, no alcohol, and unrestricted activity for 3 days before test.
- Test in morning after 10-16 hrs of fasting.
- No medication, smoking, or exercise (remain seated) during test.
- Not to be done during recovery from acute illness, emotional stress, surgery, trauma, pregnancy, inactivity due to chronic illness; therefore is of limited or no value in hospitalized patients.
- Certain drugs should be stopped several weeks before the test (e.g., oral diuretics, oral contraceptives, phenytoin).
- Loading dose of glucose consumed within 5 mins:
- For adults = 75 gm, for children = 1.75 gm/kg (of ideal body weight in obese children but never >75 gm), for pregnant women = 100 gm, glucose dissolved in about 300 ml of water is given orally
- Draw blood at fasting, 30, 60, 90, 120 mins; 30-min sample offers little additional information but can confirm adequate gastric absorption when patient is nauseous.

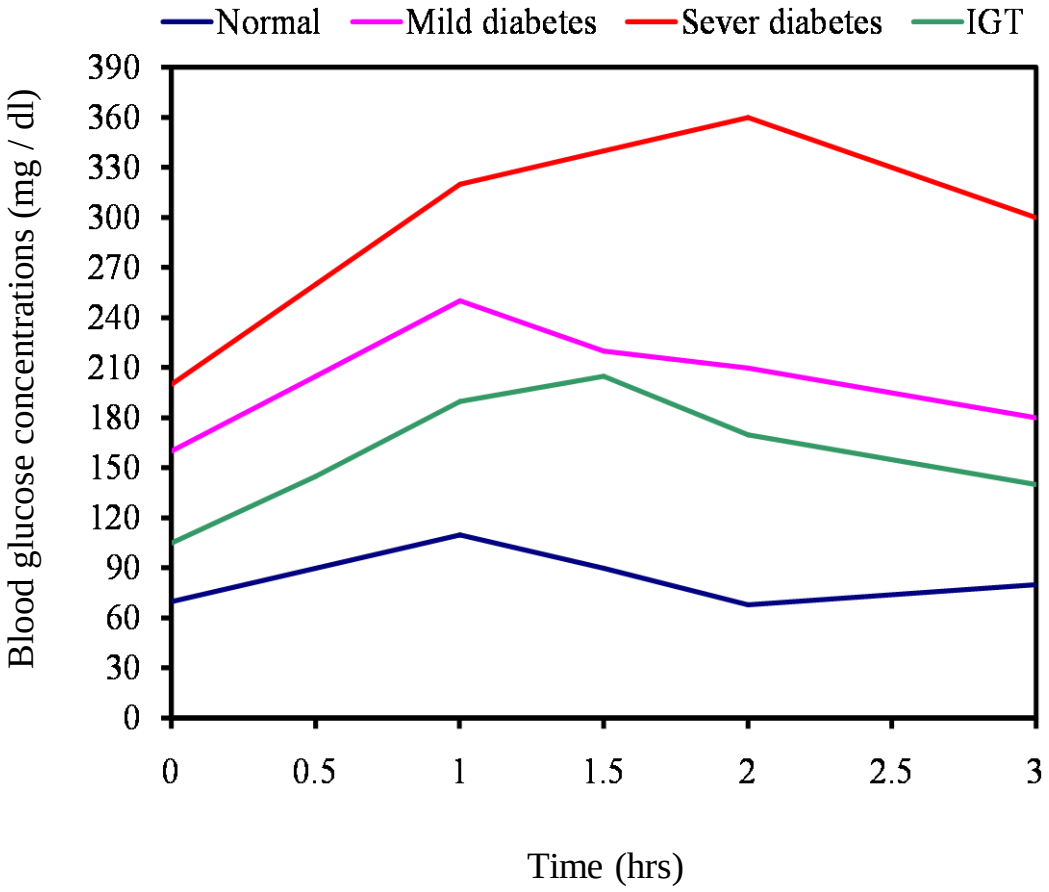
Observation:

Normal	Fasting	½	1	1 ½	2	3
Plasma glucose (mg/dl)	70	90	110	95	68	80
Urine glucose	Nil	Nil	Nil	Nil	Nil	Nil

Mild diabetes	Fasting	½	1	1 ½	2	3
Plasma glucose (mg/dl)	160	190	250	220	210	180
Urine glucose	Nil	+	++++	+++	++	+

Sever diabetes	Fasting	½	1	1 ½	2	3
Plasma glucose (mg/dl)	250	280	320	340	360	300
Urine glucose	++++	++++	++++	++++	++++	++++

Impaired glucose tolerance (IGT)	Fasting	½	1	1 ½	2	3
Plasma glucose (mg/dl)	105	130	190	205	170	140
Urine glucose	Nil	Nil	+	+	Nil	Nil



Indications for OGGT

1. Diagnosis of gestational diabetes mellitus.
2. Diagnosis of IGT.
3. Evaluation of a patient with random glucose level < 140 mg/dl & unexplained nephropathy, neuropathy or retinopathy.
4. For epidemiological studies.

WHO criteria for the diagnosis of DM:

a) Fasting plasma glucose is > 140 mg/dl, on two occasions or

b) Fasting plasma glucose is < 140 mg/dl and 2-hrs plasma glucose is > 200 mg/dl, with one intervening value > 200 mg/dl after a 75 g glucose load (GTT).

c) Normal values of non-pregnant adults are

i- Fasting plasma glucose < 115 mg/dl,

ii- 2-hrs plasma glucose < 140 mg/dl.

Some patients have impaired glucose tolerance (IGT); i.e. glucose metabolism intermediate between normal and overt diabetes, it's best to label them IGT rather than DM patients.

i- Fasting plasma glucose is < 140 mg/dl and

ii- 2-hrs plasma glucose is > 140 mg/dl but < 200 mg/dl

iii- An intervening value of > 200 mg/dl after a 75 g glucose load (GTT).

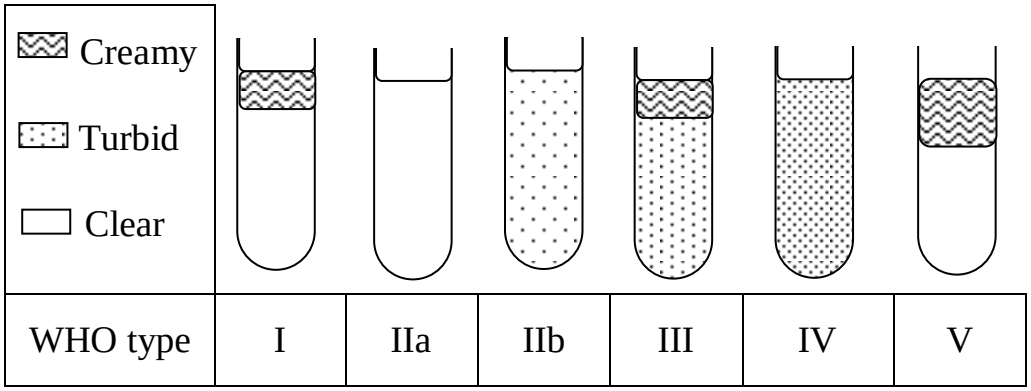
LIPID PROFILE

A lipid profile generally consists of total cholesterol, TGs, HDL-C, LDL-C and a check for the presence of CM. Lipid profiles are most commonly run on 12 hrs fasting serum.

Plasma standing (appearance) test

Blood ➔ plasma ➔ store at 4 °C ON➔ observation

- ✓ Simple visual inspection of plasma.
- ✓ CM being less dense than plasma floats to the top giving a creamy supernatant layer.
- ✓ VLDL remains in suspension and impart turbidity to the plasma.
- ✓ Both LDL and HDL are too small, with the result that even when they are present in excess the plasma remains clear.



WHO type	I	IIa	IIb	III	IV	V

PLASMA TOTAL CHOLESTEROL ESTIMATION

Introduction:

It's an important component of the body. It's used for both structural and synthetic functions e.g. cell membrane and a precursor of bile acids, steroid hormones & vitamin D.

Sources of cholesterol:

Some cholesterol is found in diet of all persons. Dietary cholesterol is called exogenous cholesterol. In our body cholesterol is synthesized by virtually all tissues (liver, gut, adrenal cortex, ovaries, testis & placenta). This cholesterol is called endogenous cholesterol. The two fractions of lipoproteins carry the cholesterol called cholesterol fractions:

1) High density lipoprotein (HDL)
2) Low density lipoprotein (LDL).

Principle:

Cholesterol and its esters are released from lipoproteins by detergents.

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

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol Oxidase}} \text{Cholesterol –3– on} + H_2O_2$$

$$H_2O_2 + \text{Phenol} + \text{4 - amino antipyrine} \xrightarrow{\text{Peroxidase}} \text{quinoneimine dye} + 4\ H_2O$$

Specimens:

Serum, heparinized or EDTA plasma. Don't use citrate, oxalate or fluoride.

Stability: 5 – 7 days ➔ 2 – 8 °C

3 months ➔ – 20 °C

Conditions:

Wavelength	:	546 nm
Cuvette	:	1 cm light path
Temperature	:	25 ºC/ 30 ºC/ 37 ºC
Measurement	:	Against reagent blank
Method	:	Enzymatic colorimetric

Procedure

	B	S	T
Standard	-	10 µl	-
Serum	-	-	10 µl
Working reagent	1000 µl	1000 µl	1000 µl

Mix well, measure after incubating at 20-25 ^{o}C for 10 min.

Calculation

$$[\text{Cholesterol (mg/dl)}] = \frac{OD\,T}{OD\,S} X\ [S]$$

Where**,** [S] = 200 mg / dl or 5.16 mmo1/1.

Normal range:

Adult: 150 – 250 mg /dl

Infant: 90 – 130 mg /dl

Child: 130 – 170 mg /dl

Clinical significance:

Hypercholesterolemia is observed in

1. Coronary heart disease
2. Hypothyroidism or myxedema
3. DM
4. Nephrosis
5. Xanthomatosis

6. Obstructive jaundice
7. High cholesterol diet

Hypocholesterolemia is observed in

1. Hyperthyroidism
2. Sever liver disease - hemolytic jaundice
3. Malabsorption

SERUM TRIGLYCERIDES ESTIMATION

Introduction:

The intestine possesses TGs from dietary FAs (exogenous), and they are transported in the blood stream as CMs which gives the serum a milky or creamy appearance after a meal rich in fats. The liver is also responsible for manufacturing TGs but travel as VLDL.

Principle:

The TGs is determined after enzymatic hydrolysis with lipases.

$$TGs + H_2O \xrightarrow{LPL} Glycerol + FAs$$

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

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

$$2\ H_2O_2 + 4\text{-aminophenazone} + 4\ Chlorophenol \xrightarrow{POD} quinoneimine + HCl + 4H_2O$$

Specimen:

Serum, heparinized or EDTA plasma.

Stability: 5 – 7 days ➔ 2 – 8 °C

3 months ➔ – 20 °C

Conditions:

Wavelength	:	546 nm
Cuvette	:	1 cm light path
Temperature	:	25 ºC/ 30 ºC/ 37 ºC
Measurement	:	Against reagent blank
Method	:	Enzymatic colorimetric

Procedure

	B	S	T
Standard	-	10 µl	-
Serum	-	-	10 µl
Working reagent	1000 µl	1000 µl	1000 µl

Mix well, measure after incubating at 20-25 ^{o}C for 10 min.

Calculation

$$[\text{TG (mg/dl)}] = \frac{\text{OD T}}{\text{OD S}} \text{ X } [\text{S}]$$

Where**,** [S] = 200 mg / dl or 2.28 mmo1/1.

Normal values:

♀: 40 – 140 mg / dl

♂: 60 – 165 mg / dl

Clinical significance:

Hypotriglyceridemia	Hypertriglyceridemia
1. Congenital β lipoproteinemia 2. Hyperthyroidism 3. Hyperparathyroidism 4. Protein malnutrition	1. Hyperlipoproteinemia 2. Acute myocardial infarction 3. Hypertension & diabetes 4. Cerebral thrombosis 5. Hypothyroidism 6. Nephritic syndrome 7. Pregnancy 8. Pancreatitis

SERUM HDL-C ESTIMATION

Introduction:

Lipoproteins are lipids bound to proteins, there are 5 classes of lipoproteins; TG–rich particles including CM, VLDL & IDL while LDL & HDL contain mostly cholesterol.

CM: it transports exogenous (dietary) and additional lipids made in intestinal mucosal cells from the intestine to the peripheral tissues.

VLDL: it carries endogenous lipids from the liver to the peripheral tissues.

IDL: it's normally undetectable in normal plasma.

LDL: its primary function is to provide cholesterol to the peripheral tissues.

HDL: it's involved in transport of cholesterol from peripheral tissues to the liver. HDL cholesterol has become an important part of evaluating a patient's risk of cardiovascular disease.

Principle:

LDL, VLDL and CM fractions in the sample are precipitated by addition of phosphotungestate in the presence of magnesium ions. After centrifugation, [cholesterol] in the HDL fraction, which remains in the supernatant, is determined.

Serum + precipitating agent $\xrightarrow{\text{Centrifugation}}$ Precipitate (LDL +VLDL + CM) + supernatant (HDL)

Procedure:

1- HDL- Separation

Serum	200 µl
Precipitating reagent	500 µl

Mix well, measure after incubating at 20-25 ^{o}C for 10 min then centrifuge for 10 min at 4000 rpm.

2- HDL-C determination

	B	S	T
DW	100 µl	-	-
Supernatant	-	-	100 µl
Standard	-	100 µl	-
Cholesterol reagent (working reagent)	1000 µl	1000 µl	1000 µl

Mix well, measure after incubating at 20-25 ^{o}C for 10 min.

Calculation:

$$[\text{HDL–C (mg/dl)}] = \frac{A\,T}{A\,S} X\ [S] X 1.1$$

The factor 1.1 is due to the serum dilution with the precipitating reagent.

Where, [S] = 200 mg/dl or 5.16 mmo1/1.

Expected values:

The average value of HDL–C for protection against CVD is:

♂: 45 mg/dl

♀: 55 mg/dl

Interpretation:

Relation between incidence of CHD and cholesterol levels

< 200 mg / dl	Low risk
200 – 250 mg / dl	Moderate risk
> 250 mg / dl	High risk specially if HDL-C < 35 mg / dl

CALCULATION & ESTIMATION OF LDL-C

Friedewald calculation or Derived beta- quantification:

The formula hinges on the assumption that VLDL-C is present in a concentration equal to 1/5 of [TGs].

When using mg/dl units, this approximation works reasonably well in most serum specimens. This assumption is valid for [TGs] < 400 mg/dl; thereafter, inconsistencies in the VLDL TG/Ch ratio occur, and the formula can not be used. Similarly the presence of CM or β–VLDL in the serum sample preclude this estimation.

[LDL-C] = [TCh] – [HDL-C] – [VLDL-C]

When concentrations are expressed in mmol/1.

$$\text{VLDL-C} = \frac{\text{Serum TG}}{2.2}$$

When concentrations are expressed in mg / d1.

$$\text{VLDL-C} = \frac{\text{Serum TG}}{5}$$

LDL-C estimation

Principle

LDLs are precipitated by heparin at their isoelectric point (PH 5.04). After centrifugation, HDL and VLDL remain in the supernatant. These can then be determined by enzymatic methods.

[LDL-C] = Total cholesterol - Cholesterol in the supernatant.

Specimen:

Serum, heparinized or EDTA plasma. Don't use citrate, oxalate or fluoride.

Stability: 7 days ➔ 2 – 8 °C

3 months ➔ – 20 °C

Conditions:

Wavelength	:	500 , 546 nm
Cuvette	:	1 cm light path
Temperature	:	25 ºC/ 30 ºC/ 37 ºC
Measurement	:	Against reagent blank
Method	:	Enzymatic colorimetric

Procedure:

Pipette into centrifuge tube

Serum	100 µl
Precipitation reagent	1000 µl

Mix well, measure after incubating at 20-25 ^{o}C for 10 min then centrifuge for 15 min at 4000 rpm.

Pipette into test tubes :			
	B	S	T
Distilled water	50 µl		
Standard		50 µl	
Supernatant			50 µl
Working reagent	1000 µl	1000 µl	1000 µl

Mix well, measure after incubating at 20-25 ^{o}C for 10 min.

Calculation:

Using a standard:

[cholesterol in the supernatant (mg / dl)] = $\frac{A\,T}{A\,S}$ X [S]

Using a factor:

Cholesterol concentration of the supernatant

Factor is given in table below

Hg	mg/dl
546 nm	1028
500 nm	690

Calculation:

LDL - C = Total cholesterol - cholesterol in the supernatant

Clinical interpretation

	mg/dl
No treatment required	< 150
Suspect range	150-190
Treatment required	> 190

Clinical significance:

↑ LDL-C is associated with ↑ risk for atherosclerosis.

PLASMA TOTAL PROTEIN ESTIMATION

Principle:

Divalent copper reacts in alkaline solution with protein peptide bonds producing a violet color biuret complex which is directly proportional to the amount of protein present.

$$\text{Protein} + Cu^{2+} \xrightarrow{\text{alkaline solution}} \text{Cu-protein complex}$$

Sodium potassium tartarate prevents precipitation of copper hydroxide & potassium prevents autoreduction of copper.

Specimen:

Serum, heparinized or EDTA plasma.

Stability: 3 days ➔ 2 – 8 °C

6 months ➔ – 20 °C

Conditions:

Wavelength	:	546 nm
Cuvette	:	1 cm light path
Temperature	:	25 ºC/ 30 ºC/ 37 ºC
Measurement	:	Against reagent blank
Method	:	Colorimetric

Procedure:

	B	S	T
DW	20 µl	-	-
Standard	-	20 µl	
Serum / plasma	-	-	20 µl
Working reagent	1 ml	1 ml	1 ml

Mix well, measure after incubating at 20-25 ^{o}C for 10 min.

Calculation:

Using a standard:

$$[\text{T.P g/dl}] = \frac{A\,T}{A\,S} X\ [S]$$

Where, [S] = 6 g/dl

Using a factor:

Factor is given in table below

Hg	g/dl
546 nm	19

Normal value:

New born: 5 – 7 g/dl

Children: 6 – 7.5 g/dl

Adult: 6.6 – 8.7 g/dl

Clinical interpretation:

Decrease in total protein is due to low albumin.

PLASMA ALBUMIN ESTIMATION

Principle:

Albumin is measured by virtue of its binding to the dye BCG " BromoCreysol Green" at PH 4.

Specimen:

Serum, heparinized or EDTA plasma.

Stability: 3 days ➔ 2 – 8 °C

6 months ➔ – 20 °C

Conditions:

Wavelength	:	620 nm
Cuvette	:	1 cm light path
Temperature	:	25 ºC/ 30 ºC/ 37 ºC
Measurement	:	Against reagent blank
Method	:	Colorimetric

Procedure:

	B	S	T
DW	20 µl	-	-
Standard	-	20 µl	
Serum / plasma	-	-	20 µl
Working reagent	1 ml	1 ml	1 ml

Mix well, measure after incubating at 20-25 ^{o}C for 10 min.

Calculation:

$$[\text{Albumin g/dl}] = \frac{A\,T}{A\,S} X\ [S]$$

Where, [S] = 6 g/dl

Normal value:

Albumin: 3.7 – 5.3 g / dl

Globulin: 1.8 – 3.6 g / dl

Total protein = albumin + total globulin

Normal Albumin: Globulin ratio = 2: 1

Clinical interpretation:

Hyperabuminemia is observed in dehydration

Hypoabuminemia is observed in:

1) Nephrosis (loss of albumin in urine)
2) Malabsorption
3) Infective hepatitis (decreased synthesis in liver)
4) Low dietary intake of protein (marasmus & kwashiorkor)
5) Generalized edema
6) Sever hemorrhage

Hyperglobulinemia is observed in

1) Multiple myeloma
2) Rheumatoid arthritis
3) Chronic infection
4) Macroglobulinemia
5) Sometimes in T.B. and bacterial endocarditis.

SERUM URIC ACID ESTIMATIOM

Principle:

$$\text{Uric acid} + 2\,H_2O + O_2 \xrightarrow{\text{Uricase}} \text{Allantoin} + CO_2 + H_2O_2$$

$$H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{POD}} \text{quinoneimine} + 4\,H_2O$$

Color intensity is directly proportional to [UA] in the specimen.

Specimen:

Serum, heparinized or EDTA plasma, urine (diluted 1:10 DW)

Stability: 5 days ➔ 2 – 8 °C

6 months ➔ – 20 °C

Conditions:

Wavelength	:	546 nm
Cuvette	:	1 cm light path
Temperature	:	25 ºC/ 30 ºC/ 37 ºC
Measurement	:	Against reagent blank
Method	:	Enzymatic colorimetric

Procedure:

	B	S	T
Standard	-	40 µl	
Serum / plasma	-	-	40 µl
Working reagent	1 ml	1 ml	1 ml

Mix well, measure after incubating at 20-25 ^{o}C for 10 min.

Calculation:

$$[\text{UA mg/dl}] = \frac{A\,T}{A\,S} \text{ X } [S]$$

Where, [S] = 6 mg/dl

Normal value:

♂: 3.4 – 7 mg/dl

♀: 2.4 – 5.7 mg/dl

Urine: 250 – 750 mg/24 hrs

Clinical interpretation:

Uric acid is the end product of purine metabolism and hence high rate of purine metabolism increases uric acid level.

High level is observed in

1. Gout
2. Arthritis
3. Cancer and leukemia
4. Polycythemia
5. Toxemia of pregnancy

Low level is seen in congenital deficiency of xanthine oxidase.

SERUM CALCIUM ESTIMATION

Introduction:

Calcium is a major cation that plays a vital role in the structure of teeth & bones. It has a functional role in neuromuscular action and clotting of blood.

Calcium component (total)	% (of total)
Ionized or free	50 – 65
Bound to plasma proteins	30 – 45
Complexed with citrate, phosphate, bicarbonate.	5 – 10

Protein binding, is blood PH dependent, decreases the amount of free calcium.

Principle:

Calcium ions bind to O – cresolphthalein complex in alkaline PH to yield colored (violet) complex. The intensity of color is proportional to [calcium].

$$\text{Calcium} + \text{O – cresolphthalein} \xrightarrow{\text{Alkaline PH}} \text{colored complex}$$

Specimen:

Serum, heparinized or EDTA plasma, urine (diluted 1+1 DW).

Conditions:

Wavelength	:	578 nm
Cuvette	:	1 cm light path
Temperature	:	25 ºC/ 30 ºC/ 37 ºC
Measurement	:	Against reagent blank
Method	:	Colorimetric

Procedure:

	B	S	T
DW	25 µl	-	-
Standard	-	25 µl	
Serum / plasma	-	-	25 µl
Working reagent	1 ml	1 ml	1 ml

Mix well, measure after incubating at 20-25 ^{o}C for 5 – 50 min.

Calculation:

$$[\text{Calcium (mg/dl)}] = \frac{A\ T}{A\ S} X\ [S]$$

Where, [S] = 10 mg/dl

Normal value:

Serum 8 – 10.4 mg /dl,

Urine 100 – 250 mg /24 hrs

Clinical interpretation:

Hypercalcemia is seen in

1- Hyperparathyroidism
2- Bone, lung, breast, bladder, or kidney malignancy
3- Multiple myeloma
4- Multiple fractures
5- Renal calculi
6- Hypervitaminosis D
7- Acidosis
8- Exercises

Hypocalcemia is seen in

1- Lack of calcium or vitamin D intake or deficiency
2- Hypoparathyroidism
3- Malabsorption
4- Diarrhea
5- Burns
6- Chronic renal failure caused by phosphorus retention
7- Alcoholism
8- Pancreatitis.

SERUM INORGANIC PHOSPHATE ESTIMATION

Introduction:

It is the principal intracellular anion; however, most of the phosphorus exists in the blood as phosphate. From 80 – 85 % of the total phosphate in the body are combined with calcium in teeth and bones. Phosphorus is the laboratory term used, since phosphates are converted into inorganic phosphorus for the test.

Principle:

Phosphate + molybdate $\xrightarrow{\text{Acidic PH}}$ phosphomolybdate complex

phosphomolybdate + ammonium sulphate $\longrightarrow$ molybdenum blue

Specimen:

Serum is the sample of choice. Anticoagulants may cause false low results.

Stability: 7 days ➔ 2 – 8 °C

2 days ➔ 20°C – 25°C

Conditions:

Wavelength	:	340 nm
Cuvette	:	1 cm light path
Temperature	:	25 ºC/ 30 ºC/ 37 ºC
Measurement	:	Against reagent blank
Method	:	Colorimetric

Procedure:

	B	S	T
Standard	-	10 µl	-
Serum / plasma	-	-	10 µl
Working reagent	1 ml	1 ml	1 ml

Mix well, measure after incubating at 20-25 ^{o}C for 1 min.

Calculation:

$$[\text{Phosphate (mg/dl)}] = \frac{A\,T}{A\,S} X\ [S]$$

Where, [S] = 10 mg/dl

Normal value:

Adult 2.5 – 5 mg / dl,

Children 4 – 7 mg / dl

Clinical interpretation:

Elevated levels are observed in

1- Hypoparathyroidism
2- Hypocalcemia
3- Hypervitaminosis D
4- Renal failure
5- Bone tumors

Decreased levels are observed in

1- Hyperparathyroidism
2- Hypercalcemia
3- Vitamin D deficiency (rickets)
4- Osteomalacia in adults

SERUM MAGNESIUM ESTIMATION

Introduction:

It is one of the major intracellular cations. Its action is closely related to that of calcium.

Magnesium component (total)	% (of total)
In the whole body	
Skeleton	53
Skeletal muscles, liver, myocardium	46
ECF & blood	1
In serum	
Ionized or free	66
Bound to plasma proteins	24
Complexed with citrate, phosphate, bicarbonate.	10

As with calcium, protein binding, is blood PH dependent, decreases the amount of free magnesium.

Principle:

Magnesium + xylidyl blue $\xrightarrow{\text{Alkaline PH}}$ purple – red chelate

The intensity of color is proportional to [magnesium]. Calcium is excluded from the reaction by complexing with EGTA (Ethylene Glycol – bis (aminoethyl ether) Tetra Acetate.

Specimen:

Serum is the recommended sample, urine (diluted 1 + 4 DW).

Stability: 7 days ➔ 2 – 8 °C

1 yr ➔ – 20 °C

Conditions:

Wavelength	:	546 nm
Cuvette	:	1 cm light path
Temperature	:	25 ºC
Measurement	:	Against reagent blank
Method	:	Colorimetric

Procedure:

	B	S	T
DW	10 µl	-	-
Standard	-	10 µl	-
Serum / plasma	-	-	10 µl
Working reagent	1 ml	1 ml	1 ml

Mix well, measure after incubating at 20-25 ^{o}C for 5 min.

Calculation:

$$[\text{Phosphate (mg/dl)}] = \frac{A\,T}{A\,S} X\ [S]$$

Where, [S] = 2 mg/dl

Normal value:

Serum 1.9 – 2.5 mg /dl,

CSF 2.4 – 3.1 mg /dl,

Urine 1 – 10 mg /dl

24 hrs 50 – 200 mg/dl

Clinical interpretation:

Hypomagnesemia is associated with

1. Hypocalcemia
2. IV therapy
3. DM

4. Alcoholism
5. Dialysis
6. Pregnancy

Hypermagnesemia is associated with

1. Dehydration
2. Sever diabetic acidosis
3. Addison's disease
4. Renal failure

ASSESSMENT OF IRON STATUS

Measurement of:

Serum iron level , total iron binding capacity (TIBC) , Unsaturated iron binding capacity (UIBC) & Transferrin saturation (% saturation)

Lab markers

Hematological

1. PCV
2. RBC indices
3. Hemoglobin

Biochemical

1. Serum iron
   - Fe^{+3} bound to serum transferrin & not iron in serum at free Hb.
   - Alone is of limited value & not good test of iron deficiency.
2. TIBC
   - Maximum concentration of iron that serum proteins principally transferrin can bind
   - An estimate of serum transferrin
3. UIBC
   - Reserve iron binding capacity of transferrin
4. Serum transferrin
   - Information regarding maximal IBC & available iron!
   - Serum transterrin (g / l) = 0.007 X TIBC!!
   - Represents most of IBC of serum.
5. % Saturation
   - Ratio of serum iron to TIBC!
6. Serum ferritin
   - Sensitive indicator of iron deficiency!

- May be misleadingly normal in some chronic disorders!

7. Free erythrocyte protoporphyrin
   - One of the most sensitive early detectors of iron deficiency!
   - Also used to diagnose iron deficiency anemia even after administration of iron is started!

Reference Ranges & Analytical Techniques

	Adult normal range		Measurements
	Male	Female	
Serum iron (µg / dl)	60 – 170	50 – 170	Colorimetric
Transferrin (µg / dl)	200 – 400	200 – 400	Immunoassay & calculated
Ferritin (µg / dl)	20 – 250	10 – 120	RIA & ELISA
% Saturation	20 – 55	15 – 55	Calculated
TIBC (µg/dl)	250 – 450	250 – 450	Precipitation & colorimetry
FEP in WB (µg/dl RBC)	17 – 77	17 – 77	Extraction & fluorometry

Note

Serum iron level (µg / dl) in:

- 0 – 5 month 40 – 250
- 6 month – 6 years 50 – 120
- 7 – 17 years 50 – 150

1. MEASUREMENT OF SERUM IRON

Principle

Ferric iron is dissociated from its carrier protein, transferrin, in an acidic medium & simultaneously reduced to the ferrous from. The ferrous iron is then complexed with the chromogen, a sensitive iron indicator, to produce a blue chromophore which absorbs maximally at 595 nm.

Specimen

- Serum but not plasma!
- Unhemolysed Specimen!
- No iron supplements at least 24hr before testing
- Morning sample!

Procedure

♦ Method = micro method ♦ Wavelength = 595 nm (590 – 510) ♦ Measurement = against reagent blank		● Temperature = 20 – 25 C^o ● Cuvette = 1 cm light path ● Standard = 200 mg / dl	
Reagents (ml)	B	S	T
Buffer R_3	1	1	1
Reductants R_2	0.05	0.05	0.05
Iron – free - water	0.25	–	–
Standard R_4	–	0.25	
Serum	–	–	0.25
Mix , read initial absorbance (A_1) for both S & T against blank			
Chromogen R_1	0.05	0.05	0.05
Mix , incubate for 5min then read final absorbance (A_2) for both S & T against blank			

Calculation

$$\triangle A \text{ (For S \& T)} = A2 - A1$$

$$\text{Concentration of iron} \quad mg/dl = \frac{\Delta OD\, T}{\Delta OD\, S} X \text{ Concentration of std.}$$

Clinical significance

Low levels

- Iron deficiency anemia?!
- Acute & chronic infection.
- Chronic inflammatory diseases as rheumatoid arthritis or renal disease
- Acute or recent hemorrhage
- Menstruation.
- Immunization.
- Malignancies as lymphoma, leukemia & breast cancer.
- Myocardial infarction.
- Viral hepatitis.

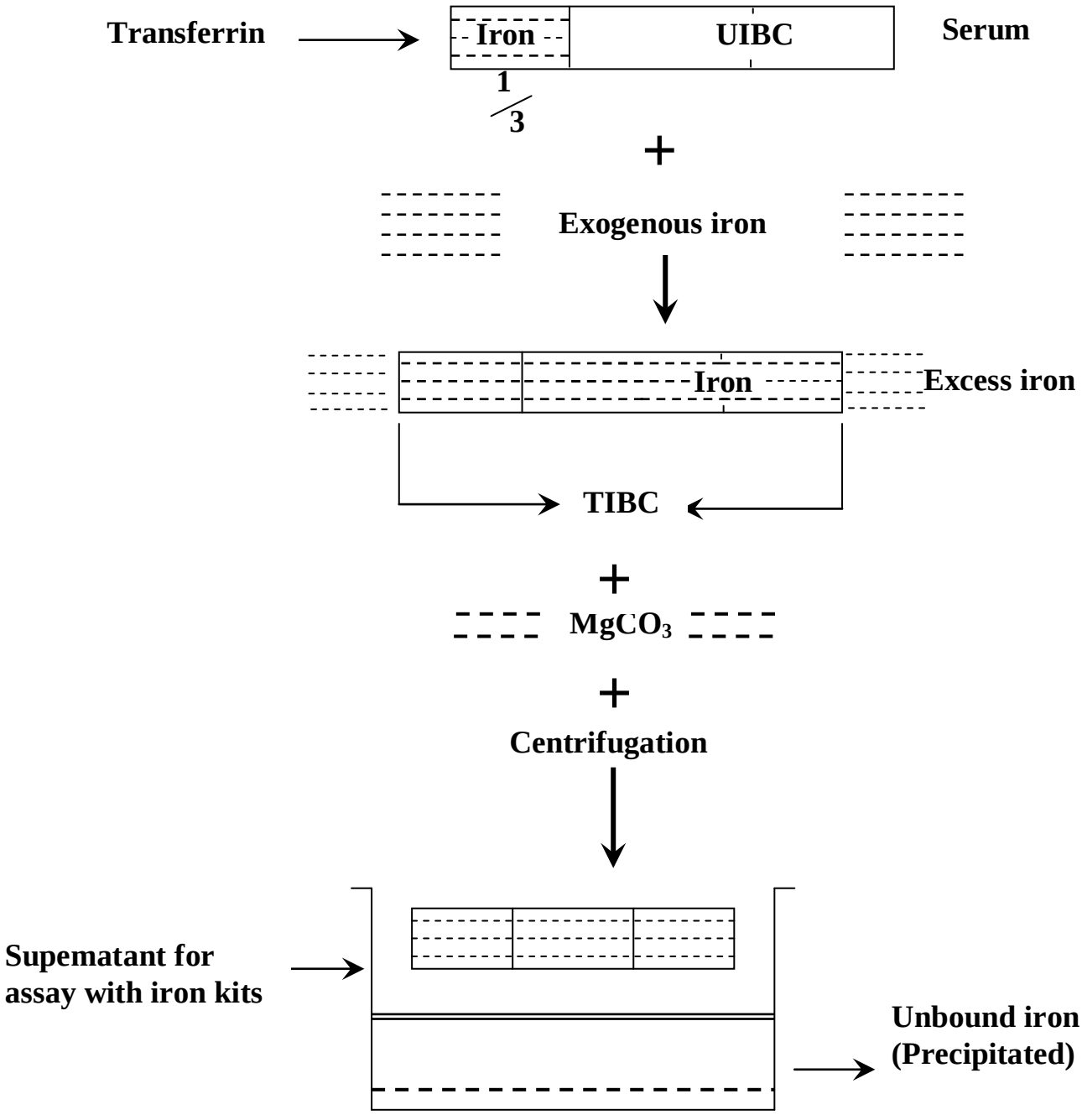
High levels

- Hemochromatosis
- Hemosiderosis.
- Recurrent blood donation.
- Acute iron poisoning.
- Use of hormonal contraceptives.

2. MESTTREMENT OF TIBC

Principle

TIBC is a measurement of the total iron binding capability of transferrin. An excess of iron is added to serum to saturate the transferrin. The unbound iron is precipitated with basic magnesium carbonate. After centrifugation the iron in the supematant is calorimetrically determined.



Specimen

- Serum , same criteria as in serum iron

Procedure

1. Pipette into 10 ml centrifuge tube 0.5 ml of serum & 1 ml of solution 5.
2. mix. Let stand for 5 – 30 min at 20 – 25 ^{o}C
3. Add one spatula full "180 mg" of solution 6.
4. Let stand for 30 – 60 min at 20 – 25 C^{o} mixing occasionally.
5. Centrifuge for 10 min at 30 rpm.
6. Remove gently 0.5 ml of supernatant & use for assay with serum iron kits.

Calculation

Using standard provided in iron kit

$$\text{TIBC} \quad \mu g/dl = \frac{\Delta OD\ T}{\Delta OD\ S} X \text{ Concentration of std. } \mu g/dl X\ 3$$

Clinical significance

High levels

- 1
- 2
- 3
- 4
- 5

Low levels

- 1
- 2
- 3

3. MESTTREMENT OF UIBC

Principle

The additional iron binding capacity of trnsferrin is known as the UIB. It can be estimated (calculated) indirectly by the following formula:

UIBC (mg/dl) = TIBC (mg/dl) – serum iron level (mg/dl)

Clinical significance

High values

- 1
- 2
- 3

Low values

- 1
- 2
- 3

4. CALCULATION OF % SATURATION

Principle

Concomitant determination of serum iron & transferrin (TIBC) allows the computation of transferrin saturation as follows:

$$\text{Transferrin saturation (\%)} = \frac{\text{serum iron (µg/dl)}}{\text{TIBC (µg/dl)}} \text{X} 100$$

Clinical significance

Low values

- 1
- 2
- 3

High values

- 1
- 2
- 3

CHEMICAL ANALYSIS OF CEREBROSPINAL FLUID

Determination of glucose and total protein

Introduction:

- CSF is a fluid that flows through and protects the subarachnoid space of the brain, and spinal cord.
- It's formed in choroid pluxes
- Its volume is 120 – 150 ml,
- It's produced at the rate of about 0.3 ml/min (500 ml / day)
- It's obtained by lumbar puncture, L 3 – L 4

Function:

I. Supports & protects brain and spinal cord
II. Acts as cushion and shock absorber
III. Reservoir to regulate contents of cranium, i.e. if the volume of brain or blood increases, CSF drains away; if the brain shrinks, more fluid is retained.
IV. It keeps the brain and spinal cord moist.
V. It may act as a medium for the interchange of metabolic substance between nerve cells and CSF.

Composition:

It consists of water, dissolved oxygen, CO_2, and a number of solids. It's specific gravity is about 1.005, PH 7.4 – 7.6.

	Plasma mg / dl	CSF mg / dl
Total protein	6000 – 8000	15 – 45
Glucose	50 – 90	50 – 80
Chlorides as NaCl	560 – 620	700 – 760

Routine CSF laboratory investigations:

- Appearance: it's clear, colorless and free of clots, sediments and blood.
- Cell count: it contains up to 5 lymphocytes per mm^3.
- Glucose
- Total protein and globulin
- Chlorides

CSF GLUCOSE ESTIMATION

Principle

The glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The formed hydrogen peroxide reacts under catalysis of peroxidase with phenol and 4-aminophenazone to a red-violet quinoneimine dye as indicator.

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

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

Contents

RGT 100 ml or 1000 ml Enzyme reagent	STD 3 ml Standard
Phosphate buffer (pH 7.5)	Glucose 100 mg/dl or 5.55 mmol/l
4-Aminophenazone	
Phenol	
Glucose oxidase	
Peroxidase	
Mutarotase	
Stabilizers	

Specimen

CSF

Procedure:

	Test (T)	Blank (B)	Standard (St)
Sample	10 µl	-	-
Standard	-	-	10 µl
Working reagent	1 ml	1 ml	1 ml

Mix well and allow to stand at room temperature for 10 minutes and read the absorbance at 546 nm.

Calculation: mg / dl = [OD for T / OD for St] X conc. of St

Clinical interpretation:

An increased CSF glucose level is seen in hyperglycemia.

Decreased CSF glucose level is observed in

1. Infection
2. Hypoglycemia
3. Increased metabolism by CNS

CSF TOTAL PROTEIN ESTIMATION

Principle:

Alkaline copper solution reacts with the peptide bonds in protein molecules producing a violet color complex compound which is directly proportional to the amount of protein present.

Procedure:

	Test (T)	Blank (B)	Standard (St)
Sample	20 µl	-	-
Standard	-	-	20 µl
Working reagent	1 ml	1 ml	1 ml

Mix well and allow to stand at room temperature for 10 minutes and read the absorbance at 546 nm.

Calculation: g / dl = [OD for T / OD for St]X conc. of St

Clinical interpretation:

Increased CSF total protein is observed in:

1. In presence of blood, due to hemoglobin and plasma proteins
2. In presence of pus, due to cell protein and to exudation from inflamed surfaces
3. In the blockage of the spinal cord by spinal tumors, by vertebral fractures or due to the spinal tuberculosis.

Decreased CSF total protein is observed in liver diseases such as cirrhosis and hepatic encephalopathy.

GLUCOSE 6 PHOSPHATE DEHYDROGENASE ESTIMATION

Introduction:

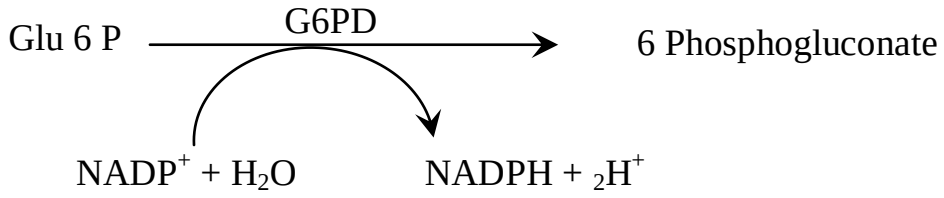
G6PD (EC 1.1.1.49) is 515 amino acids, either a dimer or a tetramer of identical subunits, each having a molecular mass of about 59 KD. It is the key enzyme of HMP; it is cytosolic and active in liver, adipose tissue, adrenal cortex, thyroid, erythrocytes, testis and lactating mammary gland. It generates NADPH which is used for a variety of synthetic reactions, to keep glutathione reduced in erythrocytes or formation of NO. The activity of G6PD is induced by insulin so that in well - fed state its activity increases whereas in starvation and diabetes decreases.

Methods for assessing the activity of G6PD

1- A screening test (qualitative test).
2- Determination of enzyme kinetics (quantitative test)
    a. Erythrocytes level
    b. Serum level

1 – Screening test:

Principle: G6PD is an enzyme that catalyzes the oxidation (dehydrogenation) of Glu 6 P.



In the presence of a suitable basic dye such as methylene blue (MB), G6PD accomplishes the transfer of hydrogen to methylene blue. A change in color indicates normal activity of the enzyme.

$$\text{Glu 6 P + MB (oxidized)} \xrightarrow{\text{G6PD}} \text{6 Phosphogluconate + MB.H}_2 \text{ (color change or colorless)}$$

Procedure:

1) Centrifuge 1 -2 ml EDTA – blood to obtain packed RBCs.
2) After discarding the supernatant (plasma) take 100 µl (0.1 ml) of packed cells into 1.25 ml of working MB reagent.
3) Incubate at 37 C° for 40 minutes.
4) Compare the final color with the original (previous) color.
5) In normal RBCs, Color changes to bluish green whereas in G6PD deficient RBCs the color doesn't change and packed cells ppt in tube.

Comment

The change in the color from blue to bluish green indicates normal activity of G6PD. The unchange in color indicates abnormal activity of the enzyme.

2 – Estimation of erythrocytes and serum G6PD

The enzyme activity was determined by measurement of the rate of absorbance change at 340 nm due to the reduction of $NADP^+$.

$$\text{G-6-P + NADP}^+ \xrightarrow{\text{G6PD}} \text{Gluconate – 6 – P + NADPH +H}^+$$

Reagents

1. Buffer – Triethanolamine buffer , EDTA
2. NADP
3. Glucose - 6 - phosphate
4. Digitonin

Preparation of solutions

1. Buffer – its contents ready for use. Stable up to the expiry date when stored at +2 to + 8 ^{o}C.
2. NADP – its contents reconstituted with 2 ml of redistilled water. Stable for 4 weeks at +2 to + 8 ^{o}C.
3. Substrate – Its contents were reconstituted with 2 ml of redistilled water. Stable for 4 weeks at +2 to + 8 ^{o}C.
4. Digitonin – contents ready for use. Stable up to the expiry date specified when stored at +2 to + 8 ^{o}C.

Preparation of sample for estimation of erythrocytes G6PD activity

1. 0.2 ml of blood was washed with 2 ml aliquots of 0.9 % NaCl solution.
2. Centrifuged after each wash for 10 minutes at around 3000 rpm. Repeated 3 times.
3. The washed centrifuged erythrocytes were suspended in 0.5 ml of solution 4 (Digitonin) and
4. Let stand for 15 minutes at + 4 ^{o}C and then centrifuged again.
5. Within 2 hours the supernatant was used in the assay.

Procedure

Pipette into cuvettes

Solution 1	1.00 ml
Solution 2	0.03 ml
Hemolysate	0.015 ml

Mixed, incubated for 5 minutes at 25 ^{o}C, then added

Solution 3	0.015 ml

Mixed, initial absorbance was read and simultaneously timer was started to read again after 1, 2, 3 minutes. The absorbance was read against air at 340 nm in spectrophotometer. The enzyme activity in hemolysate was calculated using the following formula:

$$\text{G6PD mU / erythrocytes per ml blood} = \frac{33650 \text{ X } \Delta \text{ A } 340 \text{ nm / min}}{\text{RBCs count per ml}}$$

Procedure for estimation of Serum G6PD activity

Only fresh non hemolytic serum was used in the assay.

Pipette into cuvettes:

Solution 1	1.00 ml
Solution 2	0.05 ml
Serum	0. 5 ml

Mixed, incubated for 10 minutes at 25 ^{0}C, then added

Solution 3	0.025 ml

Mixed, initial absorbance was read and simultaneously timer was started to read again after 1, 2, 3 minutes. The enzyme activity in serum in duplicate was calculated using the following formula:

$$\text{mU / ml} = 500 \text{ X } \Delta \text{ A } 340 \text{ nm / min}$$

Normal range:

In erythrocytes: 118 – 144 mU/ 10^9 erythrocytes

In serum there is virtually no detectable activity (0 – 0.18 mU/ ml)

Clinical significance:

It is established fact that G6PD deficiency is found in many cells and tissues including RBCs, WBCs, PLTs, kidneys, adrenals, skin, saliva, sperm and liver. G6PD deficiency may represent one mechanism to vascular stress and endothelial dysfunction by enhancing ROS accumulation and decreases NO bioavailability. Its deficiency is present in many diseases like DM, hypertension, and other diseases.