Chapter – II : MATERIALS AND METHODS

The present study was carried out in hypertensive subjects with and without complications. The complications were congestive cardiac failure (CCF) and hypertension with diabetes mellitus and were treated as study (test) subjects (Chapter III and IV). Chapter V and VI describe study in atherosclerosis and myocardial diseases such as ischemic heart disease (IHD), congestive heart failure (CHF) and acute myocardial infarction (AMI) (Test groups). While the healthy normotensive without any cardiovascular diseases were treated as control group for comparison.

Study Plan:

The complete study plan of the present research work was made into two parts for the selection of subjects. The first part deals with the physical (clinical) examination of the test group subjects and control group subjects which was made by the physician of medicine clinic, OPD/IPD, Government Medical College, Aurangabad. The particulars of the patients regarding the date of occurrence, symptoms of hypertension, diabetes, family history and drug of treatment.

Second part deals with biochemical analysis of the various parameter such as lipid profile, which includes total cholesterol, triglycerides, high density lipoprotein cholesterol (HDL-c), low
density lipoprotein cholesterol (LDL-c) and very low density lipoprotein cholesterol (VLDL-c), while kidney function tests were blood urea, serum creatinine and serum uric acid.

Free radical investigated were serum nitrous oxide (NO$^\cdot$), serum malondialdehyde and serum reduced glutathione, whereas the antioxidant analysed were serum Vitamin A, E and C.

**Selection of control subjects**:

For comparison with test group study purpose eighty number of healthy control subjects with normal blood pressure range less than 140/90 mmHg (SBP/DBP respectively) and with no cardiovascular diseases.

**Selection of patients for test group study**:

For selection of patients, the following criterion was applied by the physician. **Electrocardiography** (ECG), blood pressure measurement, typical angina (retrosternal chest pain on exercise, decreasing of the pain in ten minutes on rest). None of the patients had a history of prior myocardial infarction or previous cardiac surgery and also there was no important concomitant disease.

Detailed history was obtained while clinical examination was done.

**Subject Population**:

The subject population was divided into two groups i.e. eighty cases (male/female) normal healthy subjects treated as
control group and 50 cases (male/female diseased subject to each
group treated as test group. The subject population selection was
made by the “Physician”

The present study was made on fasting (10-12
hours/overnight) blood samples of both the groups.

The original serum samples were obtained by withdrawing
venous blood (after 10-12 hours of fasting) in plain bulbs. The
blood was allowed to clot. The serum was separated by
centrifugation and estimation of various parameters was done.
Simultaneously the blood is also collected in EDTA bulb to
obtained the plasma and issued for the estimation of reduced
 glutathione, after expressing with haemoglobin concentration,
vitamin ‘A’ etc.

Orally, required necessary information was given to the
subjects under study and written consent was obtained and then
blood samples were collected from the Government Medical College
and Hospital, Aurangabad.

Simultaneously the blood is also collected in EDTA bulb to
obtained plasma and is used for estimation of reduced glutathione.

Methods :

The various biochemical parameters were estimated in
fasting blood samples of the healthly control and test (diseased)
group.
1] **Estimation of Serum Total Cholesterol**: (Method – Enzymatic)

**Principle**:

The cholesterol esters are hydrolysed by cholesterol ester hydrolase to free cholesterol and fatty acids. The free cholesterol produced and pre-existing one are oxidized by cholesterol oxidase to cholestenone-4-en-3-one and hydrogen peroxide.

Peroxidase action of hydroperoxide and liberated oxygen react with the chromogen (phenol/4-amino antipyrine) to form a red colored complex (red quinone).

The intensity of the red color is directly proportional to the concentration of cholesterol present in the sample and was measured at 500 nm.\(^{34,35}\)

**Chemical Reaction**:

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

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

\[
2\text{H}_2\text{O}_2 + \text{Phenol} + 4\text{-aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Red quinone} + 4\cdot \text{H}_2\text{O}
\]

The above reaction was used for estimating test, standard and blank. Readings were taken on spectrophotometer.

- The standard concentration: 200 mg/dl
- The linearity of the method: 500 mg/dl
- Normal range: 150-250 mg/dl
The reagent kit was from “Autopak”, Bayer’s Diagnostic Ltd, Baroda, Gujarat.

2] Estimation of serum Triglycerides: (Method – Enzymatic)

**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 dihydroxy acetone phosphate and hydrogen peroxide is formed. The peroxidase (POD) uses the hydrogen peroxide formed to oxidize 4-amino antipyrine to a purple colored complex. The intensity of purple colored complex formed is directly proportional to the concentration of triglyceride in the sample. Readings were taken at 540 nm spectrophotometer. *(34,36)*

**Chemical Reaction:**

```
Triglycerides + H₂O → Glycerol + FA
```

```
Glycerol + ATP → Glycerol-3-phosphate + ADP
```

```
Glycerol-3-phosphate + O₂ → Dihydroxy acetone phosphate + H₂O₂
```

```
4H₂O₂ + 4-aminoantipyrine + ADPS → Red Quinone + 4H₂O₂
```

The above reaction was used for estimation test, standard and blank. Readings were taken on colorimeter.
The standard concentration: 200 mg/dl
The linearity of the method: 1000 mg/dl
Normal range: 40-140 mg/dl

The reagent kit was from “Autopak”, Bayer's Diagnostic Ltd, Baroda, Gujarat.

3] Estimation of Serum HDL-C : (Method : Phosphotungstate)

Principle:

Chylomicrons, VLDL-C and LDL-C fractions in serum or plasma are separated from HDL by precipitating with phosphotungstic acid and magnesium chloride. After centrifugation, the cholesterol in the HDL-C 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 color is directly proportional to the concentration of HDL-C present in the sample and is read at 500 nm.(34,37)

The above reaction was used for estimating test, standard and blank. Reading was taken on colorimeter.

The standard concentration: 50 mg/dl
The linearity of the method: 100 mg/dl
Normal range: 30-70 mg/dl
The values of LDL-C and VLDL-C can be calculated on the basis of Friedwald’s equation. Where,

\[ a) \text{LDL-C mg/dl} = \frac{\text{Total cholesterol} - \text{[Triglycerides]} + \text{HDL-C}}{5} \]

\[ b) \text{VLDL-C mg/dl} = \frac{\text{Triglycerides}}{38} \]

6] **Estimation of Vitamin A (Carr-Price Reaction)**:

**Principle**:

Proteins are precipitated with ethanol and the retinol and carotenes extracted into light petroleum. After reading the intensity of the yellow colour due to the carotenes the light petroleum is evaporated off and the residue dissolved in chloroform. Carr-Price reagent is added and the amount of blue colour produced is directly proportional to the Vitamin A concentration in the blood.\(^{39}\)

**Reagents**:

1. Absolute ethanol
2. Light petroleum, b.p. 40°C to 60°C
3. A cylinder of carbon dioxide
4. Chloroform
5. Acetic anhydride. Use good quality analytical reagent.
   
   Keep at room temperature in a tightly-stoppered brown bottle, filtering before use if necessary.
7. Stock standard, 500 mg β-carotene/l in light petroleum.
8. Working standard, 10 mg/l. Dilute the stock standard 1 in 50 with light petroleum:

**Procedure:**

Pipette 3 ml serum into a stoppered centrifuge tube and add 3 ml absolute ethanol, slowly drop by drop with shaking, in order to obtain a finely divided precipitate of protein. Add 6 ml light petroleum and shake vigorously for 10 min, then centrifuge at a low speed for about 1 min. to remove any of the watery layer with it.

**Determination of the Carotene:**

Place the light petroleum extract in the colorimeter cuvette and read at 440 nm or with a violet filter using light petroleum as blank. Prepare a standard curve from the working standard as follows:

<table>
<thead>
<tr>
<th>Serum carotenes (mg/l)</th>
<th>0</th>
<th>0.50</th>
<th>1.00</th>
<th>2.00</th>
<th>4.00</th>
<th>6.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard solution (ml)</td>
<td>0</td>
<td>0.25</td>
<td>0.50</td>
<td>1.00</td>
<td>2.00</td>
<td>3.00</td>
</tr>
<tr>
<td>Light petroleum (ml)</td>
<td>10</td>
<td>9.75</td>
<td>9.50</td>
<td>9.00</td>
<td>8.00</td>
<td>7.00</td>
</tr>
</tbody>
</table>

Serum carotene concentration is read directly from this curve. The fact that 2 ml of light petroleum contain the carotenes from 1 ml serum has been taken into account.

**Note.** Carotenes do not keep well since they easily oxidize. They then give a less yellow coloured solution in the light
petroleum. Even freshly bought specimens are not always satisfactory. We have found carotenes supplied by Roche Products Ltd. to be reliable.

7] Estimation of Plasma Ascorbic Acid

Principle:

The acid phosphotungstate (PTA) was found to be specific and sensitive for ascorbic acid (AA) determination in the lens with good reproducibility.

The PTA used serves not only as protein precipitant and ascorbic acid extractant but also colour developing agent, when compared with other methods.(40)

Reagents:

A. A mixture of 20 gms sodium tungstate (Na$_2$Wo$_4$2H$_2$O) and 10 gms disodium hydrogen phosphate (2Na$_2$HpO$_4$ 2H$_2$O) is suspended in 30 ml of water and warmed to dissolve in water bath.

B. To 15 ml of water, 5 ml of sulphuric acid (specific gravity 1.84) is added.

Solution B is poured into warm solution A and then content is boiled gently for 2 hours under reflux (vigorously boiling should be avoided). Since white precipitate may result on cooling and it should be noted that the time of reflux (2 hrs) in precipitating PTA is critical. Less than 2 hours recoveries were usually not good
probably due to the fact that insufficient reflux time would lower
the concentration of PTA (Lanol, 1971). The resulting solution is
then cooled to room temperature on its own. The solution is stable.

**Standard Ascorbic Acid Solution :**

Stock solution 50 mg L-Ascorbic acid is dissolved in 100 ml
of 0.5% oxalic acid solution.

**Working Solution :**

The stock solution is diluted 50 times for a working standard
of 1 mg/100 ml with 0.5% oxalic acid.

**Procedure :**

Take 2 ml of plasma and slowly 2 ml of colour reagent. Mix
(better with glass rod) thoroughly and allow to stand for 30 min at
room temperature (the reaction is completed within 30 minutes
and the colour is stable).

Centrifuge at 3000 rpm for 15 minutes. The blue coloured
supernant is transferred to another test tube carefully with help of
pipette without disturbing the precipitate. Absorbance at 700 nm is
read against a blank constituted with distilled water instead of
homogenate/plasma) which is subjected to all treatment
simultaneously as test samples.
Calculations:

Ascorbic acid concentration =

\[
\frac{\text{O.D. of 'T' - O.D. of 'B'}}{\text{O.D. of 'S' - O.D. of 'B'}} \times \text{Conc. of Std.} = \text{mgs%}
\]

Normal Range of plasma ascorbic acid = 0.4 – 1.5 mgs%

Levels above 0.3 are acceptable range,
0.2 – 0.29 are at risk
< 0.2 indicates deficiency

8] Estimation of Vitamin E (Serum Tocopherol) (41)

Reagent:

2. Absolute ethanol, aldehyde-free.
3. Xylene
4. \(\alpha,\alpha'\)-dipyridyl, 1.20 g/l in \(n\)-propanol
5. Ferric chloride solution, 1.20 g FeCl\(_3\), 6H\(_2\)O/l in ethanol. Keep in a brown bottle.
6. Standard solution of D-L\(\alpha\)-tocopherol, 10 mg/l in ethanol.

Procedure:

Into three stoppered centrifuge tubes measure 1.5 ml serum, 1.5 ml standard and 1.5 ml water (blank) respectively. To test and blank add 1.5 ml ethanol and to the standard 1.5 ml water. Then add 1.5 ml xylene to all the tubes, stopper, mix well, and centrifuge. Transfer 1 ml of the xylene layers into other stoppered
tubes taking care not to include any ethanol or protein. Add 1 ml
$\alpha,\alpha'$-dipyridyl reagent to each tube, stopper and mix. Pipette 1.5
ml of the mixture into colorimeter cuvettes and read the extention
of test and standard against the blank at 460 nm. Then in turn
beginning with the blank and 0.33 ml ferric chloride solution, mix
and after exactly 1.5 min read test and standard against the blank
at 520 nm.\(^{(41)}\)

**Calculation** :

Serum tocopherols (mg/l) =

$$\frac{[\text{Reading of unknown (520 nm)} – \text{Reading (460 nm)} \times 0.29]}{\text{Reading of standard (520 nm)}} \times 10$$

Since the standard contains 10 mg/l.

**Normal range** : 10 to 12 mg/l

9] **Estimation of Nitrous Oxide** :\(^{(42)}\)

**Principle** :

Ethylene diamine dihydrochloride in combination with
sulphanilamide reacts with the nitric oxide present in the
solution/fluid to form a pink coloured complex. The intensity of
this colour is directly proportional to the concentration of nitric
oxide present in the sample.\(^{(42)}\)
Reagent:

1) Ethylene Diamine Dihydrochloride:
   Dissolve 100 mg of ethylene diamine dihydrochloride powder in 100 ml distilled water. Thus concentration obtained was 0.1 mg%.

2) Sulphanilamide:
   1.0 gm of sulphanilamide powder was dissolved in 100 ml distilled water to acquire the concentration of 1 gm%. 2.0 ml of concentrated orthophosphoric acid was added to this solution to enhance the solubility.

3) Sodium Nitrate (NaNO₂) (Standard):
   50 mg of NaNO₂ powder in 50 ml distilled water to obtain the concentration of 1 mg/ml. This stock standard solution was diluted further 4 times by doubled dilution method to obtain a working of 0.125 mg/ml concentration.

   Equal volume of Reagent No. 1 and 3 were mixed to obtain the Griess Reagent (0.1 gm% and 1 gm%) 10 minutes before test.

Procedure:

Three test tubes taken, which were labeled as Blank (B), Standard (S) and Test (T). 1.0 ml distilled water was taken in a test tube labelled as ‘B’. 1.0 ml working standard solution of sodium nitrite was taken in a test tube ‘S’. 1.0 ml whole serum was taken in a test tube ‘T’. 1.0 ml reconstituted Griess reagent was added to
all the tubes ‘B’, ‘S’ and ‘T’. All these tubes were kept for 10 min. at room temperature. Then 1.0 ml distilled water was added to all the tubes to make a volume of 3.0 ml as the cuvette of instrument requires minimum volume of 3.0 ml.

Reading of blank, standard and test sample was recorded on green filter (520-580 nm). These readings were in the form of % transmission, which were then converted into optical density by following the standard conversions chart.

**Calculation** :

Nitric oxide concentration $\mu$gm%

\[
\text{O.D. of ‘T’ – O.D. of ‘B’} = \frac{\text{O.D. of ‘S’ – O.D. of ‘B’}}{\text{ Conc. of Std.}} \times \text{ O.D. of ‘B’}
\]

\[
\frac{T-B}{S-B} = \frac{\text{O.D. of ‘T’ – O.D. of ‘B’}}{\text{O.D. of ‘S’ – O.D. of ‘B’}} \times 125
\]

Nitric oxide concentration was estimated by using above formula and results were obtained.

10] **Estimation of Serum Malondialdehyde** :

**Principle** :

Serum containing lipid peroxide is treated with thiobarbituric acid in presence of 20% trichloroacetic acid. After boiling it in water bath for 15-20 minutes the resulting chromogen is extracted with n-butyl alcohol and measured at 530 nm. Malondialdehyde (MDA)
is used as standard. the lipid peroxide is expressed in terms of nmoles/ml.\textsuperscript{[43]}

**Reagent :**

1. 20% trichloroacetic acid in distilled water
2. 0.05 M H$_2$SO$_4$ – 4.904 ml concentrated H$_2$SO$_4$ per litre with distilled water.
3. 0.2% tiobarbutric acid reagent (TBA) – 200 mgs of TBA was dissolved in 2 M sodium sulphate by boiling and final volume was made to 100 ml.
4. N-butyl alcohol
5. 2 M sodium sulphate solution : In 90 ml of distilled water 28.4 gm of anhydrous sodium sulphate was dissolved by heating and stiring. After cooling volume was made up to 100 ml with water.
6. Standard Solution : Malondialdehyde (1,1,3,3- tetraethoxy propane) was used a sstandard.

**Procedure :**

0.5 ml of serum was taken in the centrifuge tube and 2.5 ml of 20% TCA was added. Tube was left to stand for 10 minutes at room temperature. Supernatant was discarded and precipitate was washed with 0.05 M H$_2$SO$_4$. Then the following additions were made:
1. 0.05 M H$_2$SO$_4$ 2.5 ml
2. 0.2 gm% TBA in 2 M sodium sulphate 3 ml. Mixed and heated boiling water for 15-20 minutes then kept in cold water and 4 ml of n-butyl alcohol was added and mixed vigorously to extract chromogen and centrifuted at 3000 rpm for 10 minutes. The absorbance of organic phase was measured at 530 nm.

From the standard curve, values were calculated.

Different dilutions were prepared from 1,1,3,3- tetraethoxy propane and readings were obtained using above procedure and graph was plotted (Concentrations in nmoles against optical density)

Working Standard :

1,1,3,3- tetraethoxy propane standard solution (10 nmoles)
Molecular weight 164.20. Thus 164.20 ml per 1000 ml distilled water – 1 mol solution.

Or

1.6 ml per 10 ml distilled water = 1 mol) solution
1.0 ml of solution A per 100 ml distilled water =1 mmol) Solution B
1.0 ml of solution B per 100 ml distilled water =1 µmol) Solution C

For 10 nmol solution :

1 ml of solution C diluted to 100 ml with distilled water (10 nmol)

This solution was used as working standard for standard calibration curve.
**Procedure for Standardization:**

Seven test tubes were taken and labeled as S1, S2, S3, S4, S5, S6 and B for standards 1, 2, 3, 4, 5, 6 and blank respectively.

To make various standard concentrations, additions were made as follows:

<table>
<thead>
<tr>
<th>Sr. No. of Std.</th>
<th>Std. No.</th>
<th>Std (10 nmol) ml</th>
<th>D.W. ml</th>
<th>Total ml</th>
<th>Conc. Nmol/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S1</td>
<td>0.5</td>
<td>2.5</td>
<td>3.0</td>
<td>1.67</td>
</tr>
<tr>
<td>2</td>
<td>S2</td>
<td>1.0</td>
<td>2.0</td>
<td>3.0</td>
<td>3.34</td>
</tr>
<tr>
<td>3</td>
<td>S3</td>
<td>105</td>
<td>1.5</td>
<td>3.0</td>
<td>5.10</td>
</tr>
<tr>
<td>4</td>
<td>S4</td>
<td>2.0</td>
<td>1.0</td>
<td>3.0</td>
<td>6.68</td>
</tr>
<tr>
<td>5</td>
<td>S5</td>
<td>205</td>
<td>0.5</td>
<td>3.0</td>
<td>8.35</td>
</tr>
<tr>
<td>6</td>
<td>S6</td>
<td>3.0</td>
<td>--</td>
<td>3.0</td>
<td>10.0</td>
</tr>
<tr>
<td>7</td>
<td>B</td>
<td>--</td>
<td>3.0</td>
<td>3.0</td>
<td>--</td>
</tr>
</tbody>
</table>

Then to each tube, 2.5 ml of 0.05 M $\text{H}_2\text{SO}_4$ was added. Then 3.0 ml of TBA was added, mixed and heated in boiling water bath for 15-20 minutes. Then kept in cold water and 4.0 ml of n-butyl alcohol was added and mixed vigorously to exact chromogen and its absorbance at 530 nm was measured. The graph of absorbance against MDA standard concentration was plotted (Graph No. 1).

Normal range of MDA in serum = 2-10 nmol/ml
11] **Estimation of Serum Reduced Glutathione:**

**Principle:**

5,5’ dithiobis (2-nitrobenzoic acid DTNB) reacts with glutathione (sulfhydryl compound) to form yellow color. Intensity of yellow color is directly proportional to glutathione concentration in the specimen.\(^{(20)}\)

**Specimen:**

EDTA blood (fasting not necessary)

**Method:**

End point reaction

**Reagents:**

1. Lysing solution : 1 g/L, disodium EDTA
2. Precipitating reagent : It contains 1.67 g of metaphosphoric acid, 0.2 g EDTA, 30 g sodium chloride in 100 ml distilled water.
3. Disodium hydrogen phosphate : 300 mmol/L. It contains 107.4 g/L of Na\(_2\)HPO\(_4\).12H\(_2\)O or 53.4 g/L of Na\(_2\)HPO\(_4\).2H\(_2\)O.
4. DTNB reagent : It contain 20 mg of DTNB in 100 ml of buffer (pH 8.0) containing trisodium citrate (1 g/dl).
5. Glutathione standard : 50 mg/dl

**Procedure:**

- Add 0.2 ml of well mixed EDTA blood of which Hb, RBC count and PCV have been determined) to 1.8 ml of lysing solution. Keep at room temperature for 5 minutes.
• Add 3.0 ml of precipitating solution, mix well and keep at room temperature (25°C ± 5°C) for 5 minutes. Filter through Whatman No. 42 filter paper to obtain clear filtrate.

• To 1 ml of clear filtrate add 4 ml of freshly prepared disodium hydrogen phosphate.

• Mix well and read absorbance at 412 nm (TA₁)

• Add 0.5 ml of DTNB reagent, mix well. Keep at room temperature for 10 minutes and read absorbance 412 nm (TA₂).

• Pipette 0.2 ml of glutathione standard in a test tube and add 1.8 ml of lysing solution. Mix well

• Filter through Whatman No. 42 filter paper.

Calculation:

Glutathione, mg/dl in hemolysate

\[
\frac{TA_2 - TA_1}{Std A_2 - Std A_1} \times 50
\]

Calculate glutathione concentration per g Hb and PCV values respectively.

12] Estimation of Blood Urea : (Method : Diacetyl Monoxime-DAM)

Principle:

Under acidic conditions when urea is heated with compounds containing two adjacent carbonyl groups, such as diacetyl (CH₃COCOCH₃), colored products are formed. DAM
(CH₃COC=NOHCH₃) has been usually been used because of greater stability. On heating it, decomposes to given hydroxylamine and diacetyl, which then condenses with urea to give diazine.⁴⁴⁻³⁴

**Chemical Reaction:**

\[
\text{CH}_3\text{COC=NOHCH}_3 + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{CO-CO-CH}_3 + \text{NH}_2\text{OH}
\]

Diacetylmonoxime   Diacetyl    Hydroxylamine

\[
\text{CH}_3\text{CO-CO-CH}_3 + \text{CO(NH}_2\text{)}_2 \rightarrow \text{CH}_3 - \text{C} - \text{C} - \text{CH}_3 + 2\text{H}_2\text{O}
\]

Diacetyl              \[\text{N} \quad \text{N}\]

\[\text{N}\]

\[\text{C}\]

\[\text{O}\]

Diazine

Thiosemicarbazide + FE³⁺ ions are added to catalyse the reaction. Pink colored complex is formed. The intensity of the color is directly proportional to the concentration of urea present in the sample and was measured at 540 nm.

The above reaction was used for estimating test, standard and blank. Readings were taken on colorimeter.

The standard concentration : 50 mg/dl
The linearity of the method : 100 mg/dl
Normal range : 15-40 mg/dl

The reagent kit was from “Autopak”, Bayer’s Diagnostic Ltd, Baroda, Gujarat.
13] **Estimation of Serum Creatinine** : (Method : Jaffe’s Reaction)

**Principle** :

Creatinine which is present in protein free filtrate reacts with picric acid in alkaline medium to form a orange red or yellow tautomer (color complex), the creatinine picrate. This is the Jaffe’s reaction.\(^{(45)}\)

The intensity of the color is directly proportional to the concentration of creatinine present in the sample and was measured at 540 nm.

The above principle was used for estimating test, standard and blank readings were taken on spectrophotometer.

- The standard concentration : 3 mg/dl
- The linearity of the method : 5 mg/dl
- Normal range : 0.7-1.1 mg/dl

The reagent kit was from “Star”, Diagnostic Ltd, Mumbai.

14] **Estimation of serum Uric Acid** : (Method (Henry-Caraway – Phosphotungstic acid))

**Principle** :

Uric acid in the protein free filtrate reacts with phosphotungstic and reagent in the presence of sodium carbonate (alkaline medium) to form a blue colored complex. The intensity of the color formed is directly proportional to the concentration of uric acid present in the sample.
The intensity of color is measured spectrophotometrically at 660 nm.\cite{34,46}

The above principle was used for estimating test, standard and blank readings were taken on spectrophotometer.

The standard concentration : 5 mg/dl

The linearity of the method : 10 mg/dl

Normal range : 3-5 mg/dl

The reagent kit was from “Star”, Diagnostic Ltd, Mumbai.

**Statistical Analysis** :

The value of various parameters obtained from the present study was statistically analyzed. The mean values were calculated and standard deviation (SD) values were obtained. 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 with healthy control subjects.

The values given the tables and the figures are of mean ± S.D.\cite{47}

The probabilities $p<0.05$ were termed as significant while the probabilities $p>0.01$ were termed as non-significant. While probabilities $p<0.001$ was termed as highly significant.\cite{47}