CHAPTER – II

MATERIAL AND METHODS

A detailed account of ‘Material and Methods’ is given in this chapter. The diabetic patients with various complications were treated as test group (Chapter III). Chapter IV and V deals with the study of chronic renal failure and iron deficiency anemia patients respectively. While the healthy normal subjects were treated as control group for comparison.

Study design:

The complete study deals with the biochemical estimation of various parameters such as fasting plasma glucose, postmeal plasma glucose, Glycosylated Hemoglobin, lipid profile which include total cholesterol (TC), triglycerides (TGS), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C), renal function test which includes blood urea and serum creatinine in diabetic and chronic renal failure patients. Diabetic patients were also tested for sensitivity of erythrocytes to peroxide hemolysis. In iron deficiency anemia patients, along with Glycosylated Hemoglobin, random plasma glucose and complete blood count of the patient were evaluated.
Selection of control subjects:

For comparison with test group, forty number of healthy control subjects without Diabetes, chronic renal failure and iron deficiency anemia were treated as control.

Subject population:

The subject population was divided into two group's i.e. forty cases of normal healthy subjects were treated as control group and forty cases of each group were treated as test group. The subject population selection was made by the physician.

Sample collection and analysis:

The present study was carried out on the fasting and postmeal blood samples for the diabetic and chronic renal failure patients. The study was also carried out in Iron Deficiency anemia patients on random sample for analysis of complete blood count, plasma glucose and Glycosylated Hemoglobin level.

For the analysis of plasma glucose, blood sample was collected in fluoride (F) bulb (sodium fluoride prevents glycolysis along with anticoagulant), while for the investigation of Glycosylated Hemoglobin and complete blood count blood sample was collected in EDTA bulb and for the investigation of renal function test and lipid profile, blood sample was collected in clean and dry plain (p) bulb (plain bulb is used to get serum from blood). For the plain bulb sample, fine clot of
blood was allowed to form to avoid the hemolysis (rupture of RBCs) of the blood sample and then only, the sample was shifted to clinical biochemistry laboratory for analysis. The investigation was carried out within one hour of duration after blood sample collection.

The availability of test group and control group subjects for study was made from Government Medical College and Hospital and Hegadewar Rugnalaya, Aurangabad.

Orally, required necessary information was given to the subjects under study and written consent was obtained and then blood samples were collected.

The biochemical estimations of the following various parameters were made on the fasting and postmeal blood samples of the control and test group.

**Statistical Analysis**

Data obtained from the present study has been statistically analyzed. Mean and standard deviations were calculated. Students’ t’ values were calculated to draw the probabilities to find out the significance and non-significance of each parameter. Statistical comparison was made between test subjects and healthy control subjects. The values given in the tables and the figures are of mean ± SD. The probability value of P<0.05 was taken to be significant while probability P<0.01 was termed as highly significant.
**Estimation of plasma glucose:**

**Method:** Glucose oxidase / per oxidase (GOD/POD).

The reagent kit used for the estimation was of ‘Autopak’(120,121).

**Principle:**

Glucose is oxidized by glucose oxidase (GOD) into gluconic acid and hydrogen peroxide. Hydrogen peroxide in presence of peroxidase (POD) oxidizes the chromogen 4-amino-antipyrine / phenolic compound to a red colored compound. The intensity of the red colored compound is directly proportional to the glucose concentration and is measured at 505 nm (490-530 mm). The final colour is stable for 2 hours.

**Chemical reaction:**

\[
\text{Glucose} + O_2 \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2.
\]

\[
H_2O_2 + \text{Phenolic compound} + 4\text{-aminoantipyrine} \xrightarrow{\text{POD}} \text{Red compound} + 2H_2O
\]

Plasma from “Fluoride bulb was used for estimation.

Estimation of plasma glucose levels were done on fully automated “CHEMWELL” computerized autoanalyser instrument. Above reaction was used for estimation. The readings of (absorbance) Blank, standard and test were taken up by the instrument and calculated results in mg% were obtained.
The standard concentration was 100 mg/dl.

The method is linear upto 500 mg/dl.

Normal range: 70-100 mg% (fasting)

**Estimation of serum Total cholesterol:**

**Method:** Enzymatic.

The reagent kit used for estimation was of ‘Autopak’(120,122).

**Principle:**

The cholesterol esters are hydrolysed by cholesterol ester hydrolase to free cholesterol and fatty acids. The free cholesterol produced and pre-existing one is oxidized by cholesterol oxidase to cholestenone – 4 – en – 3 – one and hydrogen peroxide. By the peroxidase action hydrogen peroxide and libertated oxygen react with the chromogen (Phenol / 4-aminoantipyrine) to form a red colored complex (red quinine). The intensity of the red colour is directly proportional to the concentration of cholesterol present in the sample and can be measured at 500 nm.

**Chemical reaction:**

\[
\text{Cholesterol Ester} + \text{H}_2\text{O} \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{fatty acids}
\]

\[
\text{Cholesterol Ester} + \text{O}_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholesterol} + \text{H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + \text{Phenol} + 4\text{-Aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{Red Quinone} + 4\text{H}_2\text{O}
\]
Estimation of serum total cholesterol levels were done on fully automated ‘CHEMWELL’ computerized autoanalyser instrument. Above reaction was used for estimating blank, standard and test. Readings were taken up by the instrument and calculated results in mg/dl were obtained.

The standard concentration was 200 mg/dl.

The method is linear upto 500 mg/dl.

Normal Range: 150-250 mg/dl.

**Estimation of serum triglycerides:**

**Method**: Enzymatic.

The reagent kit used for estimation was of ‘Autopak’(120,123).

**Principle:**

Lipase hydrolyses triglycerides subsequently to QI and monoglycerides and finally to glycerol. Glycerol kinase using ATP as PO4 source converts glycerol liberated to glycerol - 3 - phosphate (G-3-phosphate). G-3-Phosphate-oxidase (GPO) oxidizes G-3-phosphate formed to dihydroxyacetone phosphate and hydrogen peroxide is formed. The peroxidase (POD) uses the hydrogen peroxide formed to oxidize 4-aminoantipyrine to a purple colored complex.
**Chemical reaction:**

Triglycerides + H₂O \(\xrightarrow{\text{Lipoprotein lipase}}\) glycerol + FA

Glycerol + ATP \(\xrightarrow{\text{Glycerol kinase}}\) Glycerol – 3 – Phosphate + ADP

Glycerol–3–Phosphate+O₂ \(\xrightarrow{\text{peroxidase}}\) Dihydroxy Acetone Phosphate + H₂O₂.

\(4\text{H}_2\text{O}_2+4\ \text{– Aminoantipyrine + ADPs} \xrightarrow{\text{peroxidase}} \text{Red quinone + 4H}_2\text{O}_2\).

The intensity of purple colored complex found during the reaction is directly proportional to the concentration of triglycerides in the sample and is measured at 546 nm. Fasting serum sample was used for estimation.

The concentration of standard was 200 mg/dl.

The method is linear upto 1000 mg/dl.

Normal range: Males 60-165 mg/dl. Females: 40-140 mg/dl.

**Estimation of serum HDL-C:**

**Method:** Phosphotungstate.

The reagent kit used for estimation was of ‘Autopak’(120,124).

**Principle:**

Chylomicrons, VLDL (Very low density lipo-proteins) and LDL fractions in serum or plasma are separated from HDL by precipitating with phosphotungstic acid and magnesium chloride. After centrifugation, the cholesterol in the HDL fraction, which remains in the supernatant, is assayed with enzymatic cholesterol method, using
cholesterol esterase, cholesterol oxidase, peroxidase and the chromogen 4-aminoantipyrine/phenol.

The intensity of the colour is directly proportional to the concentration of HDL-C present in sample and is measured at 500 nm. Blank and standard were similarly treated.

The concentration of standard was 50 mg/dl.

The method is linear upto 100 mg/dl.

Fasting serum sample from ‘plain’ bulb is used for estimation.

**Procedure:** consist of 2 stages:

(1) Precipitation and  (2) Colour development.

1) **Precipitation:**

Dispense into centrifuge tube 200 microlitre of serum sample. Add 200 microlitre of precipitating reagent to it. Mix well gently. Centrifuge at 1500 g or 3500-4000 rpm for 10 minutes. separate the clear supernatant immediately and treat it as a test sample.

2) **Colour development:**

Determine the cholesterol content as given in the procedure of total cholesterol assay. Estimation of HDL-C was made on fully automated ‘CHEMWELL’ computerized autoanalyser. Above principle was used for colour development.

Normal Range: 30-70 mg/dl.
The value of LDL-C can be calculated as follows:

If the value of the Triglyceride is known, LDL-C can be calculated based on Friedwald’s equation.

$$\text{LDL-C mg/dl} = \text{Total cholesterol} - \frac{\text{(Triglycerides)}}{5} - \text{HDL-C}$$

**Estimation of Blood Urea:**

**Method:** Urease

The reagent, kit used for estimation was of ‘Autopak’(120).

The method of estimation is based on the Berthelot’s Reaction(125).

**Principle:**

Urease splits urea into ammonia and CO$_2$. The ammonia reacts with phenol in presence of hypochlorite to form indophenols, which with alkali gives a blue colored compound. The intensity of the colour is proportional to the concentration of urea present in the sample and is measured at 546 nm (530-570 nm). The colour of the reaction is stable for 8 hours.

Serum sample was used from plain bulb for estimation.

The estimation of blood urea levels were done on fully automated ‘CHEMWELL’ autoanalysers. Above principle was used for estimation. The readings (absorbance) of blank, standard and test
were taken by the instrument and calculated results in mg/dl were obtained.

The standard concentration was 40 mg/dl.

The method is linear upto 200 mg/dl.

Normal Range: 15-40 mg/dl.

**Estimation of serum creatinine:**

**Method:** Jaffe’s reaction:

The method commonly used for estimation of creatinine makes the use of Jaffe’s reaction, which leads to the production of yellowish-red colour with an alkaline picrate reagent(126).

**Principle:**

Creatinine which is present in protein free filtrate reacts with picric acid in alkaline medium to form an orange red or yellow tautomer (colour complex) the creatinine picrate. This is the Jaffe’s reaction. The intensity of the colour is directly proportional to the concentration of creatinine in the serum and can be measured at 520 nm (green filter).

The optical density of the blank, standard and test was measured on ‘UNICAM’ spectrophotometer.

The method is linear upto 5 mg/dl.

Normal range 0.7 – 1.1 mg/dl.
Estimation of Glycosylated Hemoglobin (HbA1c):

Glycosylated Hemoglobin was determined by affinity chromatography using ion mobilized aminophenylboronic acid, which binds diols selectively (52). Other methods available for estimation of Glycosylated Hemoglobin are chromatographic methods (42,127), high performance liquid chromatography (HPLC) (50,128), electrophoresis (129), isoelectric focusing (130), colorimetry (131), radioimmunoassay and mass spectrometry (31). Affinity chromatography method offers greater selectivity for diabetics than other methods: such as ion exchange chromatography, electrophoretic method etc.

0.2 ml hemolysate was applied and column was washed with buffer. The void volume and washings 25 ml were collected and pooled (unfound fraction). Adsorbed hemoglobin (bound fraction) was eluted with .1mol/l sorbitol in buffer (10ml). The absorbance of unbound and bound fraction was measured at 413 nm and percentage Glycosylated Hemoglobin was calculated.

Estimation of Glycosylated Hemoglobin is done by **NYCOCARD Glycosylated Hemoglobin KH method**.

It is rapid *in vitro* method.

Test Principle:

Nycocard Glycosylated Hemoglobin is a boronate affinity assay. The kit contains test devices with a porous membrane filter, test tubes
pre-filled with reagent and a washing solution. The reagent contains agents that lyse erythrocytes and precipitate Hb specifically as well as a blue boronic acid conjugate that binds cis-diols of Glycosylated Hemoglobin. The precipitate is evaluated by measuring the blue (Glycosylated Hemoglobin) and the red (total Hb) colour intensity with the Nycocard Reader-II, the ratio between them being proportional to the percentage of Glycosylated Hemoglobin in the sample.

Kit contents (24 test kits):

TD/Test Device 1 x 24 units
Plastic device containing a membrane filter.

R1/Reagent 1 x 24 units x 0.2 ml
Glycinamide buffer containing Zn ions, dye-bound boronic acid and detergents.

R2/Washing solution 1 x 2.0 ml
Morpholine buffered NaCl solution and detergents.

Measuring range: 3 – 18% Glycosylated Hemoglobin
Measuring interval: 0.1% Glycosylated Hemoglobin

Sample material:

Blood samples can be stored up to 10 days at 2-8°C before analysis.
**Test procedure:**

1. Precipitation of hemoglobin. Add 5 ml whole blood to the test tube pre-filled with R1/Reagent. Mix well. Leave the tube for minimum 2 minutes, maximum 3 minutes.

2. Application of sample: Remix to obtain a homogenous suspension. Apply 25 microlitre of the reaction mixture to a TD/Test Device by holding the pipette approx 0.5 cm above the test well. Empty the pipette into the membrane (approx. 10 seconds).


4. Test result measurement: Read the test result within 5 minutes using Nyocard Reader-II.

**Reference Interval:** Glycosylated hemoglobin reference ranges in diabetic patients are as below:

<table>
<thead>
<tr>
<th></th>
<th>Diabetes Glycosylated Hemoglobin values (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Glycosylated Hemoglobin values</td>
<td>4.2 to 6.2%</td>
</tr>
<tr>
<td>Good Control</td>
<td>5.5 to 6.8%</td>
</tr>
<tr>
<td>Fair Control</td>
<td>6.8 to 7.6%</td>
</tr>
<tr>
<td>Poor Control</td>
<td>&gt;7.6%</td>
</tr>
</tbody>
</table>
**Advantages**

1. With affinity chromatography, non Glycosylated Hemoglobins do not interfere.

2. The labile intermediate form of Glycosylated Hemoglobin produces negligible interference.

3. It is unaffected by variations in temperature and has reasonably good precision.

4. Hemoglobin variants, such as Hbf, Hbs, or Hbc, produce little effect.

5. Of the various methods, only Glycosylated Hemoglobin measured by affinity chromatography appears to be unaffected by CKD(132-134).

**Disadvantage**

A major disadvantage with affinity chromatography is that it produces higher values than HPLC(132). The assay detects ketoamine structures on lysine and valine residues on both α and β chains of hemoglobin. Thus it measures total Glycosylated Hemoglobin, but the system is calibrated to also report Glycosylated Hemoglobin standardized value. These values are derived from an equation obtained from correlation between total Glycosylated Hemoglobin and Glycosylated Hemoglobin analysis by HPLC.
Sensitivity of erythrocytes to peroxide hemolysis:

Standardization of the method for determination of the sensitivity of erythrocytes to peroxide hemolysis(135).

Reagents:


3. Working sodium chloride solution: 25 ml of phosphate buffer mixed with 25 ml of stock sodium chloride solution mixture was made upto 100 ml by DW. Prior to use the solution is shaken vigorously (To be saturated with atmospheric oxygen).

Procedure:

1. 0.1 ml of whole blood was added to 7.5 ml of working NaCl solution to prepare the erythrocyte suspension. The mixture is mixed on vortex to allow complete hemolysis.

2. The suspension is centrifuged at 3000 rpm for 10 minutes. The supernatant is drawn off and erythrocyte precipitate and the earlier procedure repeated.

3. 1 ml of suspension is transferred to three tubes each.

4. 4 ml of working NaCl is added to tube ‘1’ and ‘2’ and 4 ml of DW to tube ‘3’ (To achieve complete hemolysis).
5. The contents were stirred and incubated at 37°C for two hours.

6. At the end of incubation period, the contents were mixed and centrifuged at 3000 rpm for 10 minutes.

7. The supernatant was decanted into 3 tubes and the absorbance measures in a colorimeter against a DW blank at 540 nm.

**Calculations:**

The following equation is used to determine the % of erythrocyte sensitivity to peroxide hemolysis.

\[ X = \frac{(E_1 + E_2) \times 100}{2 \times E_3} \]

Where \( X \) = degree of hemolysis in %

\( E_1 \) = Absorbance of tube ‘1’

\( E_2 \) = Absorbance of tube ‘2’

\( E_3 \) = Absorbance of tube ‘3’

The results are expressed as % of the erythrocytes in the given sample, which are sensitive to peroxide hemolysis.

**Complete Blood Count**

The complete blood count was performed on auto hematology analyzer Cellenium 19 Mindryae Trivitron diagnostic:

This auto hematology analyser is a quantitative, automated hematology analyser and leukocyte differential counter for *in vitro* diagnostic use in clinical laboratories.
The purpose of this analyser is to identify the normal patient, with all normal system generated parameters, and to flag or identify patient results that require additional studies.

The analyzer is used for the quantitative determination of a maximum of 19 parameters and 3 histograms of blood samples.

White blood cell or leukocyte (WBC), Lymphocyte (Lymph#), Mid-sized cell (Mid#), Granulocyte (Gran#), Lymphocyte percentage (Lymph%), Mid-sized cell percentage (Mid%), Granulocyte percentage (Gran%), Red blood cell or erythrocyte (RBC), Hemoglobin concentration (HGB), Mean corpuscular (erythrocyte) volume (MCV), Mean cell (erythrocyte) Hemoglobin (MCH), Mean cell (erythrocyte) hemoglobin concentration (MCHC), Red blood cell (erythrocyte) distribution width (RDW-D), standard deviation, Hematocrit (HCT), Platelet (PLT), Mean Platelet volume (MPV), Platelet distribution width (PDW), Plateletcrit (PCT), White blood cell histogram (WBC histogram), Red blood cell histogram (RBC histogram), Platelet histogram (PLT histogram).

The two independent measurement methods used in this analyzer are:

- The impedance method for determining the WBC, RBC and PLT data.
- The colorimetric method for determining the Hemoglobin.
During each analysis cycle, the sample is aspirated, diluted and mixed before the determination for each parameter is performed.

**Hemoglobin measurement:**

Hemoglobin is determined by the colorimetric method. The WBC/Hb dilution is delivered to the bath where it is bubble mixed with a certain amount of lyse, which converts hemoglobin to a hemoglobin complex that is measurable at 525 nm. An LED is mounted on one side of the bath and emits a beam of monochromatic light, whose central wavelength is 525 nm, and then is measured by a photo-sensor that is mounted on the opposite side. The signal is then amplified and the voltage is measured and compared to the blank reference reading (readings taken when there is only diluent in the bath). The Hemoglobin is calculated as per the following equation and expressed in g/l.

\[
\text{Hemoglobin (g/l)} = \text{constant} \times \log_{10} \left( \frac{\text{blank photocurrent}}{\text{sample photocurrent}} \right)
\]

RBC / PLT Measurement:

Volumetric Metering:

An accurate cell count can not be obtained unless the precise volume of diluted sample that passes through the aperture during the count cycle is known. This analyzer uses a volumetric metering unit
to control the count cycle and to ensure that a precise volume of sample is analyzed for the measurement.

Measurement principles:

RBC / PLT measurement:

RBCs/PLTs are counted and sized by the impedance method. This method is based on the measurement of changes in electrical resistance produced by a particle, which in this case is a blood cell, suspended in conductive diluents as it passes through an aperture of known dimensions. An electrode is submerged in the liquid on both sides of the aperture to create an electrical pathway.

Derivation of RBC – Related parameters:

RBC

RBC \(10^{12}/l\) is the number of erythrocytes measured directly by counting the erythrocytes passing through the aperture.

MCV

Based on the RBC histogram, this analyzer calculates the mean cell volume (MCV) and expresses the result in fl.

This analyzer calculates the HCT (%), MCH (pg) and MCHC (g/L) as follows:

\[
\begin{align*}
\text{HCT} &= \frac{\text{RBC} \times \text{MCV}}{10} \\
\text{MCH} &= \frac{\text{HGB}}{\text{RBC}} \\
\text{MCHC} &= \left(\frac{\text{HGB}}{\text{HCT}}\right) \times 100
\end{align*}
\]
Where the RBC is expressed in $10^{12}$ /L, MCV in fl and Hb in g/L.

RDW – CV.

Based on the RBC histogram, this analyzer calculates the CV (coefficient of variation) of the erythrocyte distributing width.

RDB – SD:

RDW-SD (RBC distribution width – standard deviation, fl) is set on the 20% frequency level with the peak taken as 100%.

RBC Histogram:

Besides the parameters mentioned above, this analyzer also presents an RBC histogram, whose X-coordinate represents the cell volume (fl) and Y-coordinate represent the number of the cells. The histogram is presented in the analysis result area of the “count” screen when the analysis is done.