KOMAR UNIVERSITY OF SCIENCE AND TECHNOLOGY (KUST)

DEPARTMENT OF MEDICAL LABORATORY SCIENCE (MLS)

CLINICAL CHEMISTRY (MLS 3445C)
LABORATORY MANUAL

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FALL-2016

Preface

Clinical chemistry laboratory is a clinical and diagnostics subject, which aims to submit, enhance and utilize standard diagnostic methods, to monitor disease progressing and treatment by biochemical methods. Clinical biochemistry helps to make diagnosis, choice of treatment and preventive methods easier. Clinical chemistry is one of the most important parts of laboratory diagnostics. It possesses the largest number of diagnostic tests that help understand pathogenesis and etiology of different pathological processes. Information, obtained by biochemical methods help to evaluate the development of pathological process on molecular, cellular and organ level. It is essential for early diagnosis of a disease and also assessment of its therapy efficacy. Clinical biochemistry is evolving rapidly in our era. During the last ten years, more than a hundred of new analytical methods have appeared, including DNA diagnostics, determination of tumor markers, apoptosis tests. Biochemical tests are of great importance in diagnosis of endocrine, gastrointestinal, heart and renal diseases as well as in toxicology. Clinical biochemistry is closely linked to such theoretical subjects as general and bioorganic chemistry, biochemistry, histology, normal and pathological physiology, normal and pathological anatomy. This manual provides useful information about modern principles of evaluation of protein and carbohydrate metabolism, lipid profiles, liver and renal functions and enzyme tests. The information provided in this manual helps to come up with optimal schemes and calculations of laboratory diagnostics of numerous pathological conditions.

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Fall 2016

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Chapter 1: Hazards in the clinical Biochemistry Laboratory

One must always remain alert and cautious while working in the laboratory careless handling of reagents. Glassware or specimen to be tested in the laboratory to can cause serious injury and can be dangerous to life. This chapter discusses hazards encountered in clinical biochemistry la oratory and measures to be taken to ensure safe working.

Hazards from dangerous chemicals

Toxic substances in the laboratory can be absorbed either from the direct contact through skin or by inhaling vapors or fine powder or can be swallowed by mistake while pipetting.

Injury results from the effect of these chemicals on other tissues such as bone marrow, liver and kidney

Precautions for avoidance of accidents

1. All bottle containing chemicals and reagents should clearly labeled and the hazard noted
2. Never carry large bottle by the neck but hold the bottle with both hands.
3. Keep bottle in use on shelves, not higher the eye level.
4. Corrosive chemical include strong acids or alkalis, e.g. nitric, sulphuric and hydrochloric acids, sodium and potassium hydroxides. Take great care while opening these bottles or when pouring from such bottles. Always add content slowly to water, preferably while cooling and stirring.
5. Never keep acids and alkalis in bottles with ground glass stoppers as they may get stuck
6. Whenever possible use small measuring cylinders of measuring acids and alkalis. If more accurate measurement is required, use a poppet plugged with non-absorbent cotton wool or with a rubber tube attached
7. Toxic chemicals include cyanide and barbiturates. Keep these locked in a cupboard. Mouth pipetting for these should be totally forbidden.

8. Organic solvents may have toxic properties thus, benzene is toxic to bone marrow carbon tetra chloride and other halogenated hydrocarbons are toxic to the liver. So keep exposure to the minimum carry out procedure including distillation in a fume hood.
9. Many chemicals have the potential to cause cancer and the most commonly carcinogenic chemicals used are aromatic amines such as benzidine and ortho-tolidine. Precautions include keeping them in well closed bottles labeled 'carcinogenic' and avoiding any contact with the skin.
10. When handling carcinogens, rubber or plastic gloves should be used which must be washed well afterwards under cold running water.
11. In case of accidental skin contact, wash in cold running water for several minutes.
12. Explosion may be produced when using oxidizing agents, e.g. per chloric acid. Carry out work with per chloric acid in a fume cupboard.
13. Picric acid when dry, explodes on percussion. It should not be kept in ground glass stoppered bottle but should be stored underwater, in a container closed by a cork or rubber stopper
14. Ether kept in clear bottles exposed to sunlight can form peroxides, when redistilled. Peroxides reach to sufficient concentration and it may produce a violent explosion. So keep ether in dark bottles
15. While distilling a solvent a container must never be heated over naked flame and efficient condensation of solvent vapor must be ensured all the time.
16. Flammable gas stored in cylinders, constitute fire hazards, i.e. hydrogen, propane acetylene. Keep cylinders, not in use, outside the laboratory.
17. Bottles of chemicals and solutions should be handled carefully, and a cart should be used a heavy or a number of containers from one area to another.
18. All bottles containing reagents must be properly labeled before adding the reagent. The label should bear the name and concentration of the reagent, the initial of the person who made up the reagent, storage and potential hazards instructions (corrosive, toxic, and irritant, flammable, explosive).
19. Disposal of flammable solvents in sanitary sewers is not allowed.
20. Acid, caustic materials, and strong oxidizing agents should be mixed in the sink. Where water is available for cooling.

21. Do not 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
22. All the electrical equipment's should be grounded using three-point plugs and use of the extension cord should be prohibited.
23. Every laboratory should have necessary equipment to put out a fire in the laboratory.
24. All staff should familiar with the location of fuse boxes and circuit breakers. Any electrical sparking should be reported immediately.

Infection hazards

Infection hazards are mainly viral hepatitis and acquired immunodeficiency syndrome (AIDS). The guidelines for the laboratory handling of material from patients with AIDS and viral hepatitis are similar.

AIDS: it is accused by human immunodeficiency virus (HIV) which by damaging T-cell, reduces the ability of an affected individual to resist other infections. The virus has been detected in blood, breast milk, semen, vaginal fluid, and saliva, tears, urine and brain tissue. In the laboratory, the infection is most likely to occur by infection of exposed cuts and by accidental self-inoculation with blood containing virus. *Viral hepatitis:* it occurs in three forms. Hepatitis A has an incubation period of 14 to 35 days. It occurs in general population. Urine and feces contain the virus. Diagnosis requires demonstration of the virus in feces or presence of hepatitis specific lgM in the serum. In the laboratory, entry of virus is usually through ingestion of contaminated food or drink or by placing contaminated fingers or other objects in the mouth.

The second form hepatitis B has longer incubation period of 40 to 120 days. During the acute phase of illness. Blood is highly infectious but urine and feces are probably infected. Third form is non A-non B hepatitis the virus occurs in blood both hepatitis B and non A-non B hepatitis are associated with a carrier state , so blood may continue to be infectious long after the patient is well. Blood is the main source of infection in the laboratory possible routes of entry are through direct entry due to breaching of skin surface or through ingestion.

Precautions

1. Precautions include special handling of high risk samples. But this does not exclude the possibility that other specimens are not infected. Remember

that even blood from a healthy may be dangerous. It may contain microorganisms that can cause jaundice. So be just careful with blood specimen as you are with specimen of stool and CSF. So it is wise to treat all sample as potentially infectious samples. So mouth pipetting for all the samples should be avoided completely. Try not to get blood on your hand and do not spill blood in the laboratory. The AIDS virus can spread through the use of syringes, needles and instruments, which have been in contact with the blood of a person who is carrying the AIDS virus, even he is not sick. It is, therefore important to only use sterile syringes, needle and instruments. The virus is very fragile and dies only 56C^{o} or when soaked in common disinfectant. There are three ways to sterilize equipment's:

a) Completely immersing instruments in boiling water for 10 minutes.
b) Soaking for 20 minutes in disinfectant solution:
    - House hold bleach- 1% solution (available in market as 3.5% readymade solution polar R dilute it with 2.5 volumes of tap water to give 1% solution
    - Sodium hypo chloride 1% solution
    - Glutaraldehyde 2% (available commercially as cide)
    - Lysol 2.5% solution
    - Hydrogen peroxide 3% solution
    - Dettol 4% solution
    - Savlon 5% solution a solution of sodium hypo chloride prepared daily is inexpensive, effective and easily available disinfectant. Also concentration ranging from 0.1 to 2 % of chlorine if effective depending on the amount of concentration. All the linen soiled with blood/body fluid should be dipped in 1% household bleach or sodium hypo chloride for half –an- hour washing. Instruments that require sterilization or disinfection should be thoroughly cleaned before being exposed to disinfectants.
c) Sterilization and disinfection: sterilization is a process of freeing an article from all living organisms including bacterial, fungal, spores and viruses. Moist heat (steam) under pressure using different type of autoclaves is most dependable method of sterilization. Specially

modified pressure-cooker at 121 C° at 5 lbs. /sq. inch pressure for 15 to 20 minutes gives high level of sterilization.

2. Personal protective equipment's (PPE) is the primary battier of protection worn before handling potentially hazardous biological materials. PPE include laboratory coats and gloves. In case of any cut on hands, over them well with plastic tape and then wear gloves. Laboratory coats should not be kept in the locker used for personal clothes. While wearing gloves avoid touching general items like telephone, keyboard, etc. when leaving the laboratory, protective clothing should be removed.
3. Proper methods of self-decontamination after performing work are to be followed, i.e. to remove the laboratory coat first, then gloves, and then thoroughly washing the hands with soap or disinfectant, if hands are washed before removing the coat , the hands may become contaminate again while handling the coat.
4. The following are few safety precautions to be followed when pipetting in the laboratory:
    a. Do not draw reagents or specimens through pipettes directly by mouth. Use automated pipette.
    b. Pipette content should be allowed to run down along the wall of the container, making sure not to release the contents from a height.
    c. Avoid performing mouth pipetting and never blow out pipettes that contain potentially infectious material, e.g. serum
    d. Do not mix potentially infectious material by bubbling air through the liquid, which leads to aerosol formation.
5. Put needle and sharp in puncture resistant containers.
6. Never leave a discarded tube or infected material unattended or unlabeled.
7. Staff locker rooms with washing facilities should be separate from the working area.
8. Do not eat, drink or smoke in the laboratory and never store the food or drink in the refrigerator. An entirely separate staff room must be made available to enable food to be kept out of the working area.
9. Periodically clean out freezer and dry ice chests to remove broken ampules and tubes of biological samples. Use rubber gloves during this cleaning.

10. Hands should be washed as soon as possible when they come in contact with potentially infectious materials, a vigorous handwashing with a proved agent is appropriate. Hands must always be washed before leaving the laboratory, and immediately if visible contamination occurs.

FIRST-AID AND EMERGENCY TREATMENT IN THE LABORATORY

1. *Acid burns:* nitrile, sulphuric, hydrochloric and trichloroacetic acids.
   a. *For acids splashes on skin, first wash thoroughly and repeatedly with water and then bathe the affected skin with cotton wool soaked in 5%aqueous sodium carbonate.*
   b. In case acid splashes on the eye, wash the eye immediately with large quantities of water, sprayed from a wash bottle or rubber bulb or hold the eye under the running tap. After washing put 4 drops of 2% aqueous sodium bicarbonate solution in the eye until checked by the doctor.
   c. In case of accidental swallowing of acids while using a pipette, call a physician. Make the patient drink some 5% soap solution immediately. Alternatively, give him two whites of egg mixed with 500 ml of water or milk. If neither is available give ordinary water to drink. Make him gargle with the soap solution, if the lips and tongue are burned by acid, first rinse thoroughly with water and then with 2% aqueous sodium bicarbonate.

2. Alkali burns: sodium, potassium and ammonium hydroxide. Alkali burns are as serious as, and often more serious than acid burns.
   a. In case of alkali splashes on the skin, wash thought and repeatedly with water and then bathe the affected skin with cotton soaked in 5% acetic acid (or undiluted vinegar).
   b. For the alkali splash on the eye wash him immediately with large quantity of water sprayed from a wash bottle or rubber bulb. Squirt the water in to the corner of the eye, near the nose. After washing with water repeatedly, wash the eye with a saturated solution of boric acid.
   c. In case of accidental swallowing of alkalis while using a pipette, make the patient drink at once 5% solution of acetic acid or lemon juice or

dilute vinegar (1 part vinegar to 3 parts water). Make him gargle with the same acid solution. Give him 3 or 4 glasses of ordinary water. If the lip and tongue are burned by the alkali, rinse thoroughly with water and bathe with 5% acetic acid solution.

3. Burns caused by heat: they fall in to two categories:
    a. Severe burns affecting large area of skin, e.g. burns caused when burning ether or boiling water is spilled over victim. In this case if the victim is on fire, e.g. if splashed with burning ether or other inflammable solvent, roll him in a blanket or overall to smother the flames. Do not remove his clothing. Do not apply any treatment to the burns. This must be left to the physician.
    b. Minor burns affecting a small area of skin, e.g. burns caused by hot glassware or a Bunsen flame. Minor burns plunge the affect part into cold water or ice water to soothe the pain. Apply mercurochrome or acri Flavin ointment to the burns. Apply a dry gauze dressing loosely. Never tear off the blisters that form over the burns.
4. Poisoning this can be caused by :
    a. Inhaling toxic vapors or gases (e,g, chloro form)
    b. Accidental swallowing while pipetting a poisonous solutions. In all cases place the victim in the open air while waiting for physician.
5. Injuries caused by broken glass these are caused by broken test tubes, syringe or other glassware. So wash the wound immediately to remove any glass pieces and apply acre Flavin ointment to the wound. Cover with gauze and adhesive tape. If the cut bleeds heavily stop the bleeding by pressing down on it with a compressor. Refer a patient to the physician.
6. Contamination by infected material for wounds caused by broken glassware containing stools, pus, etc. wash the wound immediately , check whether the cut is bleeding. If not squeeze hard to make it bleed for several minute. Bathe the whole area, i.e. the edge s of cut and inside the cut, with antiseptic lotion. Wash thoroughly with soapy water. Bathe again with antiseptic lotion if the material involves is known to be very infective, e.g. pus, refer the patient to the physician. If infected material is accidently sucked into the mouth, spit it out immediately and go in for forced vomiting if skin is infected by highly virulent organism touch the involved part highly pure carbolic acid.

Chapter 2: Specimen collection and preservation

Most quantitative chemical investigations are carried out on blood specimens, the next most frequently examined material is urine. Other materials that can be analyzed include feces, cerebrospinal fluid, amniotic fluid, saliva, gastric, duodenal and jejunal secretions, sweat, pathological fluids obtained by paracentesis, calculi and sample of dietary intake. Analysis may be qualitative, semi-quantitative and quantitative.

TAKING CARE IN INTRA AND EXTRA LABORATORY FACTORS FOR RELIABLE RESULTS

For reliable results to be obtained and for meaningful interpretation of tests, both intra and extra laboratory factors require consideration (Table 2-1)

Table 2.1: Intra-and extra-laboratory factors interfering with the results

Patient determined variable	*Technical consideration*
1. Age	a. Site of collection of specimen, e.g. venous or capillary
2. Sex	b. Cleanliness and composition of equipment used to collect and transport specimen to the laboratory
3. Pregnancy	c. Interval between collection of specimen and its receipt in the laboratory
4. State of menstrual cycle	d. Condition of storage of specimen
5. Diet	e. Method of measurement
6. Interval since last meal	
7. Body size	
8. Ethnic groups	
9. Genetic variant	
10. Place of domicile	
11. Time of the day	
12. Posture during test	
13. Recent exercise	
14. Mental state during test	
15. Non-specific illness	
16. Medication	

Table 2.2: Effect of sex and age on various plasma constituents

Constituents	*Sex difference*	*Effect of increasing age*
Albumin	M > F	Falls
Alkaline phosphatase	M > F	Marked rise in puberty. Adult levels reach when growth ceases. Rises in adults, especially in F after menopause
ALT (Alanine aminotransferase)	M > F	Varies in different reports
AST (Aspartate aminotransferase)	M > F	Falls to minimum at 30 years, later rises especially in F
Bilirubin	M > F	Little change apart from post-menopausal rise
Calcium	M > F	Falls, especially in M
Cholesterol	M > F	Rises, especially in post-menopausal F
CK (Creatinine kinase)	M > F	Falls in M; rise in F
Creatinine	M > F	Slight rise
Glucose	M > F	Rises
GGT (Gamma-glutamyl-transpeptidase)	M > F	Falls in M; rises in F
HBD (Hydroxybutarate dehydrogenase)	None	Rises in F
Iron	M > F	Falls
β-lipoprotein	M > F	Little change
Magnesium	M > F	Rises, especially in F
5'-Nucleotidase	None	Rises in F
Phosphate	F > M	Little change apart from post-menopausal rise
Potassium	None	Rises
Sodium	None	Rises, especially in F at menopause
Thyroxine	F > M	Little change
Total protein	M > F	Little change
Triglycerides	M > F	Rises
Urate	M > F	Rises in F; steady or falls in M
Urea	M > F	Rises

Age and sex has effect on various plasma constituents **(table 2-2).** Type of the diet taken and time elapsed since last meal has effect on some of the parameters, e.g. Blood glucose concertation normally rises after a meal and then gradually falls to or below pre-prandial level, before finally returning to the fasting level. For some estimation (glucose, triglycerides) patient should be fasting overnight.

Also, fasting samples are required if lipaemia interferes with the chemical analysis (bromsulphthalein test).

Rate of reductions of many endogenous compounds varies according to the time of the day. Best known example is plasma cortisol concentration. Peak values are observed around 8-9 am and minimum level at about midnight. Other substances are plasma, total iron and inorganic phosphate concentration both of which tend to be lower in afternoon than in the morning. A moderate period of recumbency leads to hem concentration, in the order of 10-20% as a result of increased seepage of crystalloid-rich ultra-filtrate of blood from intra-to extra vascular compartment of extracellular fluid. Concentration of most proteins, albumin and small molecular weight substances such as cortisol, thyroxin, copper, bilirubin, cholesterol, triglycerides and activities of certain enzymes which are largely or completely complex to them, is increased. The increase is 8-10% plasma Ca^{2+} rises by only 4-5% as only half of the calcium is protein bound. Concentration of drug changes is a similar way depending how much is protein bound.

The use of tourniquet to be obtain blood exaggerates the effect observed with changes of posture. Moreover, prolonged stasis is said to lead to hypoxia and quantities present in erythrocytes, including phosphate, aspartate, aminotransferase and LDH can diffuse out and cause raised plasma levels. So minimum venous occlusion is advised.

In case of glucose tolerance test results obtained from recumbent subjects are not strictly speaking comparable with those obtained in siting or ambulatory ones, who when given large glucose load to drink are much more likely to exhibit reactive hypoglycemia than recumbent subjects. Mental state and exercises has effect on growth hormone secretions. Prolonged inactivity such as produced by confinement to bed can also profoundly affect metabolic responses such as ability to dispose of large glucose load.

It is important to recognize that many biochemical parameters are altered non-specifically by all sorts of diseases and medications.

In pregnancy, concentration of many substances ranging from plasma protein and enzymes to electrolytes and vitamins change.

Sex hormone values must be related to reference values appropriate to the stage of patient's menstrual cycle.

Body size has effect on total quantities such as blood volume, total body water, renal blood flow and glomerular filtration rate and has little effect on concentration of different substances.

There large differences in the mean plasma concentration of inhabitants in different parts of the world. This is due partly to ethnic difference but also due to difference in their diet and way of life.

COLLECTION AND PRESERVATION OF BIOLOGICAL FLUIDS

The different body fluids that are used for biochemical investigations are given in **(table 2-3).**

METHODS OF TAKEN BLOOD SPECIMENS AND SEPARATING THE SERUM ASEPTICALLY

Venous blood is collected usually from antecubital vein or some other prominent veins of the forearm under aseptic conditions. Arterial blood is required rarely. This may be collected from radial, brachial or femoral artery. Capillary blood may be collected from the tip of the thumb or finger or from ear lobe.

For taking blood sample tie a rubber tube around the patient's arm and choose a vein as shown the **figure 2.1** put a piece of cotton or gauge in spirit and swab the skin where you are going to put the needle. The spirit is being used as an antiseptic to kill microorganisms on skin.

Carefully unwrap the syringe from the paper. Put the needle on to syringe without touching anywhere. The arm should be straight.

Put the needle into the vein as shown in the **figure 2.1** start drawing out blood when you see some blood in the syringe. Fill the syringe with blood. Take out the needle when required amount of blood has been drawn. Press over the hole that you have made in the vein with a piece of cotton and ask the patient to keep pressing it for a minute or so. This will stop the bleeding from the vein into the tissue of the arm. Take the needle off the syringe. Put the blood into the bottle without touching any part of the bottle with any part of the syringe. Leave the bottle until the serum has separated. Take a Pasteur pipette. Fit a teat to it suck up the serum from around the blood clot.

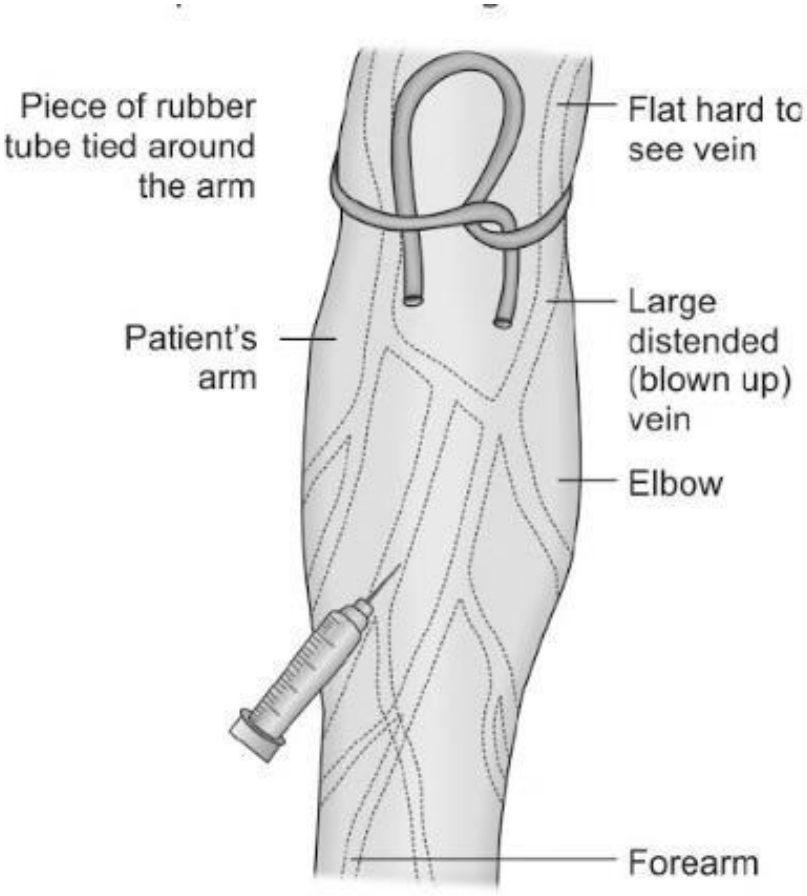


Fig. 2.1: Method of taking blood specimen

PRECAUTION FOR TAKING BLOOD SAMPLE

Blood should be collected from venipuncture after cleaning the skin with a cleansing agent. Prolonged venous stasis must avoided (as already, explained) to avoid rise in concentration of large molecules such as protein and of protein bound constituents especially calcium.

Hemolysis can occur for various reasons and renders blood unsuitable for plasma $K^{+,}$ Mg^{++} and enzyme activity measurement.

To avoid hemolysis:

1. Specimen should be collected with moderate suction and needle should be removed before transferring blood to vial or tube.
2. Avoid use of wet and dirty tubes.
3. Freezing and thawing of blood specimens causes massive hemolysis so in general whole blood should not be chilled, as besides causing hemolysis this also causes abnormal distribution of certain ions and enzymes between the cells and serum,

ANTICOAGULANTS USED IN BIOCHEMICAL ANALYSIS

Chemical agents that prevent coagulations are routinely used when whole blood or plasma is required. These anticoagulants are used for preventing coagulation of blood without diluting and without causing a change in the volume of red cells. Salts causes precipitation of Ca^{++} in the form of calcium salt and this prevent coagulation. Gentle mixing with coagulants should be done when required. Some of the commonly used anticoagulants are:

1. Heparin
2. Salts of ethylene diamine tetra-acetic acid (EDTA)
3. Oxalates.
4. Sodium fluoride.

1. ***Heparin:*** it may be considered to be natural anticoagulant because it is already present in the blood, but in concentration less than that required to prevent clotting in freshly drawn blood. Heparin prevents coagulation by increasing the activity of anti-thrombin III, an inhibitor of thrombin. This anticoagulant is used in a concentration of a **0.2 mg/ml** of blood the best anticoagulant, except for its coast, is heparin. as its molecular weight is large, it produce no

change in erythrocyte volume and does not change the composition of the blood in any way.

2. ***Salts of ethylene diamine tetra-acetic acid (EDTA):*** it may be a disodium or dipotassium salt. It is an anticoagulant which acts by virtue of removing of calcium ions by chelation. A concentration of **2mg** of the disodium salt/mL of blood is sufficient. Concentration even greater than this produce no detectable change in erythrocyte volume. EDTA has been mainly used in hematology. It is a good anticoagulant for investigation of **RBC, WBC, PCV, platelets and ESR.** It prevents the clumping of platelets.
3. ***Oxalates:*** lithium, sodium and potassium oxalate act as anticoagulants by removing calcium ions essential for blood coagulation. Potassium oxalate (**$K_2C_2O_4.H_2O$**) is commonly used. 1-2mg of salt/mL of blood is required. A stock solutions of the substance 2 mg is made and 0.5 mL of test may be collected in such vials. The disadvantage of the use of oxalate is the alteration of concentrations of plasma components. Shrinkage of erythrocytes results from a water shift from the erythrocytes to plasma. Aside from the water shift there may be alteration of erythrocyte permeability, which may explain the varied and inconsistent effects of oxalates and other salt anticoagulants on certain plasma constituents. Because of the difficulty , a balanced oxalate mixture (for use in hematocrit and sedimentations rate determinations) consists of three parts by weights of ammonium oxalate (which causes swelling of the erythrocytes) balanced by two parts of potassium oxalate (which causes shrinkage).NH4+ and K+ oxalate mixture in the ratio of 3:2, and 2 mg/mL of blood is required amount. In 100 mL of distilled water, put 2 drops of this solution in each vial and dry in hot air oven. Use it for 2 mL of blood.
4. ***Sodium fluoride:*** it is used when blood is collected for glucose estimations. In the erythrocytes (RBC), it specifically inhibits the enzyme enolase of the glycolytic pathway, preventing the consumption of glucose by the RBC's if blood is left standing at room temperature. Though, it has a weak anticoagulant action, it is usually combined with another anticoagulant such as potassium oxalate in the ratio 1:3.

Special blood containers (vacutainers)

The vacutainer system is used to collect blood specimens of diagnostic analysis in the laboratory. This system ensures specimen integrity, transportation safety, care of use and direct sample access for analysis. It is composed of three basic elements:

1. An evacuated glass tube containing a premeasured vacuum to provide a controlled pre specified draw.
2. A holder which is used to secure the needle during insertion into the tube stopper and subsequent venipuncture.
3. A sterile, disposal, blood collection needle suitable for drawing either single/multiple samples.

The evacuated blood collection pathway from the patient's vein directly into pre formulated leak-proof, sterile collection containers eliminating the transfer of blood thereby proving safely against any accident, contamination of AIDS, hepatitis and other infectious diseases to the laboratory personnel's. The evacuated blood collection system facilitate standardized blood to additive ratio thereby offering consistently high quality of blood specimens for more accurate testing, diagnosis and treatment of patients. Following benefits are offered by vacutainer system:

- Mot hygienic system of blood collection
- Avoids transfer of blood from syringe thereby avoiding spillage of blood
- Protects the laboratory and hospital staff from occupational hazards of contracting infections like HIV, etc.
- Provides accurate blood to additive ratio hence analytical results are more precise helping the clinicians in proper diagnosis
- Avoids the procedure of putting additives which may not be accurate and putting on labels on vials
- Vacutainer tubes are sterile so are especially helpful in microbiology
- Vacutainer tubes are leak-proof so transporting these tubes to laboratory is absolutely safe.

Specimen Type

Color coded vacutainer tubes are used according to the specimen type required namely serum plasma or whole blood.

Grey top: potassium oxalate and sodium fluoride

Green top: sodium heparin

Lavender top: potassium EDTA

Red top: no preservative, gel or anticoagulant

Royal blue top: acid washed metal free with EDTA (Plasma/ whole blood) and without EDTA (serum)

Red and gray: gel barrier for serum separation

Gold top: (serum separation tube)

Black top: citrate for westergren ESR

Blood is drawn in the color coded vacutainer tube. For serum or plasma about $2_{1/2}$ times the requested volume of blood is drawn. For serum the blood is allowed to clot for at least 30 minutes and is separated by centrifugation. For plasma the blood is mixed with the anticoagulant by gently inverting the tube 6-10 times and separated by centrifugation.

Then serum or plasma is transferred to a plastic transport tube.

Order of draw of blood in collection tube.

1. Blood cultures should always be drawn first to reduce the potential hazard bacterial, fungal, quantitative, CMV and/or other specimen contamination.
2. Light blue tubes must be drawn prior to collection of serum tubes to prevent contamination with clot activator and interference with the coagulation cascade.
3. Next blood should be drawn for the following serum tubes:
    a. Serum tubes that are coated on the inside wall with clot activator because the surface of a plastic tube does not promote coagulation.
    b. Red stopper tubes
    c. SST (serum separator tubes) containing a gel which separate the cells from the serum when the tube is centrifuged.
4. In the end blood should be taken in the tubes with the additives:
    a. Green stopper tubes containing lithium heparin used for most tests that require plasma.
    b. Green stopper tubes containing sodium heparin used for special testing such as cytogenetics.

c. Lavender stopper tubes contain EDTA and used for hematology studies.
d. Pink stopper tubes used for testing in blood bank, i.e. blood typing, cross-matching, and antibody screens.
e. Black ESR tube containing sodium citrate.
f. Grey tubes for sugar estimation.

PRESERVATION, STORAGE AND TRANSPORT OF BLOOD SAMPLE

Preservation of blood sample

Alteration in the concentration of a constituent in a stored specimen can result from various processes such as:

1. Adsorption onto the glass container
2. Evaporation if the constituent is volatile
3. Water shift due to the addition of anticoagulants
4. Metabolic activities of the erythrocytes and leucocytes (accelerated by hemolysis) inducing O2 consumption and CO2 production, hydrolysis, glycolysis, and finally degradation.
5. Microbial (fungal/bacterial) growth.

Changes in concentration of volatile substances such as O_2 and CO_2 are prevented or at least hindered by collection and storage of samples under anaerobic conditions.

The problem of microbial growth appears when the sample is to be stored for longer than one day at room or refrigerator temperature. This can be solved by four alternative courses of action:

a Collection and storage under sterile conditions.
b Freezing of the sample
c Extreme alteration of pH
d Addition of an antibacterial agent.

Lyophilized samples are stable with respect to many constituents for periods of at least as long as ten years. Samples can be stored at room temperature (18-37 C^o),

refrigerator temperature (4C^{o}) and frozen state (- 10 C^{o} or lower). With few exceptions, lower the temperature, greater the stability. Further, microbial growth is considerably less at refrigerator temperature than at room temperature and is completely inhibited in the frozen state. Even components of plasma deteriorate.

Chemical preservative

They can be divided in to two groups:

1. for prevention of chemical change such as glycolysis
2. for prevention of microbial growth.

The common preservatives for urine specimen are formaldehyde, thymol, toluene and chloroform. All these act primarily as antimicrobial agents.

Storage of blood sample:

The sample must be processed as early as possible. If delay is inevitable, the serum must separate and refrigerated as this delays chemical change. The concentration of most of the commonly determined constituents remain stable up to a week if kept at 4^{o}C and longer if frozen. Some enzymes are, however, unstable at 4^{o}C, and should be frozen as soon as possible.

Transport of blood sample

Specimen should be delivered as soon as possible to the laboratory, usually within 2 hours but in some cases they should be sent immediately. Passage of time affects concentration of many blood constituents once it has been shed from the body.

Common changes that occur in blood samples:

Common changes that occur in blood samples after collection are as follows:

1. Glucose is converted to lactate by glycolysis.

2. Due to hemolysis or passage through erythrocyte membrane (before separation of red cell in plasma) plasma concentration of K^+, lactate dehydrogenase and amino transferase can increase.
3. Plasma phosphates increase due to hydrolysis of organic ester phosphates because of action of enzymes on organic forms.
4. Some labile enzymes my lose activity.
5. UV irradiation or daylight destroys bilirubin remarkably quickly so it is necessary to keep the sample in dark as much as possible.
6. Some of low molecular weight polypeptide hormones adrenocorticotropic hormone (ACTH), glucagon and parathyroid hormone (PTH) are rapidly destroyed by enzymes present in the plasma and must therefore be protected by the addition of suitable antiproteolytic agent to the blood immediately after it is collected
7. At room temperature calcium is absorbed rapidly on to polystyrene containers (analyzer cups) though this is minimized if the samples are placed immediately in the refrigerator. So serum of calcium estimation is best kept in glass.
8. Sample for blood gases and blood pH, are taken in heparinized syringe and is left on with the needle bend over so that blood does not come in contact with air. This is to avoid loss of CO2 because PCO2 of blood is higher than air. Syringe containing of blood should kept on ice to low red cell metabolism and to reduce glycolysis with consequent production of lactic acid. Average composition of different substances is different in plasma or erythrocytes **(table 2-4)**. Hence, there is need for proper preservation and quick handling of specimen.
9. Formation of ammonia from urea occurs in longstanding of blood.
10. Some conversion of pyruvate to lactate occurs.

Summary chart for the collection and dispatch of specimen for bio chemical test is given in **(table 2-5)**.

Table 2.4: Average composition of different substances is different in plasma or erythrocytes

Quantity	*Units*	*Plasma*	*Erythrocytes*
Glucose	mmol/l	5.0	4.1
Calcium	mmol/l	2.50	0.25
Phosphate	mmol/l	1.20	4.20
Sodium	mmol/l	140.0	16.00
Potassium	mmol/l	4.0	100.0
Chloride	mmol/l	104.0	52.0
Bicarbonate	mmol/l	25.0	10.0
Urea	mmol/l	5.5	4.0
Aspartate aminotransferase	U/l	25.0	500.00
Lactate dehydrogenase	U/l	180.0	30000.00

Chapter 3: Quality control in clinical laboratory (QC)

It is important to recognize that laboratory investigations are liable to certain error which may mislead the clinical assessment. The most common cause of a single abnormal biochemical value is probably an error of the laboratory. The study of the sources of variation for which laboratory is responsible and procedure used to recognize and minimize them is defined as quality control (QC).the source of variation include all sources which arise within the laboratory, from the receipt of the specimen to the dispatch of reports. The main emphasis of QC is to monitor the precision and accuracy of the performances of analytical methods. Beside been important for patient care, QC is designed to give the laboratory staff confidence in their methods. Result from a laboratory that do not maintain quality control cannot be relied upon so to maintain a good standard of accuracy, laboratory should monitor its own performance continuously by keeping in mind a few things and by applying one or more quality control systems.

It is recommended that serum control with known normal and abnormal value should be run routinely in the following condition:

- With each set of assay
- At least with every working shift to monitor the validity of the reaction
- When a new bottle of reagent is used
- After preventive maintenance is performed.
- When clinical component is replaced.

It is strongly recommended that each laboratory should establish their own frequency of control determination.

TO ASSESS THE VALIDITY OF RESULTS ON PATIENT SPECIMENS

Regulatory agencies require the use of quality control (QC) materials to assess the validity of result in a laboratory. This is achieved by assaying and evaluating at least to level of control material every 24 hours. Most laboratories are require to establish their own means and standard deviations for the parameters tested and reported on patients. The published assay range for a given control is the range in which a laboratory's mean must fall to be considered acceptable.

TYPE OF MATERIAL USED FOR QUALITY CONTROL

The type of quality control -material used in the laboratory is based on the laboratory's needs. There are currently several sources from where a laboratory can obtain sufficient quantities of quality control material. These are either of the following types:

- Frozen pooled patient specimen.
- Commercial lyophilized, freeze dried pool material.
- Commercial stabilized low temperature liquid serum pools

Serum is more frequently used than plasma because it is more readily available and is less likely to have precipitated material. All pooled human material should be free of human immune deficiency virus (HIV) and hepatitis B virus. Also all control material requires refrigerator or frozen space for storage for a period of 1 to 2 year supply.

For a lyophilized material always reconstitute the material carefully following the directions given on the label. Mixing too quickly or too vigorously may interfere with solubilisation of the lyophilized material. This is especially true for enzymes and other proteins that can be denatured and inactivated by such procedures. If a frozen liquid pool is used after thawing, mix five-six times by inversion because protein and other compounds become concentrated at the bottom of the tube.

TARGET AVERAGE VALUE OF QUALITY CONTROL POOL

The target values of the quality control pool are the estimated concentration of each analyte within the pool. Each of the laboratory must establish target value of each enalyte from QC pool by the regular procedures performed by the laboratory. For this, procure a year's supply of quality control test material. It is advisable to retain 20 to 30 vials of each expiring pool for use during subsequent years as reference material. For each constituent analyse duplicate samples from each of the two separate vials for 10 days (2 vials, 40 measurements), an alternative way is to reconstitute one vial and analyse in duplicate, on each of the 20 consecutive days. Calculate average and standard deviation (SD) of the 40 analytical values

(Table 3, 1). Use the average ±2 usual standard deviations as the acceptable control limit for the new lot of quality control material.

Temporary Target Average and Final Target Average

The Initial average value calculated from preliminary set of 40 values from 20 vials is called temporary target average. A final target average can be established at the end of second month. At that time .there are three average values (i.e., a temporary target value and two monthly averages) one can take the average of three values to calculate the final target average.

Usual Standard Deviation (USD)

This is the average of six monthly standard deviation values. This represents the usual precision capability of an instrument or method. The USD is used to establish the daily control limits around the target average (AT).other uses are

1. To establish the statistical significance of the difference between the TA and monthly average.

2. To establish the statistical significance of the difference between monthly standard deviation and the USD.

Table 3.1: Levy-Jennings plot for recording daily quality control values. Values for each day are plotted sequentially along X-axis

Day	Vial 1		Vial 2	
	Sample A	Sample B	Sample A	Sample B
1	4.4	4.6	4.5	4.4
2	4.7	4.7	4.5	4.5
3	4.5	4.4	4.7	4.5
4	4.5	4.4	4.6	4.3
5	4.6	4.5	4.4	4.5
6	4.6	4.5	4.3	4.5
7	4.5	4.5	4.5	4.5
8	4.4	4.3	4.4	4.4
9	4.3	4.6	4.4	4.7
10	4.6	4.6	4.7	4.6

Temporary target average (TTA): Duplicate samples for uric acid (mg/dL) were taken from two vials for 10 days, i.e. n = 40. The average includes the variability effect of sampling, different vials and different days.

Total of all observations = 179 mg/dL
Number of observations = 40

$$TTA = \frac{Total}{n} = \frac{179}{40} = 4.475 \text{ mg/dL}$$

Final target average:
Temporary target average = 4.475 mg/dL
First month = 4.510 mg/dL
Second month = 4.495 mg/dL
Total of averages = 13.480 mg/dL
Number of averages = 3

$$\text{Average of averages} = \frac{\text{Total of avg.}}{\text{Number of avg.}} = \frac{13.48}{3}$$

$$= 4.493 \text{ mg/dL}$$

This is the target average.
Usual standard deviation (USD):

Month	Monthly standard deviation (mg/dL)
May	0.14
June	0.13
July	0.15
August	0.10
September	0.15
October	0.11

Total of SDs = 0.78
Number of SDs = 6

$$\text{Average of SDs} = \frac{\text{Total}}{\text{Number}} = \frac{0.78}{6}$$

$$= 0.13 \text{ mg/dL}$$

This is usual standard deviation (USD).

Control Limit for Quality Control Pool

The target average, plus and minus two times the usual standard deviation is taken as the control limit for each pool sample.

Frequency of analysis of Quality control Sample

Daily preparation and analysis of quality control sample should be a regular feature of the laboratory. The frequency of analysis of QC material is determined by each laboratory for each method.

For example, for some large multichannel instruments, control. Usually are analysed every 20 samples whereas in a stable analytical system quality control should be run once an 8-hour shift. Control pools used by laboratories are of the following two types:

1-Normal pool: it contains constituents at concentrations within the non-diseased reference range.

2-Abnormal pool: it contains the analytes at concentrations outside the reference range.

Calibration: And Calibration Materials

No sample can be used simultaneously for calibration and control purpose because each has a separate and important function. The calibration has an assigned value and is used to standardize the method or instrument. For calibration of standards the composition should be considered in relation to the analytical method and the type of specimen to be analysed. Thus, a primary standard solution in water may be satisfactory for some analyses and a secondary standard, which is also serum, is usually necessary for multichannel automated analysis of sera. Control materials should normally be of similar composition to that of the sample being analysed. Most biochemical methods applied to biological material show matrix effects. The difference between an aqueous and serum matrix can affect the ability of a pool to act as a calibrator. These differences include:

- Effect of surface tension which can affect sample pipetting.
- Effect of interactions between analytes and proteins.
- Effect of volume fraction occupied by protein on the actual concentration of certain analytes.

For a new calibrator the practical system is as follows:

1. Every day take 1 aliquot from one vial of calibrators the unknown. Calculate and compare the average with value assigned to the calibrator.
2. Each day, rest the instrument using manufacture's assigned value.
3. Calculate the average and standard deviation for each quality control pool level, measured with new calibrator.
4. Compare these averages with the current target average for the pool. This step will predict the average value for the quality control pool analytes using new calibrator.
5. Before beginning the use of new calibrator a decision should be made as to whether the manufacturer's value for any analyte should be changed .changes and data supporting these alterations should be recorded.

Evaluation of Daily, Weekly, Monthly Results to Maintain Accuracy and Precision

In the quality control programmer, most decisions are based on daily basis but these should be reviewed weekly and monthly to evaluate changes in average from the target average and changes in the standard deviation from the usual standard deviation.

Daily Decisions

Daily bench–level quality control testing can be used to detect only systematic errors and a decrease in precision and not random errors which occur unpredictably.

When 2 controls are used which are within 2± SD, the technologist can approve the batch and can release the analyte results. But if both the controls are

outside 2± SD, biochemist should be consulted and analysis of both quality control pools should be repeated.

A set of two controls and one calibrating material for every 20 samples allow prompt correction of specific drift in the operation of the instrument.

Cause of system failure instrument and system should be checked for the following:

1-Reagent solution.

2-Calibrating solution.

3- Control material.

4-Standard solution.

Clots in the sample interfering with the analysis.

Mechanical and flow systems of the instrument for proper delivery of reagents and sample.

Calculation error: Most laboratories have back –up methods for analysis. In case of system failure alternative method can be used. The daily results of most patient sample values fall into the familiar pattern, that is, most values within normal range and a few high and a few low values. So deviation from this pattern should alert the biochemist regarding validity of results. Also physician that does not fit the clinical picture in this opinion.

Weekly Review of values:

Short term changes in the method results from a new lot of reagents or standards or improper maintenance of instrument. Weekly review monitors short-term changes in the analytical system.

Levy-Jennings plot for recording daily quality control values: Many laboratories take either the first quality control value of each daily run or averages of the values obtained for each quality control pool for each analyte for each day and plot that on a quality control chart. Such charts, e.g. Levy- Jennings graph (Fig.3.1) gives visual presentation of the data.

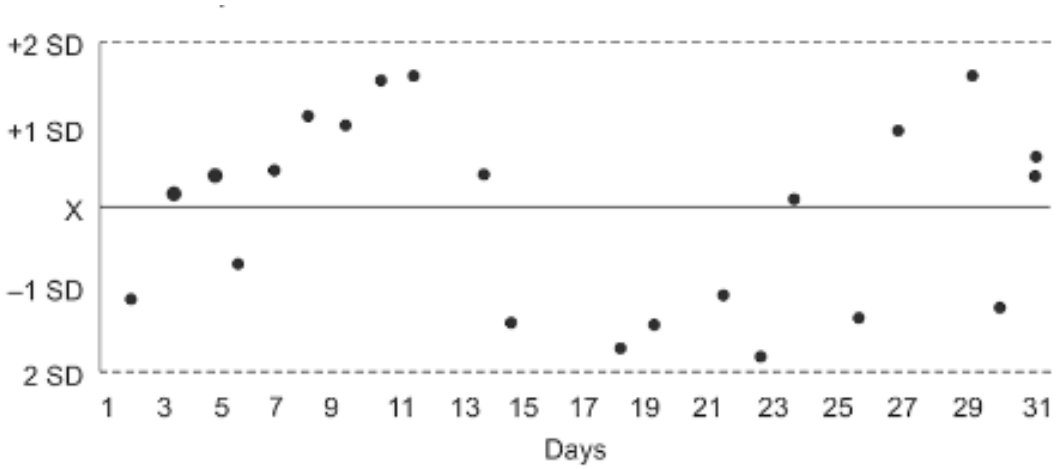


Fig. 3.1: Levy-Jennings plot for recording daily quality control values. Values for each day are plotted sequentially along X-axis

Average ±2 USD is drawn on the y-axis and the days of the month are indicated on the X-axis. Trends or shifts from the average target values are known as biases. If the Performance of the analytical method remains unchanged, succeeding points will be evenly scattered on either side of the midline indicating no change in accuracy. If the bias becomes severe and the value falls outside 2 SD limit it is called warning limit. Change in accuracy should be detectable before the graph passes beyond warning limits. If a result falls beyond the action limit, the chances of true error are much higher and there is risk that all results are erroneous.

Control Samples should be randomly inserted amongst patient's samples and analyst should not know that sample is otherwise results are unconsciously biased.

Monthly Review of Values:

Every month in the beginning, the average standard deviation of the past month's quality control analysis should be performed. Table 3.2 is a cumulative from that was developed at the New England Baptist Hospital (Boston) and the New England Deaconess Hospital (Boston).Its purpose is convenient review of previous month values and easy decision making.

Table 3.2: Table for cumulative monthly record

Month	TA 4.5[a] mg/dl			O.13[b] USD		
	$\bar{X}$	QCD	$\bar{X}$ -TA	SD	QCD	SD-USD
Initial	4.5	√		0.15	√	+ 0.02
Jan	4.6	√	+ 0.1	0.14	√	+ 0.01
Feb	4.7	√	+ 0.2	0.12	√	– 0.01
Mar	4.1	*	– 0.4[c]	0.20	*	– 0.07[d]

a. Target average (TA) calculated from (1) initial, (2) January, and (3) February months.
b. Usual standard deviation (USD) calculated from previous 6-month experience.
c. Difference of monthly X from TA greater than USD is statistically significant (NMS); it is operationally significant (OS).
d. Difference greater than 1/2 USD is statistically significant; it NMS; it is OS.

Decision: Uric acid method needs investigation during April month.

Target average (TA) is calculated from initial value and values for Jan and Feb months. Monthly average is recorded in column I. Target average is subtracted from the monthly average and the difference is recorded in the adjacent column. If the difference between the target average and the monthly average is greater than the usual standard deviation, the difference is taken as statistically significant at the 5 % level of probability.

Usual standard deviation (USD) is calculated from previous 6 months´ experience. This is subtracted from the current monthly standard deviation (SD-USD).If the difference is greater than only one-half the usual standard deviation, the monthly standard deviation is statistically different from the USD.

In the quality control decision (QCD) column two symbols are used .A check mark means that value is not statistically different and an asterisk means statistically significant difference. The decision is made regarding the medical significance of the observed change in the average and SD, i.e.

If these observed changes are medically significant for the physicians. If the decisions is that the scientifically different value is not medically significant, an operational decision should be made .An operationally significant change requires action. The persons actually using an instrument can often perceive when the instrument system or method is not operating properly. So daily, weekly, monthly and yearly quality control programmers verify that the method used in laboratory is maintaining precision and accuracy.

Internal Quality Control programme

The day to day internal quality control programme is very useful for maintenance of consistency and precision. Control samples may be prepared by the laboratory, e.g. pooling sera together and carefully analyzing them to assign a correct value. Pooled sera, carefully mixed, dispensed into small volumes, stoppered and preserved in a frozen state at -20°C are often stable for several months.

An alternative is to use a commercial product. Many companies now market products with the correct value already determined. Often these are based on animal sera which remove the danger of hepatitis infection. But animal product may not behave inexactly the same way as the humans.

This is particularly the case with enzymes and serum proteins, so a considerable caution is needed if sera other than human are used.

External Quality Control Programme

External Quality Control Programme is designed and aimed to provide comparability of results between all the laboratories using the same method anywhere .Under this QC samples of unknown value are periodically sent to the participants and they are required to analyse it along with routine samples .The sample may be liquid or freeze-dried. In each case results are reported by participating laboratories and are collected by the organizers. Usually the collected results are published to the laboratories concerned as histogram, so that each participant cane see how the results compare with those of the other laboratories .Also statistical parameters variance index score (VIS) , as recommended by WHO, is made use of by ACBI/ CMCH external quality

assessment scheme to assess the performance of different laboratories. For this purpose they make use of the term chosen co-efficient of variation (CCV). This has been recommended by WHO as the ideal precision based on the performance by many Indian laboratories in the WHO/DGH Programme. The CCV values for various analytes are listed below:

Analyte	*Chosen coefficient of variation (CCV)*
Glucose	7.7
Urea	10.0
Total protein	7.5
Albumin	7.5
Calcium	6.0
Cholesterol	7.5
Sodium	2.3
Potassium	5.0
Creatinine	10.0
Lithium	11.0
SGOT	12.5
SGPT	17.3
ALP	15.5

The following example will explain the calculation of vis for glucose external quality control sample having laboratory value of 85 mg% and designated value (DV) of 110 mg%.

Difference between participant's result and

$$\text{\% variation (\%V)} = \frac{\text{Difference between participant's result and designated value}}{\text{Designated value}} \times 100$$

$$= \frac{110 - 85}{110} \times 100$$

$$= \frac{25}{110} \times 200$$

$$= 22.7$$

$$\text{Variance index (VI)} = \frac{\%V}{CCV} \times 100$$

$$= \frac{22.7}{7.7} \times 100$$

$$= 295$$

When VI ls less than 400, it is designated VIS, therefore VIS=295.

So closes the participant's result is to DV, lower is the VIS figure. For better performance of the laboratory result should be as close to DV as possible. Because this will show a very low value for VI and VIS will be the least. One should check the VIS value for each constituent every month. If one's VIS is getting less each month, that means the laboratory is steadily improving.

Interpretations for different VIS values:

a) WS less than? 100: the objective of the laboratory should be to get the results close to the DV for each constituent so that VIS value is less than 100 which is very good.
b) VIS between 150 - 200: If VIS is in the range 150 -200 then one must look carefully at those methods for which results are high or low and improvement be made in that.
c) VIS above 250. For a VIS value above 250 means that probably many wrong results are being reported and urgent steps are needed to correct the method or standards.
d) VIS value greater than 400: indicates very erroneous results.

Each laboratory can compare its results to its closet peer group (same method, same instrument). The difference between the individual laboratory result and average value of the peer group provides the indication of a possible bias. If a laboratory's results are biased outside ±2SD from its peer group, the performance is not acceptable. The reasons for this may be reagent, method used or personal error of the technician. External quality control testing should be done quarterly (3 months' Interval) to ensure the reliability of each measurement performed on a patient sample. These programs of this type is clinical biochemistry are being run in India

So, first of all each laboratory must develop a sound internal QC and then should join some external QC too. The improvement of a laboratory after introduction of QC programme is certain.

Chapter 4: Test for carbohydrates

Carbohydrates are widespread in nature in both plant and animal kingdom. They serve as the primary source of energy, Monosaccharides, the basic compound of this series, Have one single carbon chain, having free aldehydic or ketonic group and a number of hydroxyl groups. Glucose (an aldohexose) and fructose(a keto hexose) are most common monosaccharides (Fig. 7.1).

TEST FOR CARBOHYDRATES

It can be performed with 1% solution of glucose, and 1% fructose.

Molisch Test

This reaction is a general one for all carbohydrates

Principle: Carbohydrates when treated with conc. H_2SO_4 undergo dehydration to give furfural

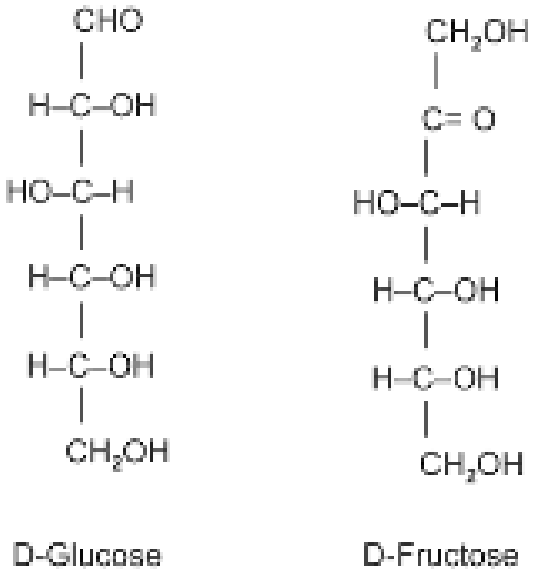


Fig. 7.1: Monosaccharides

Benedict's test

Reducing sugars under hot alkaline conditions convert to enediols. Enediols are powerful reducing agents. They can reduce cupric ions to cuprous form which is the basis for Bennedict's reaction. The cupric hydroxide formed is not easily. Soluble. In order to keep the hydroxide in solution, metal chelator like citrate (Or tartarate) included in the solution.

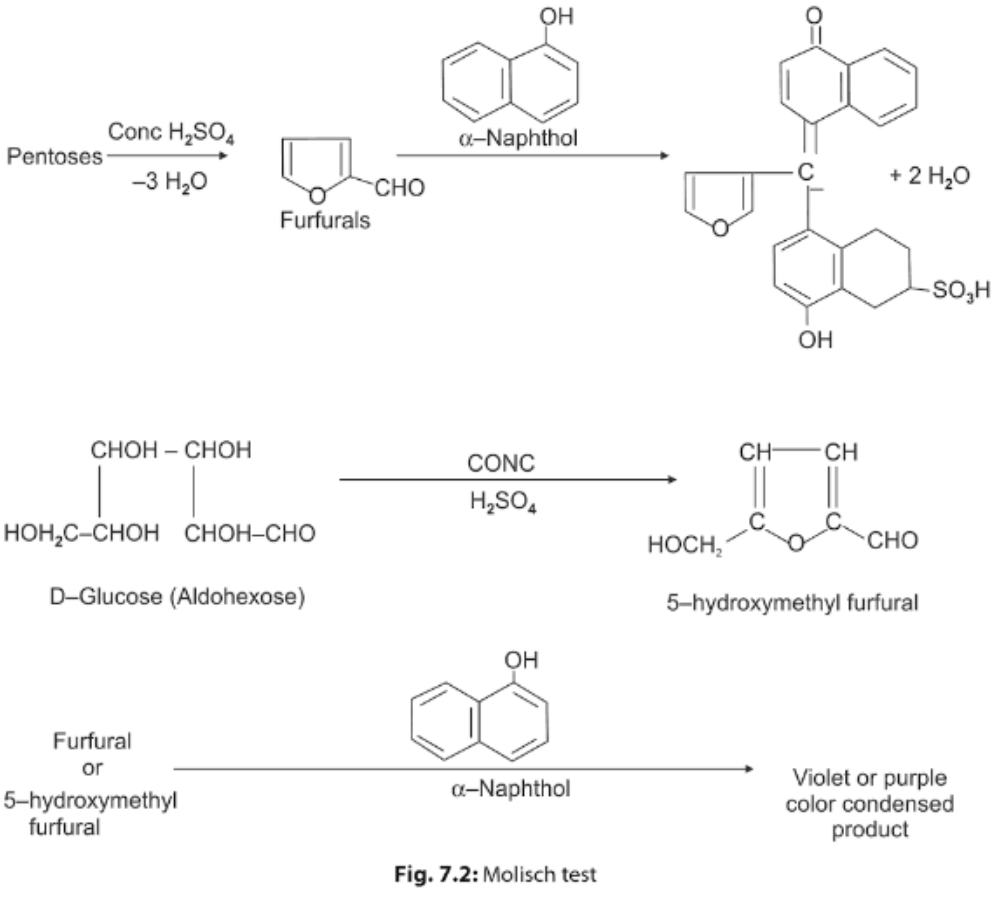
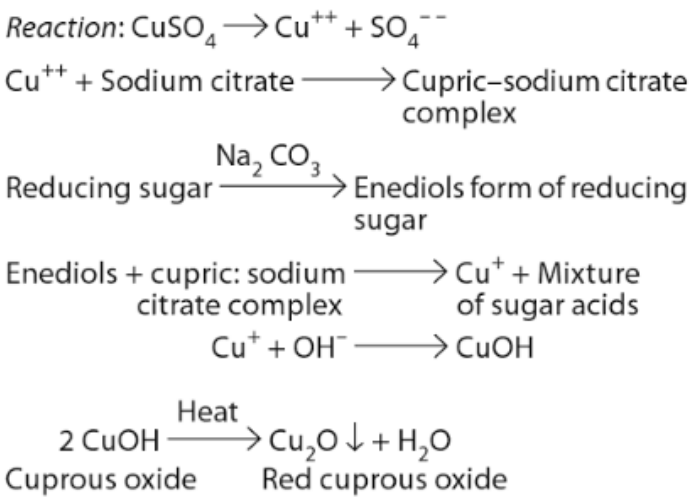


Fig. 7.2: Molisch test

Benedict's solution Contains milder alkali Na_2CO_3, Cuprous hydroxide, during the process of heating, is converted to red cuprous oxide. Benedict's test is more specific. Than fehling's test. In the former test, uric acid, creatinine and other compounds do not interfere. As much as fehling's test



Benedict's qualitative reagent contains:

1. copper sulphate: it furnishes cupric ions (Cu^{++}) in solution.
2. Sodium carbonate: It makes medium alkaline.
3. Sodium citrate: It prevents the precipitation of cupric ions as cupric hydroxide by forming a loosely bound cupric-sodium citrate complex which on dissociation gives a continuous supply of cupric ions.

Reagent: It is prepared by dissolving 173 g of sodium citrate, 90 g of anhydrous Na_2CO_3 in 500 ml of distilled water. Slightly heat, so as to dissolve the contents, filter the solution and make the volume to 850 ml. Dissolve separately 17.3 g of $CuSO_4.5H_2O$ in 150 ml of water. Add the solution slowly and with stirring to the above solution.

Technique: Take 5 mL of Benedict's reagent. Add 8 drops of given carbohydrate solution. Boil over a flame or in a boiling water bath for 2 minutes. Cool the solution. An appearance of green, yellow or red precipitate indicates the presence of reducing sugars.

Green color up to 0.5 g % (+)

Green precipitate 0.5 - 1.0 g % (++)

Green to yellow precipitate 1.0 - 1.5 g % (+++)

Yellow to red precipitate 1.5 - 2.0 g % (++++)

Brick red precipitate more than 2.0 g %.

Barfoed's Test

This test is used to distinguish reducing monosaccharide from a reducing disaccharide by controlling pH and time of heating. This is also a copper reduction test in acidic conditions. Aldoses and ketoses can reduce cupric ions even in acidic' conditions. Monosaccharides react very fast whereas reaction with reducing disaccharide is slow. However, if heating is prolonged, disaccharide may be hydrolyzed by the acid and the resulting monosaccharides, will give the test positive.

Reagent: Barfoed's reagent is prepared by dissolving 24 g of copper acetate in 400 ml of boiling water. To this add 25 ml of 8.5% glacial acetic acid solution. Stir, cool the solution and dilute to 500 ml.

Test: t o2 ml of barfoed's reagent add 2 ml of carbohydrate solution. Place the test tube in boiling water bath for 3 minutes. Cool under running water. An appearance of brick red precipitate of cuprous oxide indicates the presence of monosaccharides.

Precautions: the solution should be boiled for 3 minutes only over heating disaccharides will also give this test positive.

Seliwanoff's Test

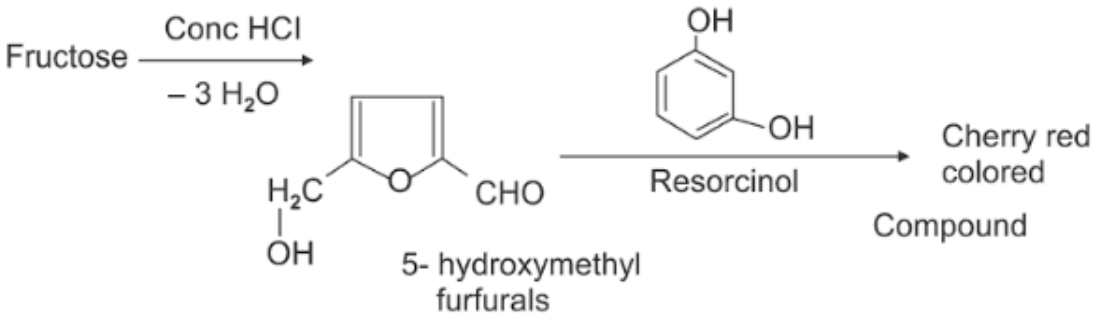
This test is positive for ketohexoses only and so is answered by fructose, sucrose and other fructose containing carbohydrates,

Principle: Ketohexoses on treatment with hydro chloric acid form 5-hydroxymethyl furfural which on condensation with resorcinol gives a cherry red colored complex.

Seliwanoff's test distinguishes between fructose and glucose. Overheating of the solution is avoided because on continuous boiling, aldoses will also give this test positive because of their conversion to ketoses by the HCI. Sucrose will also give Seliwanoff's test positive because the acidity of reagent is sufficient enough to hydrolyse sucrose to glucose and fructose but Benedict's test will be negative.

Reagent: 0.05 g resorcinol in 100 mL dilute HCI (diluted 1:1 with water).

Test: To 3 m L of Seliwanoff's reagent add 1 mL of fructose. Boil for 30 seconds only. Cool the solution. An appearance of cherry red color indicates the presence of fructose.



REACTION OF DISACCHARIDES

The most common disaccharides are maltose, lactose and sucrose maltose and lactose are reducing disaccharide.

1. **Molisch test:** All three disaccharides give positive test. Under the acid conditions disaccharides are first hydrolyzed to monosaccharides which answer the test.
2. **Benedict's test:** Maltose and lactose give Benedict's qualitative test positive whereas with sucrose, the test is negative, i.e. No reduction is observed.
3. **Barfoed's test:** Negative for disaccharides.

REACTIONS OF POLYSACCHARIDES

Starch: The most commonly available polysaccharide is starch which is a mixture of **amylose** and amylopectin. The individual glucose units in amylose are linked by α-1, 4 glycosidic linkage.

Amylopectin has branching points contributed by α -1, 6 glycosidic bonds. Starch is insoluble in cold water but forms a colloidal solution in hot water. Starch has no detectable reducing activity.

Dextrins: They are formed as intermediate products during the course of hydrolysis of starch by dilute mineral acids and also by the action of amylases, these heterogeneous compounds are grouped into amylodextrins, erythrodextrins and achrodextrins based on the color produced on reacting with iodine. These 3 groups of dextrin's which are in the order of decreasing molecular weight give respectively violet, red and no color in iodine reaction.
Dextrins are partially soluble in water. They have more reducing activity than starch.

Tests for Starch and Dextrins

For this test take 1% starch and 1% dextrin solution.

1. **Molisch test:** Both starch and dextrin give positive test. Under acidic conditions the polysaccharides are hydrolyzed to monosaccharides which answer the test.
2. **Benedict's test:** Starch gives a negative test whereas dextrin shows slight reducing activity. Note the formation of light green color with dextrins.
3. **Iodine reaction:** Iodine forms a coordinate complex between the helically coiled polysaccharides chain and iodine centrally located within the helix due to adsorption. The iodine color obtained with the polysaccharide depends upon the length of the unbranched or linear (α -1, 4 linkage) chain available for complex formation.
    a. **Amylose:** A linear chain component gives a deep blue color.
    b. **Amylopectin:** A branched chain component of starch gives a purple color.
    c. Glycogen: It gives a reddish brown color.

Chapter 5: Estimation of Blood Glucose

Diabetes Mellitus: It is a chronic disease due to disorder of carbohydrate metabolism, cause of which is either deficiency or diminished level of insulin resulting in hyperglycemia (increased blood glucose level) & glucose (presence of glucose in urine). Secondary metabolic defect is also seen. Such as metabolism of proteins & fats.

1. **Primary or Idiopathic or Essential Diabetes**
   a. Juvenile Diabetes or. Type I Diabetes or Insulin dependent Diabetes Mellitus (IDDM) Less Frequent Occurs before the age of 15 years. Due to less production of insulin from β-cells of Langerhans (Pancreas)
   b. Maturity onset diabetes or. Type II diabetes or Non-insulin dependent Diabetes mellitus (NIDDM) More frequent in population. Occurs at middle age. Ketoacidosis is rare. B-cell is degenerated to some extent but response to glucose load is seen.
2. Secondary It is secondary to some other main disease
   a. Pancreatic Diabetes. Pancreatitis Hemochromatosis Malignancy of Pancreas.
   b. Increased level of antagonistic hormone Hyperthyroidism Hypercriticism – Cushing's disease Hyperpitnatarism

Clinical Biochemical finding in diabetes

1) Presence of large amount of glucose in urine.
2) Large volume of urine & increased frequency of micturition (Polyuria)
3) Polyphagia i.e. eats more frequently.
4) Increased catabolism of fat so there is increase in free fatty acid level in blood & liver.
5) Increased acetyl coA is seen which further lead to increase formation of cholesterol & hence at formation of atherosclerosis.
6) Increased ketone bodies in blood & its appearance in urine leads to acidosis.
7) Increased catabolism of tissue protein for energy requirement lead to loss of weight & increased level of amino acid in blood & more formation of urea by deamination of amino acid.

Objective: - To estimate blood glucose.

Introduction:-

Estimation of glucose in blood was one of the first biochemical tests to be applied clinically and now it has become a routine in clinical biochemistry lab.

In blood quantitative estimation of glucose is done with either whole blood, plasma or serum and several methods have been in use. Whole blood values are 10-15% lower than plasma. Arterial blood values are higher than venous values.

The term Blood Sugar is used synonymously with blood glucose but certain other substance like glutathione, glucuronic acid, threonine, uric acid, ascorbic acid, fructose etc. give erroneously high values (5-20%) when any reduction method is adopted.

a) **Fasting blood Sugar (FBS)**: The blood sample is collected after the patient fasts for 12 hours or overnight.

b) **Post-Prandial Blood Sugar (P P B S):** After the patient fasts for 12 hours, a meal is given which contains starch and sugar (approx. 100 gms). Blood is collected 2 hours after the ingestion of the meal.

c) **Random Sample:** Blood is collected any time without prior preparation of the patient.

Collection of Blood Sample: Blood is usually collected from a vein and kept in a bottle containing sodium fluoride (Na F) and potassium oxalate mixed at proportion of 1: 3 Usually 4 mg. of the mixture is required. Both the substances act as anticoagulant and Na F prevents glycolysis in RBC's by inhibiting the enzyme 'enolase'.

Methods of Estimation:

1. **Enzymatic:** Measure only glucose in blood.

a) **Glucose Oxidase Method:**

Glucose oxidase catalysis the oxidation of glucose to gluconic acid and hydrogen peroxide. This H_2O_2 is broken down to water and oxygen by a peroxidase in the presence of an oxygen acceptor which itself is converted

to a colored compound, the amount of which can be measured colorimetric ally. This method is used in various auto analyzers.

Reaction:

Glucose oxidate

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

$$H_2O_2 + \text{reduced chromogen} \xrightarrow{\text{peroxidase}} \text{oxidized chromogen} + H_2O$$

b) Hexokinase Method:

Hexokinase

$$\text{Glucose} + ATP \xrightarrow{\text{hexokinase}} \text{glucose 6-PO}_4 + ADP$$

$$\text{Glucose 6-PO}_4 + NADP \xrightarrow{\text{G-6-PD}} NADPH + H^+ + \text{6-phosphogluconate}$$

Reaction

The rate of formation of NADH is followed spectrophotometrically at 340 nm.

2) **Reduction Methods**

a. **Alkaline Copper Reduction Methods.**

b. Asatoor & King's method

c. Folin & Wu method

3) Ortho toluidine method: O-Toluidine reacts quantitatively with the aldehyde group of glucose to from a glucosylamine and Schiff base. The color development is rapid and stable.

Estimation of Blood Glucose by method of asatoor and King (modified)
Principle : Reducing sugars in hot alkaline medium produce enediols which are strong reducing agents that convert Cu^{++} ions Cu^{+} ions combine with OH ion to form yellow CuOH which on heating gives red Cu_2O, Cu_2O produced is proportional to the amount of reducing sugar Phosphemolybdic: acid is added so that oxidation of Cu^{+} to Cu^{++} is coupled with reduction of acid to molybdenum blue which can be estimated colorimetrically.

Modified method gives values close of true glucose. This is achieved by diluting blood with isotonic $CuSO_4$ Na_2SO solution to prevent hemolysis of RBC. The glucose diffuses out of cells and is estimated while various non glucose reducing substance remain in the intact cells and are precipitated during deproteinisation and removed by centrifugation.

So method used is modification by Asatoor and King in 1954 and involves:

- Precipitation of blood proteins.
- Reduction of alkaline CuSO4 solution to cuprous oxide by glucose.
- Estimation of extent of reduction of blue colored complex by colorimetric measurement at 610 nm.

Reagents:

i) Isotonic copper sulphate solution :

$Na_2SO_4.10H_2O$ 30 gm and $CuSO_4$ $5H_2O$ 6 gm per 1 liter of solution.

ii) Sodium tungstate 10%. iii) Alkaline Tartrate solution.
Dissolve 25g $NaHCO_3$, 20gm anhydrous Na_3CO_3 and 18 gm Pot. Oxalate in about 500 ml of water. Add 12 gm of sodium potassium tartrate and make up the volume to 1 L.

iii) Phosphomolybdic acid solution: To 35 gm of molybdic acid add 5 gm. Of sodium tungstate, and 200 ml of 10% NaOH. Boil for 3040 mins to remove ammonia. Cool. Dilute to about 350ml ad add 125 ml of conc. (85%) phosphoric acid. Make volume up to to 500 ml.

iv) Stock glucose Standard solution: 1 gm% in saturated benzoic acid.

v) Working glucose standard solution: Dilute stock 1 ml to 100 ml so conc. Is 10 mg% with isotonic $CuSo_4$ solution?

Procedure

Place 0.1 ml of blood in a centrifuge tube. Add 3.8 ml of isotonic sodium sulphate, copper sulphate solution and mix. Add 1 ml of sodium tungstate solution and mix. Centrifuge at 2000 pm for 10' Use clear supernatant for test.

Solutions ml.	T	S_1	S_2	S_3	B

Supernatant	1.0	-	-	-	-
Std. Glucose	-	0.2	0.4	0.6	-
Isotonic $CuSO_4$	-	0.8	0.6	0.4	1.0
Alkaline Tartrate	1.0	1.0	1.0	1.0	1.0

Mix well. Plug the tubes with cotton wool and put in boiling water bath for 10 mins. Cool and add 3 ml of phosphomolbdic acid and 8 ml of distilled water to each tube. Mix, take O.D. at 610 nm.

Plot a graph of standard against O.D.

Calculation:

$$\text{Blood glucose (mg/dl)} = \frac{\text{OD of Test}}{\text{OD of Std}} \times \frac{\text{Amt. of Std.}}{\text{Vol. of Blood}} \times 100$$

Interpretation: The normal fasting blood glucose varies from 60-100 mg/dl and post prandial from 100-140 mg/dl. The appear limit increase with age and during pregnancy.

Hyperglycemia: Causes are:

1. Diabetes mellitus – Fasting blood sugar raised. Values of 140 mg/dl on more than one occasion or post prandial level of 200 mg/dl confirms the diagnosis. If the value is below 100 mg/dl it excludes diabetes mellitus Values in uncertain range between these figures calls for oral GTT to diagnose the condition.
2. Hyperactivity of thyroid, pituitary, or adrenal gland.
3. Surgical removal of pancreas, pancreatitis, carcinoma of pancreas, fibrocystic disease.

Hypoglycemia: When blood glucose falls below 60 mg/dl.

1. Most commonly seen due to over dosage of insulin in treatment of diabetes mellitus.
2. Hypothyroidism – cretinism.
3. Insulin secreting tumors of pancreas – rare.
4. Hypoadrenahsm (Addison's disease)
5. Severe exercise.
6. Starvation.
7. Chronic alcoholism
8. Congenital disease like – glycogen storage disorders

Normally the blood sugar levels are well maintained due to action of various hormones.

Along with estimation of blood glucose levels urine must be tested for:

1. Reducing Sugar – Commonly in diabetes mellitus.
2. Albumin – Diabetic nephropathy.
3. Ketone bodies – Diabetes ketoacidosis.

Recently quick measurement of blood glucose can be done by using only drop of blood from a finger prick using glucometer or dextrostix.

Technical contents: - kit of sugar, colorimeter or semi autoanalyser.

Methodology of its teaching: - Demonstration and estimation of sugar.

Evaluation of the session: - Asking to perform the test and taking readings.

Chapter 6: Estimation Total Protein

Object: To estimate total protein.

Introduction:

Serum contains a large variety of proteins. More than hundred different proteins have been identified so far, and perhaps many more are yet to be identified. Albumin and the various globulins constitute the bulk of the total amount of proteins present in serum.

The most widely used method is still the biuret method. (The name derives from the reaction between biuret and cupric ions in an alkaline medium.

Biuret Method

Principle: Cupric ions form chelates with the peptide bonds of proteins in an alkaline medium. Sodium potassium tartrate keeps the cupric ions in solution. The intensity of the violet color that is formed is proportional to the number of peptide bonds which, in turn, depends upon the amount of proteins in the specimen.

Reagents

(i) Biuret Reagent – 3 mg of copper sulphate is dissolved in 500 ml of water. 9 gm of sodium potassium tartrate and 5 gm of potassium iodide are added and dissolved. 24 gm of sodium hydroxide, dissolved separately in 100 ml of water is added. The volume is made up to 1 liter with water. The reagent is stored in a well-stoppered polythene bottle.

(ii) Biuret blank – this is prepared in the same way as the biuret reagent with the difference that copper sulphate is not added.

(iii) Standard protein solution – the best way is to determine the total protein concentration in pooled human serum by Kjeldahl method, dilute it to bring the

protein concentration to the desired level, say 6 gm/100 ml and use it as standard. Alternatively, a 6 gm/100 ml solution of bovine albumin in water may be prepared and used as standard.

Procedure: label 3 test tubes 'Unknown', 'Standard' and 'Blank', Measure 5 ml of biuret reagent into each. Wash 0.1 ml of serum into 'Unknown', 0.1` ml of standard protein solution into '; Standard' and 0.1 ml of water into 'Blank'. Mix and allow to stand for 30 minutes.

Read 'Unknown' and 'Standard' against 'Blank' at 540 nm or using a green filter.

Calculations

$$\text{Serum total proteins (gm/100 ml)} = \frac{Au}{As} \times 6$$

Note. The above procedure gives reliable results with clear and unhaemolysed specimens. If the serum specimen is lipaemic, icteric or haemolysed, an additional tube (Serum Blank) should be prepared. 0.1 ml of serum should be mixed with 5 ml of biuret blank in this tube and read after 30 minutes against 'Blank'. Absorbance of 'Serum Blank; should be subtracted from that of 'Unknown before calculations.

Interpretation

The normal range of serum total proteins is 6-8 gm/100 ml in adults. The values. Are lower in neonates, rise gradually in infancy and reach the adult levels in early childhood. The level decreases in pregnancy. A slight change occurs with posture also. The level is a little higher in upright posture than in recumbent posture due to shift of water from the vascular compartment into the interstices.

An increase in serum total proteins occurs in dehydration due to haemoconcentration. An increase may also occur in multiple myeloma, macroglobulinaemia, chronic infections, chronic liver disease and autoimmune diseases.

A decrease in serum total proteins may results from heavy losses of proteins in urine as in nephritic syndrome. Protein undernutrition, intestinal malabsorption and protein losing enteropathy may also lower the serum total proteins. A decrease may also occur in shock, burns, crush injuries, hemorrhage etc. due to haemodilution. Increased breakdown of proteins, as in hyperthyroidism, un-treated diabetes mellitus, wasting diseases etc. may also lower the level of proteins in serum.

Technical contents: Using kit of total protein.

Method of teaching: Demonstration and its measurement of serum total protein by kit and taking readings. Using a colorimeter or semi auto analyzer.

Evaluation: Giving exercise of total protein estimation.

Chapter 7: Estimation of Serum Albumin and Globulin

Object: To estimate of serum albumin and globulin.

Bromocresol Green Method

Principle: The method is based on the protein error of indicators. Biding of a protein to an indicator changes its color. Among serum proteins, only albumin binds to BCG this binding produces a change in the color of BCG which is measured colorimetric ally. The pH is maintained during the reaction by a buffer.

Reagents

(i) Succinate buffer - 11.8 gm of succinic acid is dissolved in about 800 ml of water. The pH is adjusted to 4.0 with 0.1 N sodium hydroxide. The volume is made up to 1 liter with water. This solution should be Stord in refrigerator.

(ii) BCG solution - 419 mg of bromocresol green is dissolved in 10 ml of water. The solution is stored in refrigerator.

(iii) Buffered BCG solution – 250 ml of BCG solutions is mixed with 750 ml of succinate buffer. The pH is adjusted to 4.2 with 0.1 N sodium hydroxide solution. 4 ml of Brij – 35 solution (30%) is added.

(iv) Standard albumin solution – an aqueous solution of human albumin with a concentration of 4 gm/100 ml can be prepared and used as a standard. Sodium azide should be included in this solution (50 mg in every 100 ml) as a preservative. Pooled human serum (preserved with sodium azide) or a control serum having an albumin concentration of 4 gm/100 ml can also be used as a standard.

Procedure: Level 3 test tubes 'Unknown', 'Standard' and 'Blank'. Measure 4 ml of buffered BCG solution into each. Wash 0.02 ml of serum into 'Unknown', 0.02 ml of standard albumin solution into 'Standard' and 0.02 ml of water into 'Blank'. Mix and allow the tubes to stand for 5 minutes.

Read 'Unknown' and 'Standard' against 'Blank' at 630 nm or using a red filter.

Calculations:

$$\text{Serum albumin (gm/100 ml)} = \frac{Au}{As} \times 4$$

Interpretation:

The normal range of serum albumin is 3.7-5.3 gm/100 ml. Serum globulin ranges from 1.8 to 3.6 gm/100 ml. the A: G ratio is roughly 2:1 though it may range from 1.2:1 to 2.5:1.

Decrease in serum albumin may occur in protein undernutrition, intestinal malabsorption, and protein-losing enteropathy, liver disease, wasting diseases, nephritic syndrome and haemodilution. A severe decrease or near – absence may be seen in analbuminaemia which is a genetic disease with autosomal recessive in-heritance. A rise in serum albumin may occur in dehydration due to haemoconcentration.

Serum globulin may decrease due to haemodilution in shock, burns, and hemorrhage etc. serum globulin increases in multiple myeloma, macroglobulinaemia, chronic liver disease, chronic infections and autoimmune diseases. A: G ratio may be decreased or reversed in these conditions.

Since the colorimetric measurement of albumin is much simpler than that of globulin, the concentrations of total proteins and albumin are measured in serum, and globulin is obtained by difference.

Technical contents: Using kit of serum albumin.

Method of teaching: Demonstration and its measurement of serum albumin by kit and taking readings. Using a colorimeter or semi auto analyzer.

Evaluation: Giving exercise of serum albumin estimation.

Chapter 8: Estimation of serum triglycerides

Lipid profile: For lipid profile estimation following tests are performed

1) Total lipids: Principle lipids react with sulphuric acid, phosphoric acid and vanillin to form pink color complex. Normal values 400-1000 mg/dl 2) Phospholipids : It is a fully enzymatic method which uses three different enzymes-phospholipase D, Choline oxidase and per oxidase to developed color which is measured at 500 nm. Normal range 160-270 mg/dl 3) Triglycerides:

4) Cholesterol :
5) HDL :
6) LDL : Cholesterol – HDL + VLDL
7) VLDL : TG/5

Object: To estimate serum triglycerides.

Introduction:

Elevated levels of triglycerides in plasma have been considered as risk factors related to atherosclerotic diseases. The hyperlipidemias can be inherited trait or they can be secondary to a variety of disorders of diseases including nephrosis, diabetes mellitus, biliary obstruction and metabolic disorders associated with endocrine disorders.

Method:

Acetyl-acetone.

Normal range 10-190 mg/dl

Principle:

The serum lipids are extracted by isopropanol, which also precipitates serum proteins. The interfering phospholipids, containing glycerol as integral part, are

removed by adsorption on alumina. Filtrate is used for saponification and glycerol is separated from triglycerides. Action of metaperiodate converts glycerol into glyceraldehyde, which forms a yellow colored complex with acetyl acetone. The intensity of the colored complex is measured at 410 nm. (Violet filter).

Reagents:

1) Alumina: (active grade: 1, for chromatography): It is washed with distilled water & dried in an oven overnight at 100^{o}C.
2) Isopropanol: AR, grade.
3) Alcoholic KOH: It is prepared by dissolving 50 g of potassium hydroxide in 600 ml of distilled water and 400 ml of isopropanol.
4) Metaperiodate: It is prepared by dissolving 77 g of ammonium acetate & 650 mg of sodium metaperiodate in 940 ml of distilled water and 60 ml of glacial acetic acid.
5) Acetyl-acetone: It is prepared by mixing 7.5 ml of acetyl acetone and 200 ml of isopropanol in 800 ml of distilled water.
6) Triglyceride standard: 100 mg/dl (It is prepared by dissolving tripalmitine (or triolein) in chloroform.

Stability of the reagents

Reagents 1, 2, 3, 4 & 5 are stable at room temperature. Reagent 6 i.e. standard is stable at 2-8^{o}C.

Procedure:

1. Take two test-tubes labeled as test and standard.
2. Pour alumina approximately 0.5 gms in both the tubes.
3. Add 4.0 ml of isopropanol to both the tubes.

4. Add 0.1 ml serum in the tube labeled as test.
5. Add 0.1 ml of triglyceride standard 100 mg/dl in the tube labeled as standard.
6. Mix the contents of test thoroughly and also mix the contents of standard.
7. Keep exactly for 15 minutes at room temperature (25^{o}C$\pm$5^{o}C) with intermittent mixing.
8. Transfer the content of test and standard to respective centrifuge tubes and centrifuge at 3000 RPM for 10 minutes. Now pipette in the tubes labeled as follows :

	Test	Std.	Blank
Filtrate (Test), ml	2.0	-	-
Filtrate (Std), ml	-	2.0	-
Isopropanol, ml	-	-	2.0
Alcoholic KOH, ml	0.6	0.6	0.6
Mix thoroughly and keep at 60-75^{o}C for 15 minutes.			
Metaperiodate, ml	1.5	1.5	1.5
Mix thoroughly and keep at room temperature for 5 minutes.			
Acetyl acetone, ml	1.5	1.5	1.5

Mix, thoroughly and keep at 70^{o}C for 15 minutes. Cool the tubes and read intensities of test and standard against blank at 420 nm (violet filter)

Calculations:

O.D. Test

$$\text{Serum triglycerides, mg/dl} = \frac{\text{O.D.}}{\text{Std.}} \times 100$$

Note:

1. Store triglyceride Std. (reagent no. 6) tightly closed at 2-8°C.
2. All glassware after washing should be rinsed finally in distilled water and dried at 100°C.

Procedure limitations:

The method is linear up to 300 mg/dl. For values higher than 300 mg/dl, dilute the specimen in saline and repeat the test. Consider appropriate dilution factor for the calculation of final result.

Enzymatic determination of serum triglycerides:

Early clinical methods for determining triglycerides involved chemical hydrolysis of a solvent extract of the serum lipids. These methods were difficult, slow, and provided numerous opportunities for error. They were not readily automatable.

Chemical principle of test

$$\text{Triglycerides} + H_2O \xrightarrow[\text{Lipase}]{\text{Lipoprotein}} \text{Glycerol} + \text{fatty acids.}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol kinase}} \text{Glycerol} - 3 - \text{phosphate} + \text{ADP}$$

$$\text{Glycerol–3-phosphate} + O_2 \xrightarrow{\text{Glycerol phosphate oxidase}} \text{Dihydroxy acetone phosphate} + H_2O_2$$

$$H_2O_2 + \text{4-amino-phenazone} + \text{p-chlorophenol} \xrightarrow{\text{Peroxidase}} \text{Colored complex}$$

it is measured at 520 nm (green filter).

Technical contents: Using kit of serum triglycerides.

Method of teaching: Demonstration and its measurement of serum triglycerides by kit and taking readings. Using a colorimeter or semi auto analyzer.

Evaluation: Giving exercise of serum triglycerides.

Chapter 9: Estimation of Serum Cholesterol (Total)

Object: To estimate serum cholesterol.

Introduction:

Cholesterol is a fat-like substance that is found in all body cells. The liver makes all of the cholesterol the body needs to form cell membranes and to make certain hormones.

The determination of serum cholesterol is one of the important tools in the diagnosis and classification of lipemia. High blood cholesterol is one of the major risk factors for heart disease.

Sackett's Method:

Principle: Proteins are precipitated by and cholesterol extracted in an alcohol ether mixture. The extract is evaporated and the residue dissolved in chloroform. By Liebermann-Burchard reaction, a green color is developed and measured colorimetric ally.

Reagents

(i) Alcohol-ether mixture – Ethyl alcohol (95%) and ether are mixed in a ratio of 3:1.

(ii) Chloroform-This should be of a high purity and absolutely anhydrous.

(iii) Acetic anhydride-sulphuric acid mixture-Acetic anhydride and conc. sulphuric acid are mixed in a ratio of 20:1 just before use.

(iv) Stock standard cholesterol solution 200 mg of chemically pure cholesterol is dissolved in and diluted to 100 ml with chloroform.

(v) Working standard cholesterol solution – 1 ml of stock standard cholesterol solution is diluted to 25 ml with chloroform. 5 ml of this solution contains 0.4 mg of cholesterol.

Procedure: Pipette 12 ml of alcohol-ether mixture in a dry centrifuge tube. Add 0.2 ml of serum slowly. Cork the tube and shake vigorously for one minute. Keep the tube in a horizontal position for half an hour. Centrifuge it at 1,500 r.p.m. for 5 minutes. Pour off the supernatant fluid completely in a small breaker, and evaporate it on a steam-bath or a hot plate. Make up to 5 ml with chloroform. Label the tube 'Unknown'.

Label two other test tubes 'Standard' and 'Break', and pipette respectively 5 ml of working standard cholesterol solution and 5 ml of chloroform the three tubes. Mix and keep in dark for 15 min.

Adjust the wavelength of the photometer to 680 nm or put in a red filter. Set the photometer to 100% transmittance (or zero absorbance) with 'Blank' and read 'Unknown' and 'standard'.

Calculations

$$\text{Serum Cholesterol (mg/100ml)} = \frac{Au}{As} \times \frac{0.4}{0.2} \times 100$$

$$= \frac{Au}{As} \times 200$$

Interpretation

Serum cholesterol varies from 150-240 mg/100 ml in healthy young adults. The level rises with age and may go up to 300 mg/100 ml in the elderly.

An increase in serum cholesterol (hypercholesterolemia) is found in diabetes mellitus, nephritic syndrome, obstructive jaundice, hypothyroidism, and

xanthomatosis and during ether anesthesia. An idiopathic hypercholesterolemia of unknown etiology occurs in some families. Hypercholesterolemia, from any cause, predisposes to atherosclerosis.

A decrease in serum cholesterol (hypocholesterolemia) is found in hyperthyroidism, hepatocellular damage, anemia (except hemorrhagic), acute infections, wasting disease, intestinal obstruction and terminal states of a variety of disease.

Recently, an enzymatic method based on the action of cholesterol oxidase on cholesterol has also been developed. Some commercial kits based on the enzymatic method are available in the market, and should be used according to the instructions of the manufacturers.

Determination of cholesterol by enzymatic method (Application on autonalysers)

Principle:

Cholesterol esters are hydrolyzed by cholesterol ester hydrolase to free cholesterol & fatty acids. The free cholesterol produced and pre-existing one are oxidized by cholesterol oxidase to Cholestenone-4-en-3-one and hydrogen peroxide. Peroxidase acts on hydrogen peroxide and liberated oxygen reacts with the chromogen (4-amino phenazone/phenol) to form a red colored compound which is read at 510 nm (505-530 nm).

Technical contents: Using kit of serum cholesterol.

Method of teaching: Demonstration and its measurement of serum cholesterol by kit and taking readings. Using a colorimeter or semi auto analyzer.

Evaluation: Giving exercise of serum cholesterol.

Chapter 10: Estimation of Serum HDL cholesterol

Object: To estimation serum HDL cholesterol Introduction:

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

Normal range Men: 30-60 mg/dl. Women: 40-70 mg/dl.

Method:

Colorimetric (Watson)

Precipitating reagents:

1) Phosphotungstie acid reagent (P.T.A.): It is prepared by dissolving 2.25 g. of phosphotungstie acid in 8.0 ml of IN sodium hydroxide and 42.0 ml of distilled water.
2) Magnesium chloride reagent: 20.34 g of magnesium chloride in distilled water. It is diluted to 50 ml.

Procedure:

Pipette in the centrifuge tubes labeled as follows:

	Test
1) Serum, ml	0.5
2) P.T.A. reagent, ml	0.05
3) $MgCl_2$ reagent, ml	0.02

Mix well, centrifuge at 3000 R.P.M. for 20 minutes separate the supernatant by using a pipette. The clear supernatant is treated in the similar way as for method for cholesterol and the absorbance is measured at 500 nm.

Calculations:

$$\text{HDL Cholesterol, mg/dl} = \frac{\text{O.D. Test}}{\text{O.D. Std.}} \times \text{Serum} \times 114$$

Additional Information:

Heparin and manganese chloride are also used as precipitating reagents for HDL-Cholesterol determination.

Technical contents: Using kit of serum HDL cholesterol.

Method of teaching: Demonstration and its measurement of serum HDL cholesterol by kit and taking readings. Using a colorimeter or semi auto analyzer.

Evaluation: Giving exercise of serum HDL cholesterol.

Chapter 11: Estimation of Serum Alkaline Phosphatase

(King & King Method)

Object: To estimate serum alkaline phosphatase.

Introduction:

Alkaline phosphatase is present in practically all tissues of the body. It occurs at high levels in the intestinal epithelium, kidney tubules, bone (osteoblasts), liver and placenta. The relative contributions of bone and liver isoenzymes to the total activity are markedly age-dependent. There is also a significant difference in levels of serum alkaline phosphatase between different sexes of same ages.

Principle: The serum is incubated with the buffer substrate under optimum, conditions of temperature and pH to release phenol. This reacts with 4animoantipyrine in alkaline medium to give a red colored compound which is estimated at 520nm against a reagent blank. Color development is rapid and is stable for at least an hour sodium hydroxide is added immediately after incubation to raise the pH and stop the reaction. Potassium ferricyanide is the oxidizing agent. Sodium Bicarbonate provides the alkaline medium.

Reagents:

1. Substrate – (Disodium phenyl phosphate – 100 mmol/L): Dissolve 2.18g (or 2.541g of the dehydrate) in water and make the volume to a liter. Bring quickly to boil; cool; add 4ml of chloroform and keep in the refrigerator.
2. Buffer (Sodium carbonate-sodium bicarbonate, 100 mmol/L): Dissolve 6.36g anhydrous sodium carbonate and 3.36 g of sodium bicarbonate in water and make the volume to a liter.
3. Buffer substrate for use-Mix equal volumes of Reagent 1 and Reagent 2. This has a pH of 10.

4. Stock standard phenol solution (1 g/L) – Dissolve 1 g of pure crystalline phenol in 100 mmol/L of HCL (8.4ml in 1 liter of d. water) and make to a liter with the acid. Keep in a brown bottle at 4°C.
5. Working standard phenol solution (1mg/100ml) Dilute the stock standard – 1 to 100.
6. Sodium hydroxide solution – 500 mmol/L (20 g/L)
7. Sodium Bicarbonate solution – 500 mmol/L (42 g/L)
8. 4-Aminoantipyrine – 6 g/L. in water. Store in a brown bottle.
9. Potassium Ferricyanide – 24 g/L in d. water. Store in a brown bottle.

Procedure: Mark test tubes and proceed as follows:

Reagent (ml)	**Blank (ml)**	**Test (ml)**	**S_1 (ml)**	**S_2 (ml)**	**S_3 (ml)**	**S_4 (ml)**
1. Buffer-Substrate	2.0	2.0	1.6	1.1	0.6	0.1
2. Working Standard	-	-	0.5	1.0	1.5	2.0
3. D. Water	0.1	-	-	-	-	-
4. Serum	-	0.1	-	-	-	-
Incubate at 37°C for 15 min.						
5. NaOH	0.8	0.8	0.8	0.8	0.8	0.8
6. $NaHCO_2$	1.2	1.2	1.2	1.2	1.2	1.2
7. 4-Aminoantipyrin	1.0	1.0	1.0	1.0	1.0	1.0
8. Potassium Ferricyanide	1.0	1.0	1.0	1.0	1.0	1.0

Mix thoroughly and read at 520 nm.

Calculation:

Alkaline phosphatase is expressed in king and Armstrong units (KA Units); King and Armstrong unit corresponds to the liberation of 1 mg of phenol by 100 ml of serum under optimum conditions.

$$\text{S. Alk. Phosphatase (KA Units/dL)} = \frac{\text{O.D. Test}}{\text{O.D. Std.}} \times \frac{\text{Amount of Std.}}{0.1} \times 100$$

Interpretations:

The normal range of serum alkaline phosphatase activity is 3-13 KA units in adults and is up to about two and a half times greater in children particularly during periods of active growth.

1. Increase in levels of serum alkaline phosphatase:-

   Physiological:-

   i. Children – during periods of bone growth.

   ii. Puberty

   iii. Pregnancy – third trimester-due to the isoenzyme of placental origin.

Pathological:

(A) Bone Diseases: - Activity is increased when osteoblasts are more actively laying down osteoid.

   i. Rickets – marked increase up to 100 KA units is seen. Levels fall upon treatment with Vitamin D.

   ii. Osteomalacia – there is an increase but not as marked as in case of rickets.

iii. Paget's disease (Osteitis deforming) - The highest levels of serum alkaline phosphatase activity are encountered. Values range from 10-25 times the upper limit of normal.

iv. Osteogenic Sarcoma

v. Secondary deposits in bone- particularly in the case of carcinoma of the prostate. Small increases may sometimes be seen in breast cancer and secondary from thyroid, pancreas and stomach.

vi. Healing of bone fractures = Transient elevation seen.

(B) Liver Disease: - The response of the liver to any form of biliary obstruction is t) syntheses more alkaline phosphatase. The main sites of new enzyme synthesis are the hepatocytes.

Technical contents: Using kit of serum Alkaline Phosphatase.

Method of teaching: Demonstration and its measurement of serum Alkaline Phosphatase by kit and taking readings. Using a colorimeter or semi auto analyzer.

Evaluation: Giving exercise of serum Alkaline Phosphatase.

Chapter 12: Estimation of Aminotransferases (Transaminases)

Object: - To estimate aminotransferases.

Aspartate Aminotransferase (AST) SGOT

Alanine Aminotransferase (ALT) SGPT

Introduction:

The aminotransferases are a group of enzymes that catalyze the inter conversions of the amino acids and α-Keto acids by transfer of amino groups. Distinct isoenzymes of AST are present in the cytoplasm and mitochondria of cells.

Method for the estimation of AST and ALT:

Colorimetric Methods:

Reitman & Frankel (1957), Tietz (1970), Bergmeyer & Brent (1974 b)

Modified Reitman & Frankel Method:

Principle:-

The amount of oxaloacetate or pyruvate produced by transamination is reacted with 2, 4 dinitrophenyl hydrazine (DNPH) to form a brown colored hydrazone, the color of which in alkaline solution is read at 520nm.

Reagents:

1. Buffer substrate – For both enzymes, 100 mmol/L phosphate buffer and 2 mmol/L 2-oxoglutarate with 100 mmol/L L-aspartate for AST and 200 mmol/L-DL alanine for ALT.
    a. For AST-add 15.7g L-aspartate monosodium salt or 13.2g L-aspartic acid.

b. For ALT-add 17.8g of DL-alanine.

Adjust pH to 7.4 with NaOH and make up the volume to 1 Liter with distilled water.

2. 2, 4 Dinitrophenylhdrazine (DNPH)-1 mmol (200 mg)/L in lmol/L HCL.
3. Sodium hydroxide solution 400 mmol (16g)/L.
4. Pyruvate solution – 2 mmol/L (22mg of sodium pyruvate in 100 ml of distilled water).

Procedure: Mark test tubes and proceed as follows:

	Blank	**Test**	**S_1**	**S_2**	**S_3**	**S_4**
Buffer-Substrate	0.5ml	0.5ml	0.9ml	0.8ml	0.7ml	0.6ml
D. Water	0.5ml	0.5ml	0.2ml	0.2ml	0.2ml	0.2ml
Serum	0.2ml	0.2ml	-	-	-	-
Pyruvate Std.	-	-	0.1ml	0.2ml	0.3ml	0.4ml

Incubate at 37^{o}C for AST-60 mins and ALT – 30 mins.

Add 0.5ml DHPH to each tube. Mix and stand at room temperature.

20 mins and add NaOH 0.5ml each tube.

Read at 520nm after 5 minutes.

	(Karmen Units)	
Conc of pyruvate	AST	ALT
S_1	24	25
S_2	61	57
S_3	114	97
S_4	190	150
1 Karmen unit=0.483 I.U.		
	AST (IU)	ALT (IU)
S_1	11.5	13.4
S_2	29.3	27.4
S_3	54.7	46.6
S_4	91.2	72.0

Interpretation:

The normal range of AST = 7-21 IU

ALT = 6-20 IU.

Transaminases are widely distributed in human tissues. Both AST and ALT are normally present in human plasma, bile cerebrospinal fluid and saliva.

Elevation in the serum levels of AST & ALT:

1. Liver disease: associated with hepatic necrosis, viral hepatitis elevated levels of AST and ALT are found even before the clinical

signs and symptoms appear. 20-25 fold elevation are encountered. Peak values are seen between the 7^{th} and 12^{th} day and values return to normal levels by 3^{rd} to 5^{th} week. AST characteristically is us high or

Higher than AST and the ALT/AST (De Rites) ration which normally is $<_1$ approaches or becomes $>_1$.

2. Extrahepatic cholestasis-moderate increase in the levels of AST and ALT activities is seen.
3. Cirrhosis-Levels vary with the status of the cirrhosis. AST activity is higher than ALT activity.
4. Primary metastatic carcinoma of the liver.

ALT is the more liver specific enzyme although both AST and ALT are raised in liver diseases.

II Myocardial infarction-Elevated AST level begins 3-8 hrs. After the onset of the attack and returns to normal within 3-6 days peak is seen 24 hrs. after the onset. ALT levels are within normal limits or only marginally increased. ALT increases in liver damage secondary to heart disease.

AST raised levels are seen in

- Dermatomyositis
- Pulmonary emboli
- Acute pancreatitis
- Crushed muscle injuries.
- Gangrene
- Hemolytic diseases

Others-Both AST and ALT levels may be elevated following

- intake of alcohol

Delirium tremens and after administration of drugs such as opiates, salicylates or ampicillin.

Technical contents: Using kit of serum SGOT and SGPT.

Method of teaching: Demonstration and its measurement of serum SGOT and SGPT by kit and taking readings. Using a colorimeter or semi auto analyzer.

Evaluation: Giving exercise of serum SGOT and SGPT.

Chapter 13: Serum Amylase

Object: To estimate serum amylase.

Method: Caraway

Introduction:

Amylase is a hydrolytic enzyme which hydrolyses starch into maltose. If present in saliva and pancreatic juice where it is secreted by parotid glands and pancreas respectively. Small amounts of leak into circulation due to where and tear of cells in these glands. The circulating enzyme is excreted by the kidneys into urine. Therefore, only a small amount of amylase in present in serum normally.

Principle:

Serum is incubated with starch substrate. The amylase in the serum hydrolyses the starch to simpler units with a resulting increase in reducing groups. In the method presented here iodine is added which reacts with the starch molecules not hydrolyzed by the amylase. The iodine-starch complex is blue in color and is measured in the spectrophotometer. The degree of loss in color is proportional to the amount of starch hydrolyzed and hence to the activity of the amylase in the serum. A substrate control is carried though the procedure to give a reference value for the amount of starch substrate present before hydrolysis.

Procedure

1. Pipette 5 ml of substrate into two 50 ml volumetric flasks.
2. Place the 'test' flask into a 34^{o}C water bath for 5 minute to warm the contents.

3. Using a pipette that deviloers between two marks add 0.1 ml of serum to the 'test' flask and mix. Do not use blow out pipettes as the smallest amount of saliva can give a large error?
4. Time the addition of serum using a stop watch.
5. After exactly 7.5 minutes add 5 ml or working iodine solution, mix and immediately remove from the water bath.
6. Similarly add 5 ml of the working iodine solution to the flask containing the 'substrate control', which has not been incubated.
7. Dilute the contents of both flasks to the 50 ml mark with distilled water and mix the flasks well.
8. Read the absorbance of both against water using the large (19 mm) cuvettes at 660 nm.

Calculation

$$\frac{\text{Absorbance of substrate control} - \text{absorbance of test} \times 800}{\text{Absorbance of control}}$$

= units of amylase activity per 100 ml of serum.

In the result is greater than 400 units per 100 ml, repeat the test using 0.1 ml of a 1 in 5 dilution of the serum or 0.02 ml of undiluted serum.

An amylase units is defined as the activity that catalysis the conversion of 10 mg of starch substrate to non-iodine reacting product in 30 minutes under the condition of the assay.

(a) Since the test is incubated for 7.5 minutes multiply by '4' to estimate for 30 minutes.

(b) Since the 5 ml of starch substrate solution added only contain 2 mg of starch divide by '5' to estimate the activity that would be indicated if 10 mg of starch were present. The activity would be less if there was more substrate to work upon, thus the need to divide.

(c) Only 0.1 ml or serum is used in the assay but the activity is reported per 100 ml. thus 0.1 must be multiplied by 1000 to give 100

I.e. 4/5 x 1000 = 800

Precautions and sources of error.

1. **SALIVA.**

Saliva has a very high content of amylase. Even the slightest contamination of the test with saliva will give large errors. As a precaution special care must be taken during pipetting. Never use blow out pipettes. If possible use automatic pipettes or dispensers or cotton plugs in the pipettes. When the same sample is for other test that for amylase must be taken first since pipetting for the other tests could give salivary contamination.

2. **SUBSTRATE**

Unless the starch substrate is carefully prepared and stored, mold of fungus may glow. If the temperature is too low the starch may come out of 'solution' giving cloudiness. Contamination with saliva is a common cause of substrate unsuitability. Any of these defects show up by variation in the 'substrate control' reading.

3. **E'ZYMES**

The precautions which apply to enzyme assays in general with regard to time, pH, temperature, substrate concentration etc. apply to this assay for amylase activity.

4. PRECIPITATE

The final blue color is really due to a fine suspension of blue particles in water rather than a true solution. Care must be taken upon dilution to 50 ml to mix well to prevent a precipitate forming, which would distort its absorption from closely obeying beer's Law, as it can under optimal conditions.

5. URI'E

Amylase in urine can be measured exactly the same as in serum, though a 24 hr. sample would be best, a random sample may also be used.

6. OTHER BODY FLUIDS

Peritoneal and duodenal fluids are sometimes analyzed for amylase. As the content is high. These fluids should be diluted 1 in 1000 by adding 0.1 ml of fluid to 9.9 of distilled water. 0.1 ml of this dilution is used for the test as described above.

Clinical interpretation

Amylase is normally secreted by the pancreas into the pancreatic juice which enters the intestine. Amylase is involved in the digestion of the polysaccharides of the diet.

If pancreatitis the inflammation of the tissue results in the amylase seeping out into the blood. Thus high levels of serum amylase indicate acute pancreatitis. The amylase molecule is a relatively small protein of low molecular weight, and this allows it to be filtered by the kidney. As a result the amylase is rapidly removed from the serum and may return to normal within 24 hours of the onset of acute pancreatitis.

Urinary amylase estimation is carried out to try and 'catch' the high levels after they have left the blood.

Serum amylase is the main basis on which severe abdominal pain can be diagnosed as pancreatitis. It is important that pancreatitis be diagnosed before surgery is attempted.

A patient with pancreatitis would suffer sever shock if surgery was carried out with fatal or near fatal consequences. Amylase is usually requested 'stat' as the decision to operate or not cannot be made until the result is known.

Chronic pancreatitis will show normal or slightly raised serum amylase levels. Mumps or other diseases of the salivary glands also cause high levels. Ulcers, intestinal or pancreatic duct obstruction will similarly cause the amylase to spill over into the blood giving raised similarly cause the amylase to spill over into the blood giving raised levels. None of these conditions give levels as high as these usually associated with acute pancreatitis.

Chapter 14: Estimation of Blood Urea

Kidney function tests: Since the kidneys perform a multitude of functions, a single test cannot give information about the entire range of renal functions. A group of tests is required to evaluate the different renal functions. Abnormal results may sometimes be obtained due to a temporary renal dysfunction. Hence the test should be performed repeatedly and interpreted on the basis of a series of results. Moreover, the results of renal function tests May some-times be affected by extra-renal factors. Therefore, the results must always be interpreted in conjunction with the clinical picture.

A large number of tests have been introduced during the past decades to assess the renal functions. Only a few of them have stood the test of time.

The more important and commonly employed tests will be discussed below.

1) Blood urea
2) Serum creatinine

Object: To estimate blood urea.

Introduction:

Urea is main end product of protein catabolism. It is formed in the liver and is excreted through urine. Urea represents about 45-50% of the non-protein nitrogen of blood and 80-90% of the total urinary nitrogen excretion. The urea concentration in the glomerular filtrate is same as that in plasma. Tubular reabsorption of urea varies inversely with the rate of urine flow and hence is not a useful measure of GFR. Blood urea nitrogen varies directly with protein intake and inversely with the rate of excretion of urea.

Estimation: Various methods are use:

(i) **Enzymatic using urease :**

a) Nessler's Method: Urea is converted to ammonia by the enzyme urease. Ammonia is made to react with Nessler's reagent (Potassium mercuric iodide) giving rise to a brown colored compound which is read at 450nm the enzyme acts optimally at 55°C pH 7.0 to 8.0 and is inhibited by ammonia and fluoride.

Disadvantages are turbidity, color instability and nonlinear, calibration beside susceptibility to contamination with NH_3 from the laboratory and endogenous ammonia in the specimen.

b) Berthelot Reaction :

In this NH_3 reacts with phenol in presence of hypochlorite to form an indophenol which gives a blue colored compound in alkali. Nitroprusside acts as a catalyst increasing the rate of reaction, intensity of color obtained and its reproducibility.

c) In the urease/glutamate dehydrogenase method, glutamate production from ammonia and 2-oxoglutarate is measured by the absorbance change at 340nm. Owing to concomitant conversion of $NADH^+$ to NAD^+. Each molecule of urea hydrolyzed causes the production of two molecules of NAD^+.

(ii) **Kinetic Method:** GLDH method

Urea is hydrolyzed in presence of urease to produce ammonia and CO2 the ammonia produced combines with alfa oxoglutarate and NADH in presence of GLDH to yield glutamate and NAD^+

The decrease in extinction due to NADH in unit time is proportional to the urea concentration.

Normal range of serum urea = 15 to 45 mg/dl

(iii) **Colorimetric Method :** Diacetyl Monoxime Method

Principal: Urea reacts with diacetyl monoxime in acidic conditions at nearly 100^{o}C to give a red colored product which is measured colorimetric ally at 520nm. Thiosemicarbazide and ferric ions are added to catalyze the reaction and increase the intensity of color. This method is linear only up to 300mg% urea. For higher values if expected, the blood sample should be diluted.

Reagents

1) **Reagent A:** Dissolve 5g of ferric chloride in 20ml of water. Transfer this to a graduated cylinder and add 100ml of orthophosphoric acid (85%) slowly with stirring. Make up the volume to 250ml with water. Keep in brown bottle at 4^{o}C.
2) **Reagent B:** Add 200 ml conc, H_2SO_4 to 800 ml water in 2L flask slowly with stirring and cooling.
3) **Acid Reagent:** Add 0.5 ml of reagent A to 1 L of reagent B. keep in brown bottle at 4^{o}C.
4) **Reagent C:** Diacetyl monoxime 20g/L of water. Filter and keep in brown bottle at 4^{o}C.
5) **Reagent D:** Thiosemicarbazide 5g/L of water.
6) **Color Reagent:** Mix 67 ml of C with 67 ml of D and make up the volume to 1000 ml with D.H_2O keep in brown bottle at 4^{o}C.
7) **Stock urea standard:** 100mg/100 ml water.
8) **Working urea standard:** Dilute 1 ml stock to 100ml with DH_2O so conc. is 1 mg/100ml.

Procedure: 0.1 ml if serum/plasma is diluted to 10 ml. set up the test tubes as follows:

	B	T	S_1	S_2	S_3	S_4	S_5	
Serum (ml) (dil 1:100)	-	1.0	-	-	-	-	-	-
Std (ml)	-	-	0.2	0.4	0.6	0.8	1.0	
D. Water (ml)	2	1.0	1.8	1.6	1.4	1.2	1.0	
Color Reagent (ml)	2.0	2.0	2.0	2.0	2.0	2.0	2.0	
Acid reagent (ml)	2.0	2.0	2.0	2.0	2.0	2.0	2.0	

Mix all the tube thoroughly. Keep in boiling water bath for exactly 30 mins. Then cool and read absorbance at 520nm.

Calculation:

$$\text{Blood urea (mg\%)} = \frac{\text{O.D. Test}}{\text{O.D. Std.}} \times \frac{\text{Amount of Std.}}{\text{Vol. of bld.}} \times 100$$

A standard curve can be plotted end value of unknown sample calculated from it.

Interpretation:

Normal blood urea in adults is 15-50 mg% when expressed as blood urea nitrogen (BON) it is 7-25 mg%. It is somewhat higher in men than women and there is gradual rise with age. The urea concentration varies with the amount of protein in the diet.

Serum urea is lower in pregnancy probably due to hoemodilution, usually between 15-20mg/100ml.

Increase in urea is significant as a measure of renal function. The causes may be

Pre Renal: When there is reduced plasma volume it leads to decreased renal blood flow and hence GFR leading to urea retention. Seen in

Reduced plasma volume:-

- Acute intestinal obstruction – prolonged vomiting.
- Severe diarrhea.
- Pyloric stenosis.
- Ulcerative colitis.
- Diabetic Ketoacidosis.
- Shocks, severe burns and hemorrhage.

Increased protein catabolism:-

- Fevers
- Thyrotoxicosis
- Cardiac failure

Renal Disease: Blood urea is increased in all forms of renal diseases like;

- Acute glomerulonephritis.
- Renal failure
- Malignant hypertension
- Chronic pyelonephritis
- Congenital cystic kidneys

Post renal: Due to obstruction to flow of urine there is retention and so reduction in effective filtration pressure at the glomeruli - Enlargement of prostrate.

- Stones in urinary tract.
- Urethral strictures.

Methodology of its teaching: - demonstration of its estimation using kits.

Evaluation of the session: - Asking to demonstrate the test.

Chapter 15: Estimation of Serum Creatinine

Object: - To estimate serum creatinine.

Introduction:

Creatinine is a waste product formed in muscle by creatine metabolism. Creatine is synthesized in the liver which then passes into circulation where it is taken up by skeletal muscle for conversion to creatine phosphate which serves as storage form of energy in skeletal muscles. Creatine and creatine phosphate are spontaneously converted to creatinine at a rate of about 2% the total per day. This is related to muscle mass and body weight.

Creatinine formed is excreted in the urine. On a normal diet almost all creatinine in urine is endogenous. Its excretion is fairly constant from day to day and has been used to check the accuracy of 24 hours urine collection. It is independent of urine flow rate and its level in plasma is quite constant.

Estimation of serum creatinine by Jaffe's Alkaline Picrate Method Principle:

Creatinine in alkaline medium reacts with picric acid to form a red tautomer of creatinine picrate the intensity of which is measured at 520nm. The two chief disadvantages with Jaffe's reaction are:

- Lack of specificity: - Only 80% of the color developed is due to creatinine in serum. Other nonspecific chromogens that react with picric acid are proteins, ketone bodies, pyruvate, glucose, ascorbate etc.
- Sensitivity to certain variables like PH temperature etc.

Reagents:

1) Picric acid – 0.04M (9.16g/L)
2) Sodium hydroxide – 0.75N
3) Sodium tungstate – 10%
4) 2/3 N H_2 SO_4
5) Creatinine standard stock – 100mg%
6) Working standard – 3mg%

Procedure: - In a centrifuge tube, take 2ml of serum with 2 ml of distilled water. Precipitate proteins by adding 2ml sodium tungstate and 2ml of 2/3 N sulphuric acid drop with constant shaking. Stand for 10 minutes and filter.

Use protein free filtrate for test.

Make the test tubes as follows and add:

	B	**S_1**	**S_2**	**S_3**	**S_4**	**T**
Filtrate	-	-	-	-	-	3 ml
Standard 3mg%	-	0.5	1.0	2.0	3.0	-
D. Water	3 ml	2.5	2.0	1.0	-	-
NaOH	1 ml	1.0	1.0	1.0	1.0	1.0
Picric acid	1.0	1.0	1.0	1.0	1.0	1.0

Mix well Allow to stand for 15 min. and read absorbance at 520nm.

Calculation:

$$\text{Serum creatinine (mg\%)} = \frac{\text{O.D. Test}}{\text{O.D. Std.}} \times \frac{\text{Amount of Std.}}{\text{Vol. of Serum}} \times 100$$

Interpretation:

Normal serum creatinine levels are males: 0.7-1 4mg/100m l

Females: 0.4-1.2mg/100m l

In is higher in males since it is related to body size.

Increased serum levels are seen in renal failure and other renal diseases in a manner similar to urea. Creatinine, however, does not increase with age, dehydration and catabolic states (e.g. fever, sepsis, hemorrhage) to the same extent as urea. It is also not affected by diet.

But the Jaffe's reaction for measuring serum creatinine is not as sensitive and reliable as method for urea. It is interfered with by Ketone bodies and glucose and hence false high values may be obtained in diabetes ketoacidosis.

How serum creatinine is not significant. It is associated with muscle wasting diseases.

Technical Contents: Kit of creatinine.

Methodology of its teaching: - demonstration of its estimation using kits.

Evaluation of the session: - Asking to demonstrate the test.

Chapter 16: Estimation of Serum Uric Acid

Object: To estimate serum uric acid.

Introduction:

Uric Acid is the end product of purine metabolism in man formed by oxidation of Purine bases.

Caraway's Method of Estimation:

Principle: - Phosphotungstic acid in alkaline medium oxidizes uric acid to allantoin and itself gets reduced to tungsten blue which is estimated colorimetrically at 700mm.

Reagents:

(1) Sodium tungstate 10%.

(2) 2/3 N Sulphuric acid.

(3) Tungstic acid: Add 50ml of 10% sodium tungstate 50ml 2/3 N H_2SO_4 and a drop of phosphoric acid with mixing to 800ml water. Discard when cloudy. Store in brown bottle.

(4) Phosphotungstic acid: Stock-Dissolve 50g sodium tungstate in about 400ml of water. Add 40ml 85% phosphoric acid and reflux gently for 2 hours, cool, make volume to 500m. Store in brown bottle. Dilute 1 to 1 for use.

(5) Na_3CO_3 10%.

(6) Standard uric acid solution stock-100mg%.

(7) Working uric acid solution-1mg%.

Procedure:

In a centrifuge tube pipette 0.6ml serum and add 5.4ml. Tungstic acid while shaking. Centrifuge and process as follows.

	B	**T**	**S**	**S_2**	**S_3**
1. Standard uric acid (1mg%) ml.	-	-	1.0	2.0	3.0
2. Supernatant (ml)	-	3.0	-	-	-
3. D. Water (ml)	3.0	-	2.0	1.0	-
4. Na_3CO_3 (ml)	0.6	0.6	0.6	0.6	0.6
5. Phosphotungstate (ml)	0.6	0.6	0.6	0.6	0.6

Mix well stand at room temperature for 30 min. Read absorbance at 700 nm or using a red filter plot a standard curve between concentration of standard and absorbance and calculate the uric acid conc, in test.

Precautions:

a) Serum must be used for test as certain substances in RBC like glutathione can also reduce and give false high color.

b) Some uric acid estimation is carried down with the protein precipitate giving low results.

c) Lithium salts may be added to prevent, turbidity in the final colored solutions.

d) Sometimes cyanide is added to increase the color intensity.

Calculations:

$$\text{Serum Uric acid} = \frac{\text{O.D. Test}}{\text{O.D. Std.}} \times \frac{\text{Conc. of Std.}}{\text{Vol. of Serum}} \times 100$$

Interpretation:

The normal serum uric acid ranges from Adult male: 4.5-8.2mg/dL

Female : 3-6.5mg/dL

Children: 2.0-5.5mg/dL

In female level rises after menopause. The levels are higher in last trimester of pregnancy and in first year of life.

Hyperuricemia:

- Seen in gout. Estimation is important in diagnosis and management of the disease. In this urates are deposited in solid form in and about the joins causing arthritis. Levels are not related to severity of disease.
- Renal failure due to decreased excretion. Blood urea also raised.
- Conditions of increased turnover of cells as in leukemia, myeloproliferative diseases, pernicious anemia, chronic hemolytic anemia.
- Toxemia of pregnancy.
- Diabetes Mellitus.
- Starvation.
- Drugs like pyrazinamide, diuretics.

Hypouricaemia:

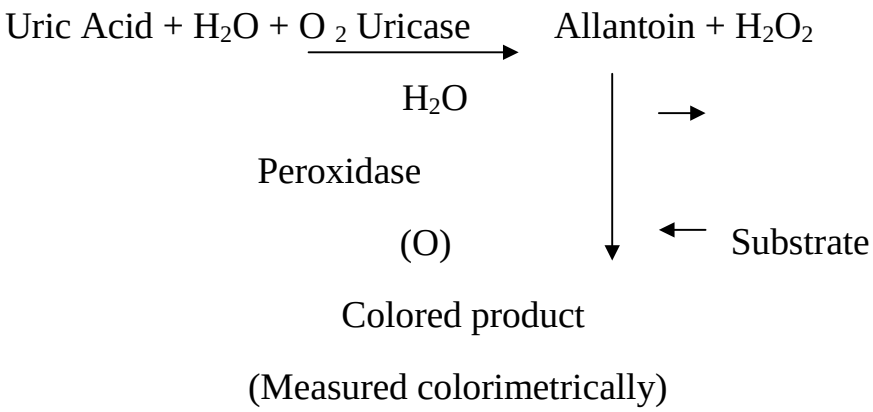
- Liver diseases (wherein maximum synthesis of uric acid occurs) like cirrhosis or Wilson's disease.
- Renal disease that decrease renal tubular resorption like Fanconi's Syndrome.
- Drugs-uricosuric in large does like salicylates, sulphinpyrazone.

Other method of estimation:

Enzymatic Method:

(1) The enzyme uricase is used to oxidize uric acid to allantoin. The amount of uric acid oxidized is obtained by observing the decrease in O.D. at 293nm after the action of uricase.

(2) An alternative approach using uricase is to measure colorimetrically the H_2O_2 formed in reaction using a peroxidase linked reaction.



Technical contents: Using kit of serum uric acid.

Method of teaching: Demonstration and its measurement of serum uric acid by kit and taking readings. Using a colorimeter or semi auto analyzer.

Evaluation: Giving exercise of serum uric acid.