Chapter 1

The Clinical Biochemistry Laboratory the use and the requirements of laboratory

Objective of the session

1. To make the students aware of the basic setup of laboratory, the procedure for sample collection, separation to the analysis.

Introduction :

Clinical Biochemistry tests comprise over one third of all hospital laboratory investigation. clinical biochemistry is that branch of laboratory medicine in which chemical and biochemical methods are applied to the study of disease while in theory this embraces all non-morphological studies, in practice it is usually, though not exclusively, confined to studies on blood and urine because of the relative ease in obtaining such specimens although analysis are made on other body fluids such as gastric aspirate and cerebrospinal fluid.

The use of Biochemical tests :-

Biochemical investigations are involved, to varying degrees, in every branch of clinical medicine.

- The results of biochemical tests may be of use in diagnosis and in the monitoring of treatment.
- Biochemical tests may also be of value in screening for disease or in assessing the prognosis once a diagnosis has been made (fig. 1)

- The biochemistry laboratory is often involved in research into the biochemical basis of disease and in clinical trials of new drugs.

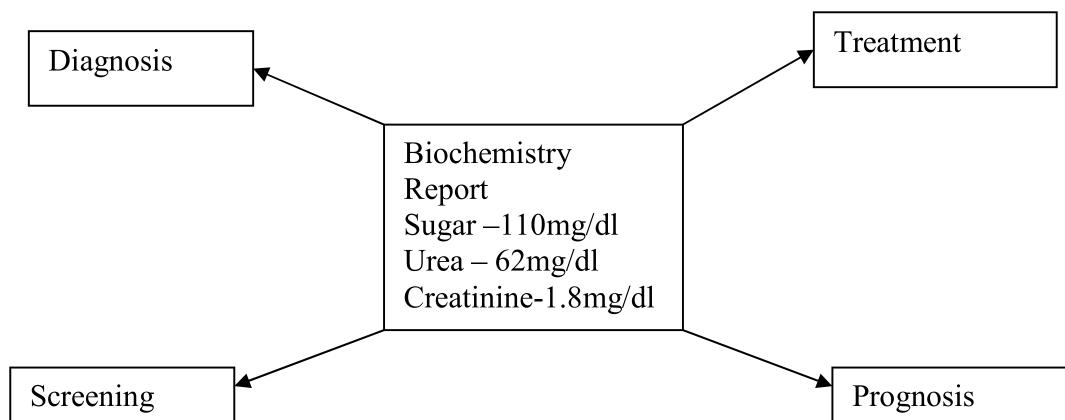


Fig. 1 How Biochemical Tests are used

The use of the laboratory :-

Every biochemistry analysis should attempts to answer a question which the clinician has posed about the patient obtaining the correct answer can often seem to be fraught with difficulty.

Basic steps for drawing a blood specimen

1. Preparation for blood collection.

   (A) The information given on the blood request form should be recorded on the specimen labels essential items include the following.

   a. Patients complete name and age.

   b. Identification number.

(B) The specimen containers should be labeled appropriated before the specimen collection.

2. Ascertaining whether the patient to fast such care is needed to ensure accurate results.
3. The technician must gain the patients confidence and assure him that, although the venipuncture will be slightly painful, it will be short duration.
4. Positioning the patient
   (a) The patient should be made to sit comfortably in a chair and should position his arm straight from the shoulder and it should not bent at the elbow.
   (b) If the patient wants to lie down, let the patient to lie comfortably on the back, the patient should extent the arm straight from the shoulder.

Requirement of Blood Collection

1. Collection tubes.
2. Sterilized syringes and needles.
3. Sprit or 70% ethanol.
4. Cotton.

Blood collection :-

- Compare the requisition form and labeling the tubes.
- Selecting Vein site.
- Applying the tourniquet.
- Cleaning the area.

- Inspecting the needles and syringes.
- Performing the venipuncture.

Separation of serum :-

1. Allow the blood to clot.
2. Loosen the clot slowly and centrifuge the supernatant fluid.
3. By using a pipette, separate the serum from blood cells and store it in a clean & day test tube.

Sampling errors

There are a number of potential errors which may contribute to the success or failure of the laboratory to provide the correct answers to the clinician's question. Some of these problems arise when a clinician first obtains specimens from the patient.

- *Blood sampling technique.* Difficulty in obtaining a blood specimen may lead to haemolysis with consequent release of potassium and other red cells constituents. results for these will be falsely elevated.
- *Prolonged stasis during venepuncture.* Plasma water diffuses into the interstitial space and the serum or plasma sample obtained will be concentrated. Proteins and protein-bound components of plasma such as calcium or thyroxine will be falsely elevated.
- *Insufficient specimen.* Each biochemical analysis requires a certain volume of specimen to enable the test to be carried out it may prove to the impossible for the laboratory to measure everything requested on a small volume specimen.

- *Errors in timing.* The biggest source of error in the measurement of any analyte in a 24-hour urine specimen is in the collection of an accurately timed volume of urine.
- *Incorrect specimen container.* For may analyses the blood must be collected into a container with anticoagulant and preservative. For example, samples for glucose should be collected into a special container containing fluoride which inhibites glycolysis; otherwise the time taken to deliver the sample to the laboratory can affect the result. If a sample is collected into the wrong container, it should never be decanted into another type of tube. for example, blood which has been exposed even briefly to EDTA (an anticoagulant used in sample containers for lipids) will have a markedly reduced calcium concentration, approaching zero.
- *Inappropriate sampling site.* Blood samples should not be taken 'down-stream' from an intravenous drip. It is not unheard of for the laboratory to receive a blood glucose request on a specimen taken from an intravenous drip. It is not unheard of for the laboratory to receive a blood glucose request on a specimen taken from the same arm into which 5% glucose is being infused. Usually the results are biochemically incredible but it is just possible that they may be acted upon, with disastrous consequences for the patient.
- *Incorrect specimen storage.* A blood sample stored overnight before being sent to the laboratory will show falsely.

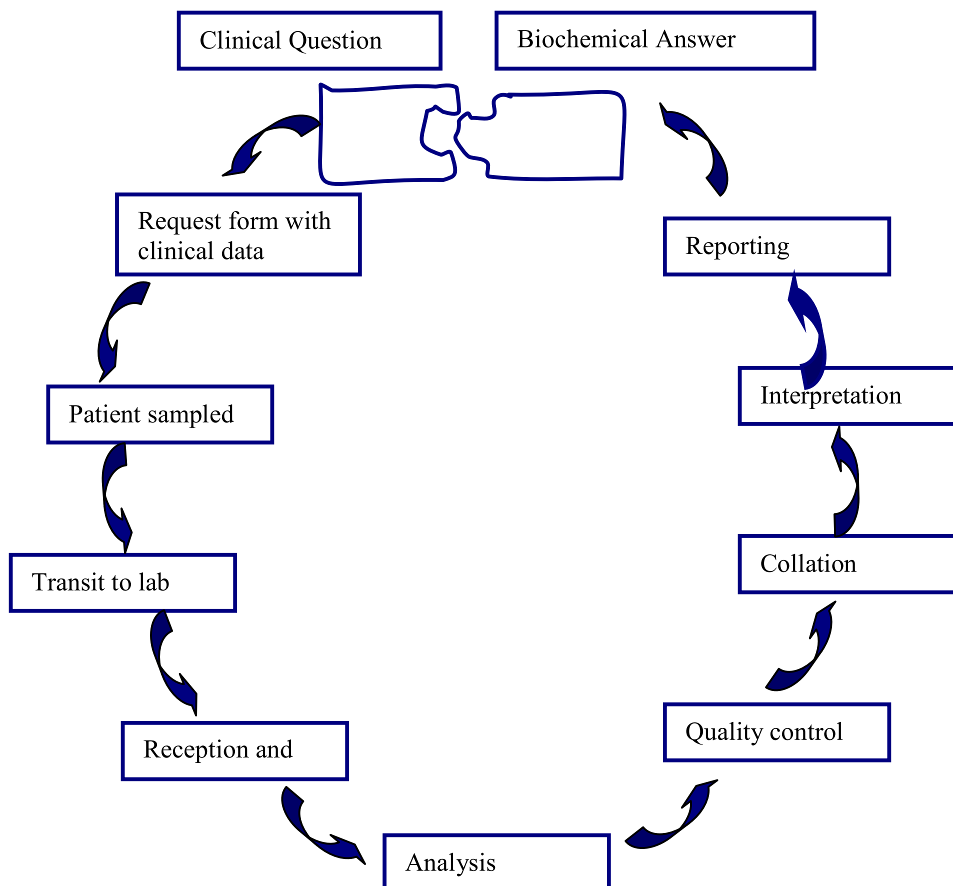


Fig. 2 Circuit diagram of clinical biochemistry process.

Analysing the specimen

Once the form and specimen arrive at the laboratory reception, they are matched with a unique identifying number or bar code. The average lab receives many thousands of requests and samples each day and it is important that all are clearly identified and never mixed up. Samples proceed through the laboratory as shown in figure 2. All analytical procedures are quality controlled and the laboratory strives for reliability.

Once the results are available they are collated and a report is issued. Cumulative reports allow the clinician to see at a glance how the most recent result (s) compare with those tests performed previously, providing an aid to the monitoring of treatment.

Methodology of its teaching :- lecture with few C.D. demonstration.

Evaluation of the session :- Asking queries regarding the lecture.

Chapter 2

The Interpretation Of Results

Objective of the session :-

1. **To teach the interpretation of results.**

The laboratory report

It can take considerable effort, and expense, to produce what may seen to be just numbers on pieces of paper, understanding what these numbers mean is of crucial importance if the correct diagnosis is to be made, or if the patients treatment is to be changed. Usually results of tests carried out in clinical chemistry are reported in units of concentration or of activity.

CONCENTRATION

Units of concentration contain both units of quantity and units of volume. The amount of substance present can be expressed in grams, equivalents, or moles or divisions of these ie. milligrams, milliequivalents millimoles etc. similarly the volume can be expressed in liters milliliters or deciliters (100 ml).

For many substances the usual units of concentration has been in mg per 100 ml. This form had replaced the earlier less defined form of mg% which should no longer be used as the use of % to mean per 100 ml here differs from the general use of (%) to more recently deciliter has been proposed as a term for 100 ml. Just as a milliliter is one thousand of a liter so a 100 ml. is one tenth of a liter (a deci-litre) so units have changed from mg % to mg/100 ml to mg (dl) without any numerical change.

Serum electrolytes are usually expressed in mill equivalent per liter and protein and albumin in grams per 100 ml.

"S. I. " UNITS

For many purpose e.g., calculation of osmolarity or electrolyte balance it would be convenient to have all results expressed in the same units, this would also help avoid confusion when results are compared from one laboratory to another or in the evaluation of results by medical staff are used to different units, for these reasons standard international units for reporting results have been recommended. As with many changes there has been resistance to the change over to the new units especially among the medical profession. But as the newer text books and reviews use these units. they are gaining international acceptance.

The S.I. (Standard International) units apply to clinical chemistry as follows.

1. Where the molecular weight of the substance being measured in known, the units of quantity should be the mole_submultiple of a mole. e.g., milimoles and Micromoles.
2. The units of volume should be the liter. Units of concentration. will there fore be millimoles per liter etc.
   e.g., sodium of .140 m eg/l in S.I. units is 140 m mol/l.
   glucose of 180 mg/100 ml in S.I. units is 10 m mol/l
3. When the molecular weight is not known, the for example for serum protein or albumin determinations the concentration should be expressed in grams per liter i.e. 7.0 g/100 ml becomes 70 g/l.

Units of activity

Usually activities are measured in clinical chemistry as an index of the concentration of certain enzymes, or enzymatic processes e.g. prothrombin activity.

Activity is a measure of the rate at which a process takes place. Enzymatic activity is usually estimated by measuring the rate at which a substrate is converted to a product. This activity is affected by many things e.g. temperature, time over which the activity is measured, incubation conditions etc. All of these must be defined when reporting the activity and so units or activity include fixed values for each variant. As the combination of the variations as large so many different such units are in use. International standardization of all these variables is almost impossible and as such the international unit of enzyme activity has not been widely accepted the units for each such test in the laboratory should be clear to all concerned.

Reporting results

There is a certain error in all results. Usually laboratory will claim 95 % confidence in its result i.e. plus or minus two standard deviation. Thus a blood sugar report of 100 mg/100 ml with a standard deviation of 1 mg per 100 ml would really be 100 $\pm$ 2 mg/100 ml. For simplicity the variation is not usually reported with the individual result. The standard deviation for the method should be indicated and the result given with the understanding that the variation is understood.

Significant figures

From mathematical calculations results may be obtained to many decimal places but these will not usually be significant. The final result cannot be accurate to a greater degree than the sum of the errors through the test allow e.g. error in pipetting sample. or standard error in reading spectrophotometer etc. When reporting a result then each figure given should have a meaning. A blood sugar is not a significant part of the result would not usually be reported as 100.2 mg/100 ml. As 0.2 mg is not a significant part of the result for most blood sugar methods. Thus result should be rounded off to the nearest significant figure. before reporting. What is or is not significant must be established for each test.

Use of zeros after a decimal point can give misleading information if they are not significant.

e.g. 7.0 g means between 6.95 and 7.05 g.

7 g means between 6.5 and 7.5 g.

Methodology of its teaching :- lecture with few C.D. demonstration.

Evaluation of the session :- Asking queries regarding the lecture.

Chapter 3

Quality Control

Objective of the session : To teach the external and internal quality control programme

Introduction :-

Variation in results

Biochemical measurements vary for two reasons. There is analytical variation and also biological variation.

Laboratory analytical performance

A number of terms describe biochemical results. these include:

- precision and accuracy
- Sensitivity and specificity

Concentration is always dependent on two factors: the amount of solute and the amount of solvent. The concentration of the sugar solution in the beaker can be increased from 1 spoon/beaker **(a)** to 2 spoons beaker by either decreasing the volume of solvent **(b)** or increasing the amount of solute **(c).**

- quality assurance
- reference ranges.

Precision and Accuracy

Precision is the reproducibility of an analytical method. Accuracy defines how close the measured value is to the actual value. A good analogy is that of the shooting target figure 3 shows the scatter of results which might be

obtained by someone with little skill, compared with that of someone with good precision where the results are closely grouped together. Even when the results are all close, they may not hit the centre of the target. Accuracy is therefore poor, as if the 'sights' are off. It is the objective in very biochemical method to have good precision and accuracy.

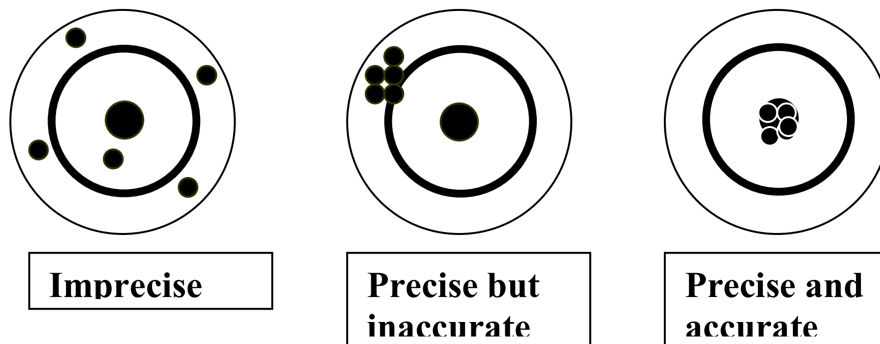


Fig. 3 Precision and accuracy

Sensitivity and specificity

Sensitivity of an assay in a measure of how little of the analyte the method can detect, As new methods are developed they may offer improved detection limits which may help in the discrimination between normal results and those in patients with the suspected disease. Specificity of a assay related to how good the assay is at discriminating between the requested analyte and potentially interfering substances.

Quality assurance

Every laboratory takes great pains to ensure that the methods in use continue to produce reliable results. laboratory staff monitor performance or assay using quality control samples to give reassurance that the method is

performing satisfactorily with the patients' specimens. These are internal quality controls which are analysed every day or every time an assay is run. The expected values are known and the actual results obtained are compared with previous values to monitor performance. In external quality assurance schemes, identical samples are distributed to laboratories; results are then compared.

in this way, the laboratory's own internal standards are themselves assessed.

Reference ranges

Analytical variation is generally less than that from biological variables. Biochemical test results are usually compared to a reference range considered to represent the normal healthy state (fig. 3) Most reference range are chosen arbitrarily to include 95% of the values found in healthy volunteers, and hence, by definition, 5% of the population will have a result out with the reference range. In practice there are no rigid limits demarcating the diseased population from the healthy; however, the further a result is from the limits of the range, the more likely it is to represent pathology. In some situations it is useful to define 'action limits', where appropriate intervention should be made in response to a biochemical result.

There is often a degree of overlap between the disease state and the 'normal value' (fig. 4) A patient with an abnormal result who is found not to have the disease is a *false positive.* A patient who has the disease but has a 'normal' result is a *false negative.*

Biological factors affecting the interpretation of results

The discrimination between normal and abnormal results is affected by various physiological factors which must be considered when interpreting any given result. These include:

- *Sex of the patient.* Reference ranges for some analytes such as serum creatinine are different for men and women.
- *Age of the patient.* There may be different reference range for neonates, children, adults and the elderly.
- *Effect of diet.* The sample may be inappropriate if taken when the patient is fasting or after a meal.
- *Time when sample was taken.* There may be variations during the day and night.
- *Stress and anxiety.* They may affect the analyte of interest.
- *Posture of the patient.* Redistribution of fluid may affect the result.
- *Effects of exercise.* Strenuous exercise can release enzymes from tissues.
- *Medical history.* Infection and/or tissue injury can affect biochemical values independently of the disease process being investigated.
- *Pregnancy.* The alters some reference ranges.
- *Menstrual cycle.* Hormone measurements will vary through the menstrual cycle.
- *Drug history.* Drugs may have specific effects on the plasma concentration of some analytes.

Others factors

When the numbers has been printed on the report from, they still have to be interpreted in the light of a host of variable. Analytical and biological variations have already been considered. Other factors relate to the patient. The clinician can refer to the patient or to the clinical notes, whereas the biochemist has only the information on the request from to consult. The cumulation of biochemistry results is often helpful in patient management.

Technical contents :- kits of calibrators, kits of normal and elevated quality control samples.

Methodology of its teaching :- lecture with few C.D. demonstration.

Evaluation of the session :- Asking queries regarding the lecture.

Chapter No. 4

Photometery

Objective:- To give the brief idea about the principles applied in several kinds of analytical measurements.

Introduction of the topic:-

Photometery is the most common analytical technique used in clinical biochemistry. The principle of photometery is base on the physical laws of radiant energy or light. In this method, the intensity absorbed transmitted or reflected, light is measured and related to the concentration of the test substance.

Photometeric principles are applied in several kinds of analytical measurements.

1. Measurement of absorbed or transmitted light:
   Colorimetery, spectrophotometery, atomic absorption, turbidometery.
2. Measurement of emitted light, Flame emission photometery : Flourometery.

Colorimetry:

Many methods for quantitative analysis of blood, urine and other biological materials are based upon the production of a coloured compounds in solution, the intensity of which is used as a measure of concentration.

Laws of Absorption of Light:

There are two common methods of expressing the amount of light absorbed by a solution.

1. By % transmittance.

2. By optical Density (O.D) Absorbancy (A) or Extinction (E) of the solution.

OPTICAL DENSITY-LOG T.%=Log 100/1

Lambert Beer's Law:

The Lambert Beer law governing these relationships states that light absorption is proportional to the number of molecules of absorbing material through which light passes. Absorbance, therefore changes with the thickness of the solution and with the concentration of absorbent in a manner characteristic for each absorbing material of a given wavelength.

The mathematical expression at a given wavelength is $\frac{I_E}{I_o}$ C-Kct

Where-

I_E = Intensity of emergent light.

I_o = Intensity of incident light.

K = A constant.

C = Concentration of coloured substance.

t = Thickness of the layer of solution.

e = Base of natural logrithim (2.718)

I_E/I_O is knows as transmittance.

By assuming that the cell which is used to measure absorbance, is of constant thickness, it is possible to simplify the mathematical expression for this law in logarithmic form. The mathematical expression at a given wavelength is absorbance / Opticaldensity / Extinetion [E]=Lo (I/T) = Log 100%T so that E=2-Log%. T.

Beer Lambert's law is applied for

a) Only monochromatic Light

b) There should be no changes in ionization, dissociation association or solvation of the solution with concentration.

When A (absorbance) is plotted against C (concentration) straight line passing through the origin should be obtained because absorbance is directly proportional to concentration. With the aid of a standard curve the concentration of an unknown solution can then be readily determined.

Parts of photo colorimeter

A) **Light Sources :-** This usually on tungsten lamp (420-760m) for U.V. range Hydrogen lamp is used.

B) **Monochromator/Filters :-** Complimentry filters should be used in order to increase the sensitivity of photometeric instrument.

Table :

Colour of solution	Filter used	Peak transmission range (nm)		
Bluesh Green	Red	580	-	680
Blue	Yellow	520	-	580
Purple	Green	490	-	520
Red	Blue-green	470	-	490
Yellow	Blue	430	-	470
Yellowish-green	Violet	400	-	430

In the spectrophotometers grits are used. These provide narrow spectrum of light. Grits have practically replaced by prism as they are inexpensive.

C) **Cuvettes :-** Cuvette holds the solution whose absorbance is to be measured. The cuvette must be optically-transparent, scrupulously clean deoid of any scratch and free form contamination. the optical

path in the cuvette is always one cm. Glass cuvettes are used in visible range colorimetery while quartz/silca cuvwettes are used in U.V. range.

Glvanometer :- This is used for measuring the output of the photosensitive element. In most of the instruments a sensitive galvanometer is used.

Prepration of solutions for measurement:

1. **Blank :-** This used to set the pnotometer to zero absorbance (A) or 100% transmittance (%T).
2. **Standard :-** These are solutions of known concentration which range within limits found in the specimen (normal and test).
3. An unknown solution.

Determination of absorpition maxima :

For conc/O.D. relation to be linear the wavelength chosen to measure the coloured solution should be that, at which, light is maximally absorbed. This is determined by using fixed concentration of coloured solution and measuring it at different wave lengths. Plot the graph taking O.D. on Y axis and wavelength on X axis & show results Graphically.

Verification of Beer-Lambert's Law :

To study Lambert Beer's law different concentration of dye, Bromophenol blue are taken. The optical density (O.D.) of these samples are measured in colorimeter. The corrected O.D. (after substracting the blank) readings are plotted on Y axis against the concentration of the dye on the X axis. It gives a liner graph. (i.e. straight line passing through origin) up to certain concentration.

Reagents: Bromophenol. Blue standard solution – 1.5mg%-

Procedure :-

Take 7 test tubes and number them B,S_1,S_2,S_3,S_4,S_5, and Test-

Tube No.	Vol of dye (in ml)	Distilled water (in ml)
1. Blank	0	5
2. S_1	1	4
3. S_2	2	3
4. S_3	3	2
5. S_4	4	1
6. S_5	5	0
7. Test	5ml (unknown)	0

Now mix tubes & take O.D. using a suitable filter or wavelength as determined by absorption maxima experiment. Plot a graph of O.D. against concentration of dye. From the graph determine the amount of dye present in unknown solution & express as mg% of the dye in solution.

Calculations :

If a suitable standard is prepared the extinctions of this and test are read, then.

$$\frac{\text{Concentration of unknown}}{\text{Concentration of standard}} = \frac{\text{Extinction of unknown}}{\text{Extinction of standard}}$$

and therefore,

$$\text{concentration of unknown} = \frac{\text{Extinction of unknown}}{\text{Concentration of standard}} \text{ X conc. of standard}$$

This can be expressed as :-

$$\text{concentration of unknown} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \text{ X conc. of standard}$$

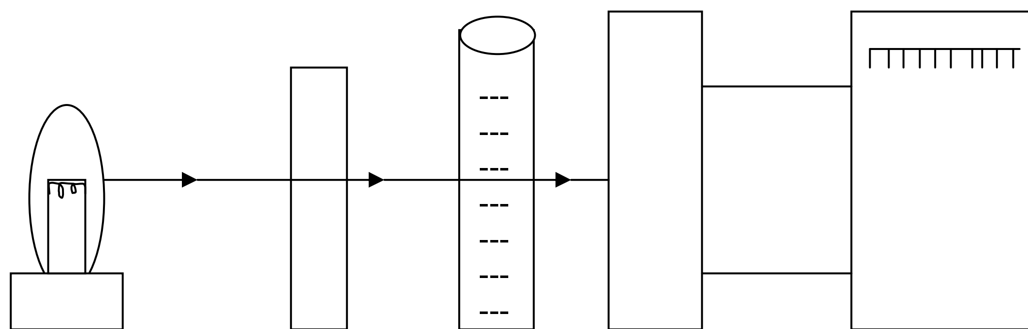


Fig.4 (diagrammatic) photoelectric colorimeter

Technical contents :- Photoelectric colorimeter

Methodology of its teaching :- lecture with few C.D. demonstration.

Evaluation of the session :- Asking queries regarding the lecture.

Chapter - 5

Automation

Objective :- Advances in diagnostic methodologies and instrumentation have been impressive but most of these are deleted in the teaching programme this chapter tries to cover the automation required for the analysis of samples.

Introduction

During the past few year, in clinical biochemistry there has been a considerable increase in clinical demand for laboratory investigations. When the volume of work increased, there arose a need for work simplification. Monostep methods are introduced to replace multistep cumbersome and inaccurate methods like Folin-Wu's blood sugar determination. The efficiency of monostep methods was further increased by the introduction of automatic dispensers and diluters. For the common test like blood glucose, blood urea etc., however, most large laboratories found this approach still inadequate to deal with work load and instruments designed to handle the whole analytical process in mechanized fashion have become common place in last decade. This procedure is called **automation.** It is a self regulating process, where the specimen is accurately pipetted by a mechanical probe and mixed with a particular volume of the reagent and results are displayed in digital forms and also printed by a printer. There is an element of feed back which detects any tendency to malfunction. The function of autoanalyser is to replace with automated devices the steps of pipetting, preparation of protein free filtrated, heating the colour forming reagents in a waterbath and increase the accuracy and precision of the methods.

The automated instruments not only save the labour and time but allow reliable quality control, reduce subjective crrors and work economically by using smaller quantities of samples and reagents. Following are the different types of autoanalysers used in clinical chemistry laboratories.

Continuous flow analysers

The early form of automation was introduced by Technicon Instrument Corporation. It was based on continuous flow analysis. In these systems, the samples and reagents are passed sequentially through the same analytical pathway and separated by means of air bubbles. The relative proportions of sample and reagents were determined by their individual flow rates. Missing occurred when tubes joined to form a common pathway.

The disadvantage of these automated systems is that the physician may not be interested in all the test reported by the instrument, and the may be interested in some other tests which the instrument does not report.

discrete analysers

The various types of discrete autoanalysers used in the clinical chemistry laboratories are

(A) **Batch analysers** and (B) **'Stat'** (means immediate reporting or emergency determination analysers.

(A) Batch analysers

These are convenient to analyse specimen in batches such as of sugar, urea creatinine etc. state testing may not be conveniently carried out on these analysers. The batch analysers can be further differentiated as (1) semiautomated and (2) fully automated.

(1) Semi automated (batch) discrete analysers

In the case of these analysers the initial part of the procedure i.e. pipetting of reagent and specimen, mixing and incubation is carried out by the technician. Rest of the procedure i.e. setting of incubation temperature (for kinetic determinations), zero setting, photometric readings, result display, automatic printing and data management and processing is carried out by the analyser.

Advantage of semiautoanalysers

1) The semiautoanalysers are cheap and compact, compared to other fully automated analyses.
2) Specimen analysis is cheap, since volume of reagent used is 0.5 to 1.0ml.
3) Enzyme determinations by kinetic methods are performed accurately in 1 to 3 minutes.
4) The enzymatic reagents are not corrosive and involve monostep testing.

Fully automated batch analysers

These analysers carry out all the function of a semiautomated analyzer, in addition to the pipetting of specimen and reagents and also the mixing of the reaction mixtures. The basic working stages of these analysers, after selecting general system parameters are as follows

1) the specimen cups are placed on the sampler.
2) The required quantity of reagent is dispensed by a reagent probe, in the reaction cups.

3) The respective specimens from the sampler are pipetted into the appropriate reaction cups by another sample probe.

4) The reaction cups are shaken mechanically to mix the contents.

5) After observing the required incubation time (for delay time in the case of kinetic determinations) the reaction mixture is aspirated by a probe for photometric readings.

6) The resulted values are printed and displayed in appropriate units by digital display.

(B) Stat Analysers (Random access analysers)

In the case of these analysers many reagents (8 to 20 or more) can be pipetted one after another, so that various biochemical determinations can be performed on one specimen, according to the number of tests ordered for the patient. Hence, these are patient (or specimen) orientated autoanalysers. For examples, if serum specimen No. 1 requires following tests to be performed; 1) Urea nitrogen 2) Serum creatinine 3) Total proteins 4) Albumin 5) SGPT and 6) SGOT

- The analyzer is programmed for these tests with respective system parameters.
- The reagents for urea nitrogen, creatinine, total proteins, albumin, SGPT and SGOT are pipetted automatically by a reagent probe in the respective reaction cups.
- The required specific serum quantities are added to the respective reaction cups by a specimen probe.
- The analyzer identifies various reagents and specimen.
- The photometric determinations are carried out by the autoanalyser.

- The values of the respective tests are displayed on the computer screen as well as printed on a paper, after the specific test incubation periods.

The advantages of a fully automatic 'stat' (or random access) analyzer are as follows

1) The advantages the various chemistry tests from the file.
2) It performs a single test, a profile, an organ panel or a 'stat' determination.
3) It reduces the cost per test by utilization of micro-volumes of a reagent.
4) It performs automatic monitoring of specimen and reagent volumes.
5) It can perform various methodologies such as End point, kinetic, initial rate and bichromatic (readings at two different wavelengths) to eliminate errors which may arise due to haemolytic, icteric or lipemic sera.
6) The analyser can perform repeatation of tests with or without automatic dilution.

Technical contents :- semi autoanalyser and fullyautomatic analyser.

Methodology of its teaching :- lecture with few C.D. demonstration. Live demonstration of the semi and fully automatic analyser.

Evaluation of the session :- Asking to demonstrate the basic functioning of the above instruments.

Chapter No. 6

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 hyperglyeemia (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 Insuline dependent Diabetes Mewlitus (IDDM)

- Less Frequent
- Occures before the age of 15 years.
- Due to less production of insulin from b cells of langerhans (Pancrea)

(b) Maturity onset diabetes or. Type II diabetes or Non-insulin dependent Diabetes melitus (NIDDM)

- More frequent in population.
- Occures 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 Pancrease.

(b) Inereased level of antagonistic hormone

- Hyperthyrodism
- Hypercorticism – Cushings disease
- Hyperpitnatarism

Clinical Biochemical fnding 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 catabolisim 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 apperance 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 glutation, 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 prepration of the patient.

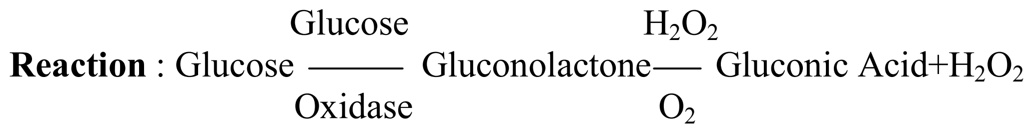
Collection of Blood Sample : Blood is usually collected from a vein and kept in a bottle containing sodium fluoride (Na F) and potassium oxalate mixed at proportion of 1 : 3 Usually 4 mg. of the mixture is required. Both the substances act as anticoagulant and Na F prevents glycolysis in RBC's by inhibiting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

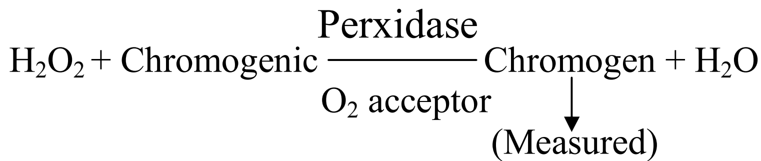
Methods of Estimation :

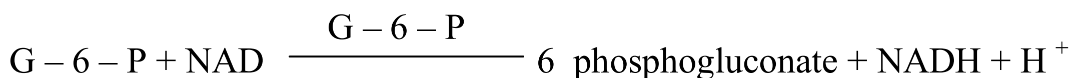
1. Enzymatic : Measure only glucose in blood.

a) **Glucose Oxidase Method :**

Glucose oxidase catalyses the oxidation of glucose to gluconic acid and hydrogen peroxide. This H_2 O_2 is broken down to water and oxygen by a peroxidase in the presence of an oxygen acceptor which itself is converted to a coloured compound, the amount of which can be measured colorimetrically. This method is used in various autoanalyzers.







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

II. Reduction Methods

1) **Alkaline Copper Reduction Methods.**

- Asatoor & King's method
- Folin & Wu method

2) **Ortho toludine method** : O-Toludine reacts quantitatively with the aldehyde group of glucose to from a ghcosylamine and schiff base. The colour development is rapid and stable.

Estimation of Blood Glucose by method of asatoor and King (modified)

Principle : Reducing sugars in hot alkaline medium produce cnediols 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 coloured complex by colorimetric measurement at 6.10 nm.

Reagents :

i) Isotonic copper sulphate solution :

Na_2SO4 IOH_2O – 30 gm and CuSO4 $5H_2$ 0-6 gm per 1 litre of solution.

ii) Sodium tungstate 10%.

iii) Alkaline Tratrate 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.

iv) Phosphomolybdic acid solution : To 35 gm of molybdic acid add 5 gm. Of sodium tungsta'e, and 200 ml of 10% NaOH. Boil for 30-40 mins to remove ammonia. Cool. dilute to about 350ml ad add 125 ml of conc. (85%) phosphoric acid. Make volume upto to 500 ml.

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

vi) 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 3 add mol 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 :

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

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.

Hyperglycagmia : 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 pancrease, pancreatitis, carcinoma of pancrease, fibrocystic disease or baemachromatosis of pancrease.
4. Intracranial diseases like meningitis, encephalitis, tumors and haemorrhage show a moderate hyperglycaemia.
5. Drug induced eg : thiazide diuretics, steroids, ACTH, thyroid extracts.

   Diabetes mellitus is the commonest cause of hyperglycaemia and its early detection is of vital importance.

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

1. Most commonly seen due to overdosage of insulin in treatment of diabetes mellitus.
2. Hypothroidism – cretinism, myxoedema.
3. Insulin secreting tumours of pancrease – rare.
4. Hypoadrenahsm (Addison's disease)
5. Hypopitruitism.
6. Severe exercise.
7. Starvation.
8. Chronic alcoholism
9. 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 No. 7

Glucose Tolerance Test

Object :- To estimate the glucose tolerance.

Introduction :

Glucose Tolerance is defined as the capacity of the body to tolerate an extra load of glucose. Normally the blood glucose level remains relatively constant the fasting being 63-100mg% which returns to normal within 2 hours. The definitive diagnostic test for DM is the G.T.T.

Oral G T T : Procedure : After an overnight fast of 12-16 hrs. fasting blood sample is taken. Then 75gm of glucose dissolved in 250-300ml of water is given orally. Blood samples are collected at 30 mins interval for 2-3 hours but 2 hours sample is most important for interpretation of result according to WHO criteria. Corresponding urine samples can also be collected and presence of reducing sugar tested by Benedicts qualitative test. Blood sugar in each sample is estimated by King and Asatoor method. A curve between time and blood glucose concentration, is plotted.

Precautions :

1) The patient should be on a diet of 300 gm of carbohydrate per day for at least last 3 days.
2) Fasting should not be less than 10 hrs. and not more than 16 hours. Only water is permitted after dinner.
3) The patient should not be taking drags that affect carbohydrate metabolism.

4) Durings the test patient activity should be normal (mild to moderate) and he should abstain from smoking. The patient should be at rest mentally.

Factors affecting GTT :

1) Starvation/Ingestion of high fat diet.
2) Exercise.
3) Pregnancy-tolerance is decreased.
4) Illness-stress causes decreased tolerance, so patient recovered from surgery, burns or child birth should be allowed 2 weeks time before the test is carried out.
5) Physiologically decreased tolerance with age.
6) Endocrine disorders.
7) Drugs-certain drugs must be withdrawn before the test eg-Oral contraceptives, thiazide diuretics, insulin, oral hypoglyan mic agents, salicylates etc.
8) Liver diseases.

Interpretation:

The following important type of response are seen commonly :

a) Normal Response : Fasting blood sugar is normal. After 1 hour level rises, but remain below the renal threshold of 180%. It returns to normal fasting level within 2 hours.

b) Diabetic curve : Fasting level are 7.8 mmol/L (140mg dL) and 2 hour venous blood glucose of 200mg/dl (11 lmmol/L) or more are diagnostic of diabetes. Glycosuria is usually seen.

c) Impaired GTT: 2 hours values of blood glucose between 140mg/dl and 200mg/dl are not abnormal and must be followed up for DM.

d) Renal Glycosuria : Curve is normal Due to lowered renal threshold one or more samples of urine contain glucose.

e) Lag storage/Alimentary Type: Fasting blood glucose is normal. Due to rapid absorption, maximum level is found at 30 mins which crosses 180mg/dl (80 glycosuria seen) and hypoglycaemic levels may be reached at end of 2 hours.

f) Flat curve of enchanced glucose tolerance : the fasting blood glucose level is normal.

Throughout the test the level does not vary $\pm$ 20mg%.

Extended GTT :

In this blood samples are taken for 4-5 hrs after giving glucose to see how the curve behaves below the normal fasting glucose limits. Done in some conditions causing hypoglycaemia.

Cortisone Stressed GTT :

Can be used for detecting latent Diabetes mellitus.

Intrevenous GTT :

In is done if oral glucose is not tolerated or oral GTT curve is flat. In these cases 20% glucose as 0.5g glucose/Kg body weight is infused. Blood samples are collected hourly. Usually peak occurs within 30 mins after infusion and returns to normal after 90 mins.

Methodology of its teaching :- Plotting the glucose tolerance graph.

Evaluation of the session :- Asking queries regarding the above test.

Chapter No. 8

Glycated hemoglobin

Object : To estimate Glycated hemoglobin.

Introduction : Human Hemoglobin inside erythrocytes undergoes a non enzymatic chemical reaction with glucose. The rate and extant of this reaction are thought to be depended on the average blood glucose concentration during the life time of the erythrocytes there are several reaction procedure, “Glycated hemoglobin”, which collectively Hb A_1. The most abundant of these is Hb A_{1c} the ratio of Hb A1c or HbA1 to the total HbA concentration has been suggested as a reliable measure of the degree of metabolic control in diabetic patients.

Technical Contents :-

Using kit of Glycated hemoglobin and its measurement by semi autoanalyser or by using Ghb analyser.

Principle and procedure:

1. **Using kit of Glycated Hemoglobin an its measurement by semi autoanalyser**. A hemolysed preparation of whole blood is mixed continuously for 5 minutes with a weak binding cation-exchange resin during this time the non glycated hemoglobin, which consists of the bulk of the hemoglobin (Hb_{A0}) binds to the resin. After the mixing period a filter is used to seprate the supernatant containing the glycohemoglobin from the resin. The percent glycohemoglobin is determined by measuring the absorbance at 415 nm (405-420nm acceptable) of the glycohemoglobin fraction and the total hemoglobin fraction. The ratio of

the two absorbances gives the percent glycohemoglobin. Normal range is 6.0% to 8.3%

2. Using Ghb analyser.

It uses low pressur cation exchange chromatography in conguction with gradient elution to separate human hemoglobin sub types and variants from hemolysed whole blood. The separated hemoglobin fraction are monitored by means of absorption of light at 415nm the chromatogram obtained is recorded and stored by the onboard computer. The analyser performs the analysis of the chromatogram and generates a printed report.

Expected range of Hb A_{1c}

Sugar – 90-150	5-0% to 7.0%
Sugar – 150-180	7.0% to 8.0%
Sugar – 180-360	9.0% to 14.0%.

Method of teaching : Using kit of Ghb flowing its procedure and giving demonstration.

Evaluation of session: To calculate the percent of glycated hemoglobin after doing the experiment.

Chapter No. 9

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 exeretion. The urea concentration in the glomerular filtrate is same as that in plasma. Tublar reabsorption of urea varies inversely with the rate of urine flow ad hence is not a useful measure of GFR. Blood urea nitrogen varies directly with protein intake and inversely with the rate of excertion of urea.

Estimation : Various methods are use :

(i) **Enzymatic using urease :**

a) **Neseler's Method :** Urea is converted to ammonia by the enzyme urease. Ammonia is made to react with Neseler's reagent (Potassium mercurie iodide) giving rise to a brown coloured 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, colour instability and non linear, 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 coloured compound in alkali. Nitroprusside acts as a catalyst increasing the rate of reaction, intensity of colour obtained and its reproducibility.

c) **In the urease/glutamate dehydrogenose method**, glutamate production from ammonia and 2.0 x 0- glutarate is measured by the absorbance change at 340nm. Owing to concomitant conversion of $NADH^+$ to NAD^+. Each molecule of urea hydrolysed causes the production of two molecules of NAD^+.

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

Urea is hydrolysed 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 extintion 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°C to give a red coloured product which is measured colorimetrically at 520nm. Thiosemicarbazide and ferric ions are added to catalyse the reaction and increase the intensity of colour. This method is linear only upto 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 strring. Make up the volume to 250ml with water. Keep in brown bottle at 4°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°C.
4) **Reagent C :** Diacetyl monoxime 20g/L of water. Filter and keep in brown bottle at 4°C.
5) **Reagent D :** Thiosemicarbazide 5g/L of water.
6) **Colour 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°C.
7) **Stock urea standard :** 100mg/100 nl 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	
Colour 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 diarrhoea.
- Pyloric stenosis.
- Ulcerative colitis.
- Diabetic Keloacidosis.
- Shocks, severe burns and haemorrhage.

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 pyclonephritis
- Congenital cystic kidneys

Post renal : Due to obstruction to flow of urine there is retention and so reduction in effective filteration 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 No. 10

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 colour developed is due to creatinine in serum. other non specific 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 centrigure 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 filterate 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 (eg fever, sepsis, haemorrhage) 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 andreliable 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 No. 11

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 colour that is formed is proportional to the number of peptide bonds which, in turn, depends upon the amount f 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 litre with water. The reagent is stored in a well-stoppered polythene bottle.

(ii) Biuret blank – this is prepared in the same way as the buiret 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 occure in multiple myeloma, macroglobulinaemia, chronic infections, chronic liver disease and auto-immune 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, haemorrage 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 analyser.

Evaluation : Giving exercise of total protein estimation.

Chapter No. 12

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 colour. Among serum proteins, only albumin binds to BCG this binding produces a change in the colour of BCG which is measured colorimetrically. The pH is maintained during the reaction by a buffer.

Reagents

(i) Succinate buffer - 11.8 gm of soccinic 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 litre 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 undermutrition, intestinal malabsorption, 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-heritence. A rise in serum albumin may occur in dehydration due to haemoconcentration.

Serum globulin may decrease due to haemodilution in shock, burns, haemorrhage etc. serum globulin increases in multiple myeloma, macroglobulinaemia, chronic liver disease, chronic infections and auto-immune 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 analyser.

Evaluation : Giving exercise of serum albumin estimation.

Chapter No. 13

Estimation Of Aminotransferases (Transaminases)

Object :- To estimate aminotransferases.

Asparate Aminotransferase (AST) SGOT

Alanine Aminotransferase (ALT) SGPT

Introduction :

The aminotransferases are a group of enzymes that catalyse the inter conversions of the aminoacids 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 :

Colorimatric 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 coloured hydrazone, the colour 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 jwith 100 mmol/L L-asparate for AST and 200 mmol/L-DL alanine for ALT.
    a. for AST-add 15.7g L-asparate 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 Litre 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°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 7th and 12th day and values return to normal levels by 3rd to 5th 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
- Haemolytic diseases

Others-Both AST and ALT levels may be elevated following

- intake of alcohoal

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 analyser.

Evaluation : Giving exercise of serum SGOT and SGPT.

Chapter NO. 14

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 makedly 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 4-animoantipyrine in alkaline medium to give a red coloured compound which is estimated at 520nm against a reagent blank. Colour 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 oxidising 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 litre 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.

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

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 upto 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 deformans)- 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 secondaries from thyroid, pancress 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) synthese 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 analyser.

Evaluation : Giving exercise of serum Alkaline Phosphatase.

Chapter No. 15

Estimation of Serum Bilirubin

(Estimation by method of Malloy and Evelyn, 1937, Modified.)

Jaundice

Jaundice is yellow discoloration of the skin or sclera (fig. 1) this is due to the presence of bilirubin in the plasma and is not usually detectable until the concentration is greater than about 1.1 mg/dl. Normally the bilirubin concentration in serum ranges from 0.2-1.1 mg/dl.

Biliburin is derived from the terapyrrole prosthetic group found in haemoglobin and the cytochromes. It is normally conjugated with glucuronic acid to make it more soluble, and excreted in the bile (fig. 2) there are three main reasons why bilirubin levels in the blood may rise (fig. 3)

- *Haemolysis.* The increased haemoglobin breakdown produces bilirubin which overloads the conjugating mechanism.
- *Failure of the conjugating mechanism within the hepatocyte.*
- *Obstruction in the biliary system.*

Both conjugated bilirubin and unconjugated bilirubin may be present in plasma. Conjugated bilirubin is water soluble. Unconjugated bilirubin is not water soluble and binds to albumin from which it may be transferred to other proteins such as those in cells membranes. It is neurotoxic, and if levels rise too high in neonates, permanent brain damage can occur.

Biochemical tests

Bilirubin metabolities are responsible for the brown coloration of faeces. If bilirubin does not reach the gut, stools become pale in colour. Bilirubin in

the gut is metabolized by bacteria to produce stercobilinogen. And may be detected by simple biochemical tests. When high levels of conjugated bilirubin are being excreted, urine may be a deep orange colour, particularly if allowed to stand.

Biochemical test (LFTs) include measurement of serum AST and ALT as markers of hepatocellular damage, as well as bilirubin and alkaline phosphatase which are indicators of cholestasis.

Object :- To estimate serum bilirubin.

Introduction:

Most of bilirubin is formed in reticule-endothelial system from breakdown of red blood cell precursors. This bilirubin is unconjugated and is almost insoluble in water. Bilirubin is transported to serum albumin mainly and is not filtered through glomerulus. Bilirubin is actively taken up by carrier mediated process and binds to intracellular proteins eg. Ligandin and Z protein. At the endoplasmic reticulum, UDP-glucuronyl transferase conjugates bilirubin forming mainly bilirubin onide with some monoglucuronide. This conjugated bilirubin is water soluble and when present can be filtered through glomerulur appearing in urine. Secretion of conjugated bilirubin in biliary canalicular membrance is rate limiting process and is sensitive to liver damage. Bile is stored in gall bladder from where it passes into-duodenum via cystic and common bile duct.

Principle: When reacted with diazotized sulfanilic acid (Ehrlich's Reagent), bilirubin is converted to azobilirubin molecules which give a red purple colour in acid the intensity of which is read colorimetrically .

Both conjugated and unconjugated bilirubins give purple azobilirubins with diazotized acid. Conjugated bilirubin can react in aquous solution

(Direct Reaction), whereas unconjugated requires an accelerator or solubiliser , such as methanol (Indirect Reaction-which gives total bilirubin i.e. conjugated + unconjugated bilirubin).

Reagents :

1. Diazo reagent : Make freshly before use the mixing 10ml of solution A and 0.3ml of solution B.

   Solution A : 1g of sulphanilic acid and 15ml of concentrated HCL per litre in water.

   Solution B : 0.5g of sodium nitrite/100ml in water. This solution should be kept in refrigerator renewed monthly.
2. Diazo Blank : 15ml of conc. HCL/litre in water.
3. Methanol
4. Bilirubin standard : Dissolve 10mg bilirubin in a minimum (about 5ml) of 0.1N sodium solution, as quickly as possible (since it is unstable in alkaline solution) and make volume with human citrated plasma (obtained from blood bank from outdated bottles. Plasma is left in sunlight for some hours before use to destroy bilirubin present). To be kept frozen in small fractions (for practicals methanol may replace plasma for making volume)

Procedure : Label test-tubes and proceed as follows :

Reagent	Standard		Total Bilirubin		Conj. Bilirubin	
	S	S_C	T (tot)	T $(tot)_C$	T (Conj)	T $(conj)_C$
Serum	-	-	0.2ml	0.2ml	0.2ml	0.2ml
Standard Soln. (10mg/dl)	0.2ml	0.2ml	-	-	-	-
D. Water	1.8ml	1.8ml	1.8ml	1.8ml	4.3ml	4.3ml
Diaze Reagent	0.5ml	-	0.5ml	-	0.5ml	-
Diazo Blank	-	0.5ml	-	0.5ml	-	0.5ml
Methanol	2.5ml	2.5ml	2.5ml	2.5ml	-	-

Mix and allow to stand in dark for 30 min.

Read absorbance in next 10 min at 540nm using D. water as blank.

Calculation :

$$\text{Total S. Bilirubin (mg/dL)} = \frac{\text{T(tot)-T (tot)c}}{\text{S} - \text{Sc}} \times \text{Amount of std,} \times \frac{100}{\text{Vol. of Serum}}$$

$$\text{Conj. S. Bilirubin (mg/dL)} = \frac{\text{T(tot)-T (tot)c}}{\text{S} - \text{Sc}} \times \text{Amount of std,} \times \frac{100}{\text{Vol. of Serum}}$$

Serum value of unconjugated bilirubin may be derived by subtracting the value of conjugated bilirubin from total bilirubin.

Interpretations :

Normal serum bilirubin is less than 1 mg/dL. Hyperbilirubinaemia of more than 3 mg/dL results into clinical jaundice.

Hyperbilirubinaemia may be due to :-

I. **Unconjugated Hyperbilirubinaemia (Retention Jaundice):** Due to retention in circulation of increased amount of unconjugated bilirubin.

(A) Haemolytic (Pre-hepatic Jaundice) due to:

i) Increased haemolysis : increased breakdown of haemoglobin to bilirubin at a rate exceeding the ability of normal liver cells remove it from circulation.

- abnormal R.B.C. e.g. in spherocytocis, haemoglobinopathies, G-6-p dehydrogenase deficiency etc.
- Antibodies eg. Incompatible blood transfusion.
- Organisms e.g. Plasmodium in malaria etc.

ii) Inteffective eryghropoiesis eg. in pernicious anaemia.

(B) Non haemolytic Hepatie : due to

i) impaired hepatic uptake eg. in Gilbert's syndrome.

ii) impaired glucuronyl transferase activity in conditions such as :

- Crigler_Najjar Syndrome
- Gilbert's syndrome (in addition to impaired uptake)
- Physiological jaundice of new born. Particularly in premature infants not having fully developed microsomal conjugating enzyme syste.

II **Conjugated Hyperbilirubinaemia (Regurgitation Jaundice) :**

Due to regurgitation into circulation of conjugated Bilirubin which would normally pass along the bilirubin system.

Technical contents : Using kit of serum Bilirubin.

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

Evaluation : Giving exercise of serum bilirubin.

Chapter No 16

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 colour complex. Normal values 400-1000 mg/dl

2) Phospholipids : It is a fully enzymatic method which uses three different enzuymes- phospholipase D, Choline oxidase and per oxidase to developed colour which is measured at 500 nn. 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 glyceraldehydes, which forms a yellow coloured complex with acetyl acetone. The intensity of the coloured 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 overn 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 acetyle 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. Pur 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°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°C for 15 minutes. Cool the tubes and read intensities of test and standard against blank at 420 nm (violet filter)

Calculations :

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

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.

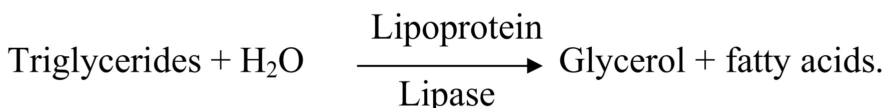
Procedure limitations:

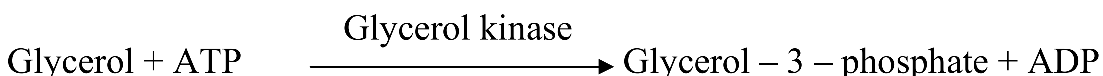
The method is linear up to 300 mg/dl. For values higher than 300 mg/dl, dilute the specimen in saline and repeat the test. Consider appropriate dilution factor for the calculation of final result.

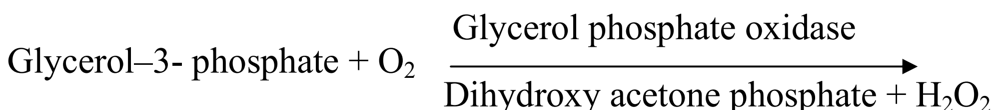
Enzymatic determination of serum triglycerides:

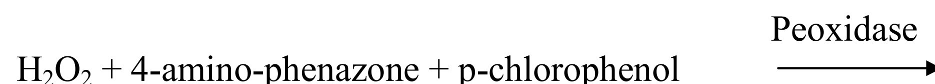
Early clinical methods for determining triglycerides involved chemical hydrolysis of a solvent extract of the serum lipids. These methods were difficult, slow, and provided numerous opportunities for error. They were not readily automatable.

Chemical principle of test









Coloured 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 analyser.

Evaluation : Giving exercise of serum triglycerides.

Chapter No 17

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 an 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 alcoholether mixture. The extract is evaporated and the residue dissolved in chloroform. By Liebermann-Burchard reaction, a green colour is developed and measured colorimetrically.

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 ration 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 obsorbance) with 'Blank' and read 'Unknown' and 'Standard'.

Calculations

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 upto 300 mg/100 ml in the elderly.

An increase in serum cholesterol (hypercholesterolaemia) is found in diabetes mellitus, nephritic syndrome, obstructive jaundice, hypothyroidism, xanthomatosis and during ether anaesthesia. An idiopathic

hypercholesterolaemia of unknown actiology occurs in some families. Hypercholesterolaemia, from any cause, predisposes to atherosclerosis.

A decrease in serum cholesterol (hypocholesterolaemia) is found in hyperthyroidism, hepatocellular damage, anaemia (except haemorrhagic), 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 hydrolysed by cholesterol ester hydrolase to free cholesterol & fatty acids. the free cholesterol produced and pre-existingh one are oxidised 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 coloured 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 analyser.

Evaluation : Giving exercise of serum cholesterol.

Chapter No 18

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 supernatent. 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 supernatent by using a pipette. The clear supernatent is treated in the similar way as for method for cholesterol and the absorbance is measured at 500 nm.

Calculations :

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

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 analyser.

Evaluation : Giving exercise of serum HDL cholesterol.

Chapter No. 19

Estimation of Enzymes in diagnosis of Myocardial infraction

Object : To make aware of LDH, CK, CKMB and their clinical significance.

Introduction:

Three enzymes are commonly used in the diagnosis and follow-up of the MI. these are :

- Creatinine kinase (CK)
- Aspartate aminotransferase (AST)
- Lactate dehydrogenase (LDH).

Creatinine kinase levels rise rapidly in serum peaking at 24h., with slower rises being shown by AST and LDH.

Creatine kinase

CK is also released from damaged skeletal muscle. Although the predominant isoenzyme in cardiac muscle cells is the same as that in skeletal muscle (CK-MM), heart cells contain another CK isoenzyme, CK-MB. This isoenzyme is a more specific indicator of cardiac muscle damage, and is increasingly used (rather than total CK) in the investigation of MI.

Aspartate and alanine aminotransterases

AST release is not specific to MI. but is also found in many acute pathologies affecting the liver and skeletal muscle. The enzyme alanine aminotransferase (ALT) is sometimes measured as part of the 'cardiac

enzyme' profile of test (fig. 1) ALT is found in high concentration in liver rather than muscle, and normal ALT in the face of a raised AST confirms that liver pathology is not contributing to the raised enzyme levels. Approximately 25% of patients with pulmonary embolus, which features in the differential diagnosis of MI and which may or may not cause chest pain, show a raised AST concentration, and most also show an elevated LDH.

Lacate dehydrogenase

Like the other enzymes used in the diagnosis of MI. LDH. Is not specific to cardiac muscle, being found in liver and red cells. Although there is a measurable difference between the LDH_1. Iosenzyme in heart and the LDH_5, isoenzyme in liver, the red cells can also release LDH_1. blood for LDH measurement must therefore not be allowed to remain unseparated before analysis, or in vitro haemolysis will invalidate the LDH measurements.

Commercially available kits are now available for the detection of these enzymes which can be assessed by semi and fully automatic analysers.

Technical contents : Using kit of serum LDH, CK and CKMB.

Method of teaching : Demonstration.

Evaluation :discussion on the above exercise.

Chapter no. 20

Estimation of Serum Calcium

Object : To estimation of serum calcium.

Introduction :

Calcium is present in serum in three forms – calcium bound to proteins, calcium bount to other organic substances, e.g. citrate, and ionized calcium. Most of the physiological functions of calcium depend upon the ionized fraction, but for routine work only total calcium in serum is estimated. A convenient and commonly used method is that of Clark and Collip*.

Principle:

Serum is treated with ammonium oxalate to precipitate calcium as calcium oxalate. The precipitate is washed with ammonia to remove excess oxalate and then treated with sulphuric acid to convert calcium oxalate into oxalic acid. The latter is titrated with standard potassium permanganate.

Reagents:

1) Ammonium oxalate, 4% - 4 gm of ammonium oxalate is dissolved in water and diluted to 100 ml.
2) Ammonia, 2% - 2 ml of conc. ammonia (S.G. 0.880) is diluted to 100 ml with water.
3) Sulphuric acid, 1 N-28 ml of conc. sulphuric acid is diluted to 1,000 ml with water.

4) Stock potassium permanganate, 0.1 N-3.158 gm of potassium permanaganate is dissolved in water and diluted to 1,000 ml. This is allowed to stand for one week and then filtered through asbestos.
5) Potassium permanganate, 0.01 N-10 ml of 0.1 N potassium permanganate is diluted to 100 ml with water and standardized against 0.01 N sodium oxalate. 1 ml of 0.01 N potassium permanaganate is equivalent to 0.2 mg of calcium.
6) Sodium oxalate, 0.01 N-0.67 gm of sodium oxalate is dissolved in water. 5 ml conc. sulphuric acid is added. The volume is made up to 1,000 ml with water (1 ml of 0.01 N sodium oxalate $\equiv$ 1 ml of 0.01 N potassium permanganate.

Procedure:

Measure 2 ml of serum, 2 ml of water and 1 ml of 4% ammonium oxalate in a centrifuge tube. Mix thoroughly and allow to stand for at least half an hour. Mix again and centrifuge at 1,500 r.p.m. for 15 minutes. Pour off the supernatant fluid and drain the tube by keeping it inverted on a filter paper for a few minutes. Wipe the mouth of the tube dry with a filer paper. Add 3 ml of 2% ammonia, shake, centrifuge and drain as before. Add 2 ml of 1 N sulphuric acid and shake vigorously. Keep the tube in a boiling water-bath shaking intermittently, until the precipitate completely dissolves. While the tube is still hot, titrate with 0.01 N potassium permanganate until a pink colour develops and persists for at least one minute. Note the volume of 0.01 N potassium permanganate used. Suppose it is x ml. For blank, take 2 ml of 1 N sulphuric acid and titrate it with 0.01 N potassium permanganate exactly as before, suppose the volume of potassium permanganate used in y ml.

$$\text{Serum calcium (mg/100 ml)} = (x-y) \times \frac{0.2}{2} \times 100$$

$$= (x-y) \times 10$$

Interpretation :

Serum calcium varies from 9 – 11 mg/100 ml in healthy persons. The product of serum calcium and serum inorganic phosphorus is around 40 in adults and 50 in children. A change in the concentration of one is usually accompanied by an opposite change in the concentration of the other.

A rise in serum calcium is seen in hyperparathyroidism, hypervitaminosis D, multiple myeloma, extensive metastatic involvement of bones, sarcoidosis, idiopathic infantile hypercalcaemia, milk and alkali syndrome, and polycythaemia.

Serum calcium decreases in hypoparathyroidism, rickets, osteomalacia, steatorrhoea, nephritic syndrome, renal failure, acute pancreatitis and starvation. In rickets, the product of serum calcium and phosphorus decreases, usually bellow 30.

Technical contents : Using kit of serum Calcium.

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

Evaluation : Giving exercise of serum Calcium.

Chapter No. 21

Estimation of Serum Phosphorus (Inorganic)

Object : To estimation serum phosphorus.

Introduction :

Phosphorus is present in blood as inorganic phosphate and in combination with several organic compounds including carbohydrates, lipids and nucleotides. Inorganic phosphorus is present mainly in serum. Two methods are described below for its estimation.

Method of Fiske and Subbarow*

Principle:

Serum is deproteinized with trichloracetic acid. Protein-free filtrate is treated with acid molybdate which combines with phosphate to form phosphomolybdic acid. This is reduced by 1, 2, 4-aminonaphtholsulphonic acid to blue coloured phosphomolybdous acid (molybdenum blue). The intensity of the colour is measured colorimetrically.

Reagents:

1) Trichloracetic acid, 10% - 10 gm of trichloracetic acid is dissolved in water and dilute to 100 ml.
2) Molybdate reagent – 25 gm of ammonium molybdate is dissolved in 200 ml of water and added to 300 ml of 10 N sulphuric acid. The volume is made up to 1,000 ml with water.
3) Aminonaphtholsulphonic acid – 59.5 gm of sodium bisulphate and 2 gm of anhydrous sodium sulphite are dissolved in water and dilute to

250 ml. 1 gm of recrystallized 1, 2, 4-aminonaphtholsuphonic acid is dissolved in the above solution and dilute to 400 ml with water.

4) Stock standard phosphorus solution – 0.351 gm of potassium dihydrogen phosphate is dissolved in water. 10 ml of 10 N sulphuric acid is added. The volume is made up to 1,000 ml with water. 5 ml of this solution contains 0.4 mg of phosphorus.
5) Working standard phosphorus solution – 5 ml of the stock standard phosphorus solution is dilute to 100 ml with 10% trichloracetic acid. 5 ml of this solution contains 0.02 mg of phosphorus.

Procedure :

Measure 9 ml of 10 % trichloracetic acid in a test tube. Add 1 ml of serum drop by drop with constant shaking. Filter. Transfer 5 ml of the filtrate to a tube labeled 'Unknown'. Pipette 5 ml of the working standard phosphorus solution into a tube labeled 'Standard' and 5 ml of 10% trichloracetic acid into a tube labeled 'Blank'. To each tube, and 3.6 ml of water. Mix after each addition. Let the tubes stand for 5 minutes.

Read 'Unknown' and 'Standard' against 'Blank' at 680 nm or using a red filter.

Calculation :

Serum inorganic phosphorus (mg/100 ml)

$$= \frac{Au}{As} \times \frac{0.02}{0.5} \times 100$$

$$= \frac{Au}{As} \times 4$$

Interpretation :

The normal range of serum inorganic phosphorus is 2.5 – 4.5 mg/100 ml in adults and 4 – 6 mg/100 ml in children.

Serum inorganic phosphorus rises in hypervitaminosis D, hypoparathyroidism, renal failure and during healing of fractures.

Serum inorganic phosphorus falls in rickets, osteomalacia, steatorrhoea, hyperparathyroidism, fanconi syndrome, renal tubular acidosis and after injection of insulin.

Technical contents : Using kit of serum phosphorus.

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

Evaluation : Giving exercise of serum phosphorus.

Chapter No. 22

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 allantion 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 colour.

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 coloured solutions.

d) Sometimes cyanide is added to increase the colour 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

Femal : 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.

Hyperuricaemia :

- 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 anaemia, chronic hemolytic anaemia.
- Toxaemia of pregnancy.
- Diabetes Mellitues.
- Starvation.
- Drugs like pyrazinamide, diuretics.

Hypouricaemia :

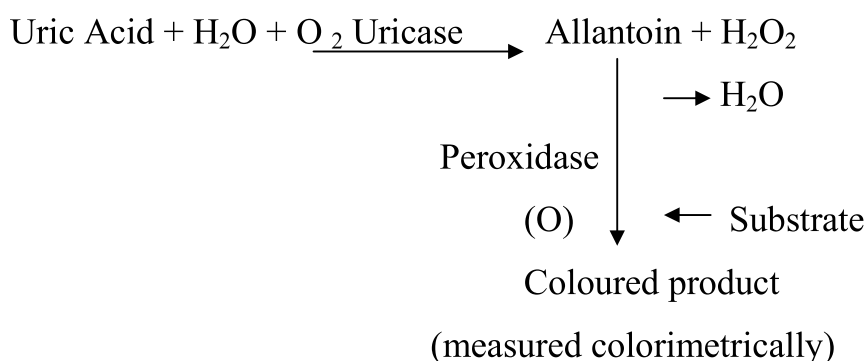
- Liver diseases (wherein maximum synthesis of uric acid occurs) like cirrhosis or Wilson's Disease.
- Renal disease that decrease renal tubular resorption like Fanconi's Syndrome.
- Drugs-uriconsuric in large does like salicylates, sulphinpyrazone.

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 analyser.

Evaluation : Giving exercise of serum uric acid.

Chapter No. 23

Blood Gas Analyser

Object: To estimate blood gas.

Introduction :

The homeostatic mechanism of the body is governed largely by the acid base and electrolyte balance Blood gases and pH are determined electrochemically by the blood gas analyzer. The clinical significance PO_2 determination is to assess the oxygen carrying capacity of the Hb. An elevated PO_2 may be related to decreased oxygen affinity of Hb, low plasma pH and high PCO_3.

The blood gas analyser is designed to quantitatively determine 125 μl of blood sample for pH, PCO PO_2 and Hb by means of electrode and a potentiometer. On the basis of these it can (calculate addition parameters i.e. plasma HCO_2. Std HCO_2 actual base excess, std. base excess, total CO_2 oxygen saturation and oxygen content in the blood so pH or blood gas parameters of whole blood, plasma or expired is calculated. In some instruments a single blood sample is in contact with pH, PCO_2 and PO_2 electrodes simultaneously. In others the electrodes are in contact with separate sub samples.

Capillary samples in addition to arterial blood samples are suitable. Sample required in 50 μl More accurate results are obtained using arterial blood collected with small amount of heparin (1 mg/ml) as anticoagulant and read as soon as possible. If necessary blood is stored at $0.4^{o}C$.

Calibration :- Primary stds. for pH measurement are phosphate buffers of pH 6/8 and pH 7.3—PCO_2 the primary calibrating standards are gas mixture of known CO_2 content after 5% of 10% PO_2 electrolytes is also calibrated using two gases of known composition. An oxygen mixture of 12% or 20% is commonly use for higher level and nitrogen (oxygen free) for the others. Again the gases must humidified and the PO_2 calculated. PO_2 electrodes respond differently to dissolved and gaseous O_2.

Additional metabolic parameters calculated with the help of blood gas analyzer are :

a) Actual Bilarboate :- this can be calculated for pH and PCO_2 using Henderson Hasselbach equation.

b) Standard Bicarbonate : It is HCO_3 in plasma from whole blood which has been equilibrated at 37°C at PCO_2 of 40mm Hg and with O_2 to give full saturation of Hb.

c) Base exess: Is defined as titrable base on titration to normal pH (7.4) at normal PCO_2 (40mm H and normal temperature (37°C) and is expressed in mmol/L. It is positive if there is actual excess base or defecit of acid.

d) Total CO_2 : Total Co_2 is that liberated from dissolved CO_2 and HCO_3 present in plasma when blood drawn anaegrobically and not further treated. This method relies on the colour change of an indicate in a carbonate-bicarbonate buffer following the release of CO_2 on acidification of the sample.

Reference Values :

Base excess of the blood is – 2.3 to 2.3mmol/L.

Actual bicarbonate 24-32 mmol/L.

Std. bicarbonate 22 4-25.8 mmol/L.

Arterial PCO_3=34-45mmol Hg Or 1.02-1.35mmol/Lt

pH=7.36-7.45

when the sample light turns on open the flap and inject the sample by pressing the aspirator button. The ready light turns on and flapis closed. Details of sample are fed with the help of key board i.e. Body temp in centigrades, arterial/venous blood and identification of patient. Wait for the machine to get results on the screen.

Precautions :

1. Arterial blood is to be used. Capillary blood is similar to arterial. However, it cannot be assumed to be so if circulation is poor. If hands are cold warm them to 45-50^oC for at least 4 minutes, before taking the sample.
2. Blood must not be exposed to air. This can be avoided when collecting directly into capillary tubes.
3. Fear and painful arterial puncture may induce hyperventilation.
4. In hyporthermia a correction can be made for low body temp. the measurements are made at 37^oC then, if T is the actual body temp.

   pH at T^oC = pH at 37^oC + 0.014 (37-T)

Type of possible acid base disturbances :

(b) Metabolic acidosis :- Primary decrease in HCO_3 arise either from increased production of acids or decreased removal of acids which deplete HCO_3.

- Common causes are :
- Deabetic Ketoaoido is.
- Mild Ketoacidosis.

- Lactic Acidosis.
- Chronic renal failure and renal tubular acidosis Type I
- Ingestion of NH_4Cl produces acidosis since NH_3 gets coverted to urea liberating H^+ which neutralize HCO_3.
- In watery diarrehoea and in patients with intestinal billiary or pancreatic fistula and renal tubular acidosis type II direct loss of HCO_3 Is there.

Respiratory acidosis :- Increased PCO_2 arises when there is impaired excretion of CO_2 or form breathing and rebreathing air containing CO_2.

- It may occur in chronic lung disease that may be primary or secondary to some heart condition. Common causes are chronic bronchitis, pulmonary fibrosis or acute form of bronchial asthama.

Metabolic alkalosis : Primary increase in plasma HCO_3 is due to accumulation of base or loss of acid other than $H2CO_3$. This can occur if, excessive dose of $NaHCO_3$ is given in the treatment of metabolic acidosis or with massive blood tansfusion with blood containing sodium citrate.

Respiratory alkalosis :- The primary decrease in PCO_2 is due to excessive ventilation. This occurs physiologically at high altitude. The stimulus is

oxygen lack but it can be produced by excessively rapid and deep respiration in normal persons. Clinically seen in patients with

- Encephalitis
- Hysterical attacks
- Fevers with septicaemia
- Cerebral tumors
- Salicylate poisoning

Tetany may also develop in alkalosis and when there is reduced ionized Ca_3^+ proportion of ionized Ca_2^+ falls as pH rises.

Combined respiratory and Metabolic acidosis causes are :

- Cardiac failure
- Sever barbiturate poisoning
- Respiratory distress in new borne.

This occurs when there is profound circulatory failure leading to severe hyposia. PCO_3 increases because of depressed respiration while formation of latic acid in tissue neutralizes HCO_3.

Combined respiratory and metabolic alkalosis :

Salicylates may cause vemitting and sufficient acid may be lost from stomach to produce alkalosis which has both respiratory and metabolic component. It may develop during treatment of primary respiratory acidosis. In salicylate poisoning poisoning respiration is stimulated so that PCO_3 falls while an accompanying primary metabolic acidosis produces a fall in HCO_3. the two changes may counteract each other causing little change in pH if at all.

Chapter No 24

Examination Of Cerbrospinal Fluid (CSF)

Object: To estimate sugar, protein and chloride in CSF.

Introduction:

CSF is formed as an ultra filterate of the plasma by the choroids plexuses of the brain. The process is not one of simple filtration since active secretory processes are involved. The normal fluid is watery clear, with a specific gravity of 1.003 to 1.008. the chemical determinations most frequently of value in the examination of CSF are those of protein, chloride and sugar.

Physical Examination

1) Colour - Normally CSF is colourless

2) Appearance - Note the appearance of CSF i.e. turbidity, suspension etc.

Chemical Examination –

Estimation of sugar in CSF

The CSF sample is treated directly like protein free filterate of blood.

Mark Folin Wu tubes as Test Std. and Blank

	Test (ml)	**Std. (ml)**	**Blank (ml)**	
CSF	0.2	-	-	
Std. Glucose	-	2.0	-	
Sion. (10 mg%)				
Distilled water	1.8	-	2.0	
Alkaline well Cuso4 Reagent	2.0	2.0	2.0	
Mix and keep the Folin Wu tubes in boiling water bath for 8 minutes. Remove the tubes and cool.				
Phosphe molybdic acid	2.0	2.0	2.0	

Dilute up to 25 ml. Mark with distilled water.

Mix well and take the reading on colorimeter using blue filter.

Calculations :

$$\frac{T\text{-}B}{S\text{-}B} \times \frac{\text{Conc. Of Std. } (0.2)}{\text{Effective vol. of sample } (0.2)} \times 100$$

$$\frac{T\text{-}B}{S\text{-}B} \times 100 = \text{mg\%}$$

Estimation of Protein in CSF

	Test (ml)	Std. (ml)	Blank (ml)	
CSF Sample	1.0	-	-	
Std. (20 mg%)	-	1.0	-	
D.W.	-	-	1.0	
Sulphosalisylic acid Reagent	3.0	3.0	3.0	

Mix by inversion keep it at R.T. for 10 min. Read on red filter

Calculations :

$$\frac{T.B.}{S.B.} \times 20 = \text{CSF Proteins in mg\%}$$

Estimation of chlorides in CSF

Rinse all the glass ware with distilled water. Take CSF 1ml. in a test tube.

Dish add 5% potassium chromate 5 drops.

Titrate against std. $AgNo_3$ (0.29%) End point Lemon Yellow to Bolt Red coloured precipitate Reading in ml. x 100^0 = mg% chloride.

CSF chloride estuation can also be done directly as other tests using standard units by calorimetric estuation.

Clinical Interpretation :

Constituents	Normal range	Clinical condition in which variations may be found.
Sugar	60-65 mg/100 ml	Raised in encephalitis CNS syphilis, abscesses, tumors, diabetes, Lowered in purulent meningitis.
Protein conditions,	20-40 mg/100 ml	Raised in inflammatory meningitis, syphilitic Froin's Syndrome, various diseases of the brain (Neurosyphillus encephalitis, abscess, tumors)
Chlorides	700-750 mg/100 ml (Expressed as NaCl) or 120-127 mEq/L.	Raised in nephritis, Lowered in meningitis particularly tuberculous meningitis.

Colour

In pathological conditions it tums to various colours

i) In severe obstructive jaundice – Yellow colouration

ii) Due to obstruction and hemorrhage in subarachnoid space – Yellow colouration due to conversion of Hb to bilirubin.

iii) Yellow colouration of CSF is called as Xanthochromia.

iv) In pueumococal meningitis – greenish colouration.

v) Reddish colouration of CSF obtained due to traumatic injury of subarachnoid space.

vi) Obstruction and spinal tumour – erythrochromia (reddish colour)

Appearance –

In various of meningitis, due to tremendous increase in cells, CSF turns coloudy, turbid is possibility of presence of fibrinogen which turns into fibrin clot on standing.

All these estimations can be done by using kits.

Chapter No. 25

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 amalyse 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 hydrolysed by the amylase. The iodine-starch complex is blue in colour and is measured in the spectrophotometer. The degree of loss in colour is proportional to the amount of starch hydrolysed 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°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

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

= 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 catalyses 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 devide 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 devide.

(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. **ENZYMES**

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 colour 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 absorbtion from closely obeying beer's Law, as it can under optimal conditions.

5. **URINE**

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 analysed 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 No. 26

Serum Electrolytes

Object : To estimate electrolytes.

Introduction :

Sodium and potassium are the principle cations of clinical interest. Chloride and bicarbonate being the anions. Other electrolytes in serum include Calcium, Magnesium cations, phosphate, sulphate, organic acids and protein as anions. When allowance is made for these other ions a the relationship between the principle ions can be considered as follows :-

$$(Na + K) – (Cl + HCO_3) = 16 \pm 2$$

this relationship holds true in most clinical states except when major alterations occur in that the other ions of the serum if in sharp rapid change in the concentration of one of the ions of the equation above. This relationship is a convenient check in laboratory estimation of electrolytes.

Laboratory determination

Sodium and potassium – earlier determined by precipitation of their salts and subsequent calorimetery. The procedures were very time consuming, laborious and open to many errors. Flame photometry (Flame omission spectroscopy) is the method most commonly used for their determination.

Principle of flame photometry :

Alkali metals, when raised to a sufficiently high temperature will absorb energy form the source of heat and be raised to an excited state in their atomic form. As each atom cools to its original unexcited state it will reemit the absorbed energy by way of radiation at specific wavelength, some of which is in the visible region. Therefore, if an alkali metal in solution is

aspirated into a low temperature flame in an aerosol form, it will alter excitation by the flame, emit a discrete frequency which is isolated by an optical filter. The emission is proportional (for low concentrations only) to the number of atoms returning to the ground state, which is, in turn, proportional to the number of atoms excited i.e. the concentration of the sample. Photodetector placed behind the filter converts it to an electric current of the proportional amount which is measured.

Type of Flame Photometers :

1. Direct reading instruments: Here the intensity of the emitted light is compared to that obtained from standards treated similar to the sample.
2. Internal Standard Instruments: The light intensity emitted form the element under investigation is compared with that from an element which acts as an internal standard.

Other methods in use for serum electrolyte determinations include atomic absorption spectrophotometry and the use of ion specific electrodes. As with many methods the introduction of automation has improved the speed and accuracy of each of the methods.

1. **Atomic absorption spectroscopy**: It is a modification of the flame photometer in that it measures the amount of light absorbed by the nebulized sample while moving from ground state to excited state.
2. **Ion selective electrodes:** The potentometric techniques measure the potential difference between two electrodes. Specific electrodes are used for the determination of sodium, potassium and chlorides. The

requirement is for an ion selective membrane to separate the soluation of known activity from the detecting system. The membrane consists of special glass, a disk of crystalline material or an organic ion exchanger saturating a water-immiscible solvent held in a gel or plastic. The sodium electrode is sensitive to changes in sodium ion concentration and potassium electrode is sensitive to changes in potassium ion concentration.

Normal range of electrolytes :

Sodium : 135 - 145 mMol/L

Potassium : 3.5 – 4.5 mMol/L

Chloride : 95 – 105 mMol/L

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The reforece range depends on the method of analysis.

The reference range of common biochemical tests in whole blood/serum are as follows:

Test	S.I. Units	Other Units
ALT (SGPT)	Up to 12 U/L	Upto 40 karmen Units/L
AST (SGOT)	Up to 15 U/L	Upto 35 karmen Units/L
Albumin	35-47 g/L	3.5-5.0 g/100 ml
Alkaline Phosphatase	20-90 U/L Children upto 350 U/L	3-13 King Armstrong units /100 ml
Acid Phosphatase	0-0.3 U/L	1-3.5 King Armstrong units /100 ml
Amylase	70-340 U/L	60-200 Somogyi Units/100 ml
Bilirubin (total)	adults 3-20 µmol/L	0.2-1.3 mg/100 ml
	Newborns 8-67 µmol/L	0.5-4.0 mg/100 ml
Calcium	adults 2 25-2.6 mmol/L	9-11 mg/100 ml
	Newborns 1.85-3.45 mmol/L	7.4-13.8 mg/100 ml
Cholesterol	3-7.8 mmol/L	140-260 mg/100 ml
Bicarbonate	23-31 mmol/L	23-31 meq/L
PCO 2	4 6-6.1 K Pa	35-46 mm Hg
Creatinine	55-110 umol/L	0.7-1.4 mg/100 ml
Electrolyte Na	134-146 mmol/L	134-146 meq/L
K adults	3 6-5.2 mmol/L	3.6-5 2 meq/L
Newborn	4.0-5.9 mmol/L	4.0-5.9 meq/L
Cl	96-107 mmol/L	96-107 meq/L
Glucose fasting	< 6.4 mmol/L	60-100 mg/100 ml
Inorganic Phosphorus	0.8-1.5 mmol/L	2 5-4.5 mg/100 ml
Protein (total)	60-80 g/L	6-8 g/100 ml
Uric Acid males	206-460 µmol/L	2-7 mg/100 ml
female	135-382 µmol/L	
Urea	2.5-8 mmol/L	15-50 mg/100 ml
Triglycerides	0.5-1.5 mmol/L	10-190 mg/dl
Serum LDH		70-240 IU
Serum CPK		15-130 IU Men 15-110 IU Women