MATERIAL AND METHODS
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The present study was carried out at the Department of Biochemistry, Government Medical College & Wanless Hospital Cardiac Care Unit. Laboratory work was carried out at the Biochemistry Laboratory Padmabhusan Vasantdada Patil General Hospital Sangli, and Government Medical College Miraj and Wanless Hospital Miraj. The study protocol was approved by Ethical Committee of Government Medical College Miraj.

Total numbers of subjects included in the study were 216 subjects the distribution was made as follows

1) Normal healthy controls 100 subjects
2) Coronary heart disease without diabetes 24 subjects.
3) Coronary heart disease with diabetes 24 subjects.
4) Ischaemic heart disease without diabetes 34 subjects
5) Ischaemic heart disease with diabetes 34 subjects.

The subjects of the above groups were in the age group of 45-60 years and all of them were male.

The diagnosis was made by senior Cardiologist after detailed clinical examination, Echo cardiogram, blood sugar, lipid profile were the criteria for the diagnosis.

Sample Collection

Blood samples from patients and controls were collected from anticubital vein with all aseptic precautions, using 10 ml polythene disposable syringe. 3.0ml blood was collected in the bulb with heparin as anticoagulant and remaining 7.0ml blood was collected in polythene tube to avoid glass containing, for the estimation of Vit C, Vit E, copper, zinc and selenium.
Biochemical investigations were carried out with sample of each subject according to the protocol mentioned below-

**Protocol for Laboratory Investigation**

<table>
<thead>
<tr>
<th>No</th>
<th>Estimation</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lipid peroxidation</td>
<td>K.Satoh</td>
</tr>
<tr>
<td>2</td>
<td>Glycosylated Hb</td>
<td>Latex turbidimeter</td>
</tr>
<tr>
<td>3</td>
<td>Blood sugar</td>
<td>GOD POD</td>
</tr>
<tr>
<td>4</td>
<td>Superoxide dismutase</td>
<td>Ransod Kit</td>
</tr>
<tr>
<td>5</td>
<td>Glutathione Peroxidase</td>
<td>Ransel Kit</td>
</tr>
<tr>
<td>6</td>
<td>Ascorbic Acid</td>
<td>Ayekyew Method</td>
</tr>
<tr>
<td>7</td>
<td>Alpha Tocopherol</td>
<td>Baker and Frank method</td>
</tr>
<tr>
<td>8</td>
<td>Cholesterol</td>
<td>CHOD-POD Liquid</td>
</tr>
<tr>
<td>9</td>
<td>Triglycerides</td>
<td>GPO-POD liquid</td>
</tr>
<tr>
<td>10</td>
<td>HDL</td>
<td>Auto HDL</td>
</tr>
<tr>
<td>11</td>
<td>Copper</td>
<td>Atomic Absorption</td>
</tr>
<tr>
<td>12</td>
<td>Zinc</td>
<td>Spectrophotometer</td>
</tr>
<tr>
<td>13</td>
<td>Selenium</td>
<td></td>
</tr>
</tbody>
</table>

Oxidative stress, lipoproteins in cardiovascular dysfunctions
**GLUCOSE**

By Method DPCC – GOD / POD

**Principle**

Glucose oxidase

\[
\text{Glucose} + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{Gluconic acid} + \text{H}_2\text{O}_2
\]

Peroxidase

\[
\text{H}_2\text{O}_2 + \text{Phenol} + 4\text{- Aminoantipyrine} \rightarrow \text{coloured complex} + \text{H}_2\text{O}_2
\]

The red coloured complex formed is measured at 505 nm.

**Content**

Regent 1: Enzyme powder

- Glucose oxidase $\geq 6.7 \ \mu\text{ml}$
- 4- Aminoantipyrine $0.2 \ \text{mM}$
- Phosphate Buffer $8.\text{mM}, \text{PH} \ 7.0$

Regent 2: Phenol

- Phenol $86 \ \text{mM}$

Regent 3: Glucose standard

- Glucose $100 \ \text{mg/dl}$

**Test Procedure: End Point**

<table>
<thead>
<tr>
<th>Pipette into test tube</th>
<th>Blank</th>
<th>Std</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working solution</td>
<td>1.0 ml</td>
<td>1.0 ml 1.0ml</td>
<td></td>
</tr>
<tr>
<td>Std</td>
<td>--</td>
<td>10 $\mu$l</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>10 $\mu$l</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10 $\mu$l</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Mix and incubate at 37°C for 15 minutes at room temperature for 30 minute. Mix and read absorbance of the test (AT) standard (As) and Reagent Blank (AB) at 505 nm or with Green filter against distilled water.

**Calculations**

\[
\text{Glucose (mg/dl) } = \frac{AT - AB}{AS - AB} \times 100
\]

GLYCOHOEMOBLOBIN (HbA1c)

CATION EXCHANGE RESIN METHOD.

Reagents

120 ml bottle 8 mg/ml cation –exchange resin in borate buffer, PH 6.9.
30 ml Bottle Glycohemoglobin lysing Resin, 10 mH Pottasium cyanide surfactant added

40 serum separators.

Procedure

A] Hemolysate Preparation

1. Dispense 500 µl lysing reagent into tubes labeled standard, control etc.
2. Place 100 µ of the well mixed blood sample, standard or control into the opportunity labeled lysing reagent tube mix.
3. Allow to stand for 5 minutes or until completely lysis is evident.

Plastic or glass tubes appropriate size are acceptable.

B] Glycohemoglobin

1. Dispense 3.0 ml of glycohemoglobin cation- exchange resin into 13 x 100 mm glass tubes labeled standard control etc.
2. Add 100 µl of the hemolysate to resin reagent.
3. Position the filter separators in the tubes so that the rubber sleeve is approximately 1 cm above the liquid level.
4. Place the tubes on the rocket or rotator and mix continuously for 5 minutes.
5. Remove the tubes from the rocker or rotator.
6. Push the filter separator into the tubes until the resin is firmly packed.
7. The supernatant may be poured into another tube or directly into a cuvette for absorbance measurement.
8. Zero the instrument at 415 nm (405- nm) with deionized water us the blank.
9. Read the record the absorbance values for standard control etc. These readings are for glycohemoglobin.

**Total Hemoglobin Fraction**

1. Dispense 5.0 ml deionized water into plastic or glass tubes labeled standard control etc.

2. Place 20 µl of the hemolysate (from Step A-3) into the appropriately labeled tube of total hemoglobin diluent Mix.

3. Adjust the instrument to zero absorbance at 415 nm (405-420 nm acceptable) with deionized water as the blank.

4. Read and record the absorbance values for standard control etc.

These readings are for total hemoglobin.

**Calculation**

\[
\text{Unknown} \, (\%) = \frac{R \, (\text{unk})}{R \, (\text{std})} \times \text{std conc.} \, (\%)
\]
ASSAY OF LIPID PEROXIDATION IN SERUM

PRINCIPAL

Trichloro acetic acid acts on serum gives precipitate, which was heated with thiobarbituric acid [TBA], by which coupling of lipid peroxide with TBA gives pink coloured chromogen. The chromogen formed was then extracted with n- butanol and the observation reading of organic phase was noted at 530 nm filters. The result was expressed in terms of n moles of malondialdiyde (MDA) per ml. Malondialdehyde was used as reference standard.

Reagent

1. Trichloroacitic acid [TCA] 20%.
   Dissolve 20gms of TCA in 100 ml distilled water.
2. N/12 H$_2$SO$_4$
3. Thiobarbuituric acid [TBA] 0.2% or 0.67% in 2 m sodium sulphate.
   Dissolved 28.4gms of sodium sulphate in 100ml distilled water. 200 mgs of TBA dissolved in 2M sodium sulphate solutions by heating and final volume was made 100ml.
4. N.Butyle alcohol.
   164.2 mgm MDA [1, 1 ,3, 3 tetramethoxypropane sigma] was dissolved in 1 lit. D.W. that was 1 mole solution. Using these serial dilution were made to prepare solutions of concentration 1 n mol to 8 n mol.

Procedure

A series of standard containing 1.0 to 8.0 n mole of MDA. The volume was made up to 3ml with 0.05 M H$_2$SO$_4$ These tubes were treated exactly like the sample tubes as described below.
**Tubes as described below:**

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum sample</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>20% TCA</td>
<td>2.5 ml</td>
</tr>
</tbody>
</table>

Mixed and allow to stand for 10 minutes.

The tubes were centrifuged at 3500 rpm. After removing the supernant, the precipitate was washed with sulphuric acid [0.05 M] and centrifuged to the ppt.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulphuric acid</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>TBA</td>
<td>1.0 ml [3 ml]</td>
</tr>
</tbody>
</table>

Tube was heated for 30 minutes in boiling water both and cooled.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>N – butanol</td>
<td>4.0 ml</td>
</tr>
</tbody>
</table>

Extraction was done by shaking, followed by centrifugation. Absorbance of colored organic phase was measured on colorimeter at wavelength 530 nm using n – butanol as blank.

By using above procedure absorbance of standard solutions with different concentrations were measured and graph was plotted with concentration in n moles of MDA per ml against absorbance. From graph, values of lipid peroxide in serum were determined.
RANSOD SUPEROXIDE DISMUTASE

Assay Principle

The role of superoxide dismutase (SOD) is to accelerate the dismutatio of the toxic superoxide radical ($O_2^-$) produced during oxidative energy process, to hydrogen peroxide and molecular oxygen.

This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl) 3-(4-nitrophenol)-5 phenylthtraxolium chloride (I.N.T) to form a red formazon dye. The superoxide dismutase activity is then measured by the degree of inhibition of this reaction.

\[
\begin{align*}
\text{XOD} & \\
\text{Xanthine} & \rightarrow \text{uric acid} + O_2^- \\
O_2^- & \rightarrow \text{I.N.T.} \rightarrow \text{formazon dye} \\
SOD & \rightarrow O_2^- + 2H^+ \rightarrow O_2 + H_2O_2
\end{align*}
\]

Sample Preparation

Use heparinized or EDTA whole blood sample. It is recommended that erythrocytes should be washed four times with 0.9% NaCl Solution.

Centrifuge 0.5 ml of whole blood for 10 minutes at 3000 rpm and then aspirate off the plasma.

Then wash erythrocytes four times with 3 ml of 0.9% NaCl solution centrifuging for 10 minutes at 3000 rpm after each wash.

The washed centrifuged erythrocytes should then be made upto 2.0 ml with cold redistilled water, mixed and left to stand at +4°C for is
The lysate is diluted with 0.01 mmol/L phosphate buffer PH 7.0 so that the % inhibition falls between 30% and 60%.

A 25 fold dilution of lysate is recommended for human samples (final dilution factor = 100) and a 50 fold dilution for bavine samples (final dilution factor = 200).

**Reagent composition**

<table>
<thead>
<tr>
<th>Contents</th>
<th>Initial concentration of solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed substrate</td>
<td></td>
</tr>
<tr>
<td>Xanthine</td>
<td>0.05 mmol/l</td>
</tr>
<tr>
<td>I.N.T.</td>
<td>0.025 mmol/l</td>
</tr>
<tr>
<td>Buffer</td>
<td></td>
</tr>
<tr>
<td>CapS</td>
<td>40 mmol/l PH 10.2</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.92 mmol/l</td>
</tr>
<tr>
<td>Xanthine oxidase</td>
<td>80 µ/l</td>
</tr>
<tr>
<td>Standard</td>
<td></td>
</tr>
</tbody>
</table>

**Additional reagents**

Sample diluent Cat No. SD 124 (0.01 mol/l phosphate Buffer, PH 7.0)

**Stability and preparation of reagents**

1. **Mixed substrate**

   Reconstitute the contents of one vial of mixed substrate 1 with 20 ml of buffer 2, stable for 10 days when stored at +2 to +8°C.

2. **Buffer**

   Contents ready for use stable up to the expiry date when stored at +2 to 8°C.

3. **Xanthine oxidase**

   Reconstitute one vial of xanthine oxidase 3 ml 10 ml of redistilled water stable for 2 weeks when stored at +2 to 8°C.
4. Standards:

Reconstitute one vial of standard 4 with 10 ml redistilled water. Subsequent dilutions of the standard should be prepared with ransod sample diluent.

<table>
<thead>
<tr>
<th>Volume of standard soln.</th>
<th>Volume of sample diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>S 6 Undiluted standard</td>
<td>-</td>
</tr>
<tr>
<td>S 5 5 ml of S 6</td>
<td>5 ml</td>
</tr>
<tr>
<td>S 4 5 ml of S 5</td>
<td>5 ml</td>
</tr>
<tr>
<td>S 3 5 ml of S 4</td>
<td>5 ml</td>
</tr>
<tr>
<td>S 2 5 ml of S 3</td>
<td>6 ml</td>
</tr>
</tbody>
</table>

Procedure

<table>
<thead>
<tr>
<th>Wavelength</th>
<th>505 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cuvette</td>
<td>1 cm path length</td>
</tr>
<tr>
<td>Temperature</td>
<td>37°C</td>
</tr>
<tr>
<td>Measurement</td>
<td>Against air</td>
</tr>
</tbody>
</table>

Pipette in cuvette

<table>
<thead>
<tr>
<th></th>
<th>Sample diluents</th>
<th>Standards S2-S6</th>
<th>Diluted sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluted sample</td>
<td>--</td>
<td>--</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>Standard</td>
<td>--</td>
<td>0.05 ml</td>
<td>--</td>
</tr>
<tr>
<td>Ransod sample diluent</td>
<td>0.05 ml</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Mixed substrate</td>
<td>1.7 ml</td>
<td>1.7 ml</td>
<td>1.7 ml</td>
</tr>
</tbody>
</table>

Mix well

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthine oxidase</td>
<td>0.25 ml</td>
<td>0.25 ml</td>
<td>0.25 ml</td>
</tr>
</tbody>
</table>
Mix. read initial absorbance A1, after 30 seconds and start timer simultaneously. Read final absorbance A2 after 3 minutes

**Calculation**

\[
\frac{A_2 - A_1}{3} = \Delta A/ \text{min of standard or sample}
\]

\[
\frac{(\Delta A_{\text{std.}}/\text{min} \times 100)}{100} - \frac{\Delta A_{\text{std.}}}{\text{min}} = \% \text{ inhibition}
\]

\[
\frac{(\Delta A_{\text{sample}}/\text{min} \times 100)}{100} - \frac{\Delta A_{\text{sample}}}{\text{min}} = \% \text{ inhibition}
\]

Plot percentage inhibition for each standard against 10 g 10. Standard concentration in SOD units/ml.

Use percentage inhibition of sample to obtain units of SOD from standard curve.

SOD units/ml of whole blood = SOD units/ml from std curve x dilution factor

Converting to SOD units/gm Haemoglobin,

\[
\frac{\text{SOD units/ml}}{\text{gm Haemoglobin/ml}} = \text{SOD units/gm aemoglobin}
\]
RANSEL GLUTATHIONE PEROXIDASE

UV Method

This method is based on that of Paglia and Valentine. Glutathione peroxidase (GPX) catalyses the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH the oxidized glutathione (GSSG) is immediately converted to the reduced form with a concomitant of NADPH to NADP+. The decrease in absorbance at 340 nm is measured.

Reaction Principle

\[
\text{GSHPx} : 2 \text{GSH} + \text{ROOH} \rightarrow \text{ROH} + \text{GSSG} + \text{H}_2\text{O}
\]

\[
\text{GR} : \text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow \text{NADP}^+ + 2\text{GSH}
\]

Sample preparation:

Use heparinized whole blood

Reagent Composition:

<table>
<thead>
<tr>
<th>Contents</th>
<th>Concentration in the test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent</td>
<td>4 mmol/l</td>
</tr>
<tr>
<td>Glutathione</td>
<td>≥ 0.5 μmol/l</td>
</tr>
<tr>
<td>Glutathione reductase</td>
<td>0.28 mol/L</td>
</tr>
<tr>
<td>NADPH</td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td></td>
</tr>
<tr>
<td>Phosphate Buffer</td>
<td>0.05 mmol/l, PH 7.2</td>
</tr>
<tr>
<td>EDTA</td>
<td>4.3 mol/L</td>
</tr>
<tr>
<td>Cumene hydroperoxide</td>
<td>0.18 mmol/L</td>
</tr>
<tr>
<td>Diluting Agent</td>
<td></td>
</tr>
</tbody>
</table>
STABILITY AND PREPARETATION OF REAGENT

1. **Reagent**
   
   Reconstitute one vial of reagent 1 with the appropriate volume of:
   
   - 6.5 ml for the 8 x 6.5 ml kit
   - 10 ml for the 8 x 10 ml kit
   - 30 ml for the 8 x 30 ml kit
   
   Stable for 48 hrs. at +2 to +8°C for 8 hours at +15 to 25°C.

2. **Buffer**

   Contents ready for use stable up to expiry date when stored at +2 to +8°C.

3. **Cumene Hydroperoxide**

   Dilute 10 µl with 10 ml of redistilled water and mix thoroughly by shaking vigorously as the cumene is difficult to dissolve. Prepare fresh daily. Concentrate stable up to the expiry date when stored at + to +8°C.

   A pipette with a positive displacement action and using glass capillaries, should be used to measure the cumene hydroperoxide volume.

**Procedure**

<table>
<thead>
<tr>
<th>Wavelength</th>
<th>350 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cuvette</td>
<td>1 cm light path</td>
</tr>
<tr>
<td>Temperature</td>
<td>37°C</td>
</tr>
<tr>
<td>Measurement</td>
<td>Against air</td>
</tr>
</tbody>
</table>
Pipette into cuvette

<table>
<thead>
<tr>
<th></th>
<th>Macro</th>
<th>Semi-micro</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diluted sample</td>
<td>Reagent blank</td>
</tr>
<tr>
<td>Diluted sample</td>
<td>0.05 ml</td>
<td>--</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>-</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>Reagent</td>
<td>2.50 ml</td>
<td>2.50 ml</td>
</tr>
<tr>
<td>Cumene</td>
<td>0.10 ml</td>
<td>0.10 ml</td>
</tr>
</tbody>
</table>

Mix, read initial absorbance of sample and reagent blank after one minute and start timer simultaneously. Read again after 1 and 2 minutes. Subtract reagent blank value from that of the sample.

**Calculation**

Glutathione peroxidase concentration may be calculated from the following formula.

\[ \text{U/L of Haemolysate} = 8412 \times \Delta 340 \text{ nm / minute.} \]
Alpha –tocopherol Vitamin-E

The estimation of serum Alpha-Tocopherol was done by colorimeter method of Baker and Frank (1949)

**Principle:**

The serum Alpha –tocopherol is determined by Emmerie-Emmerie Engel reaction which is based on the reduction by tocopherol of ferric to ferrous ions which is then form a red complex with α,α’-Dipyridyl. Tocopherols and carotene are first extracted into xylene and the extinction read at 460 nm to measure carotenes. A correction is made for these after adding ferric chloride and reading at 520 nm.

**Reagents**

1) Absolute ethanol,aldehyde free
2) Xylene
3) α,α'-Dipyridyl Reagents -1.20 g, α,α'-Dipyridyl dissolved in 10000ml n propanol
4) Ferric Chloride solution -120 grm Fecl3, H2O dissolved in 100 ml ethanol.(kept in a brown bottle)

**Procedure:**

Into three stopper centrifuge tubes was measure 1.5 ml serum,1.5 standard, and 1.5 ml water (blank) respectively. Then in test and blank 1.5 ml Xylene was added to all the tubes, stopper mixed well,and centrifuged,1ml of the xylene layers was transferred in to other stopered tubes taking care not to include any ethanol or protein.1ml α,α’-Dipyridyl reagents was added to each tube was stoppered and mixed 1.5 ml of the mixture was pipetted into colorimeter cuvettes and extinction of test and standard was read against the blank at 460 nm. Then in turn beginning with the blank 0.33 ml ferric chloride
solution was added mixed and after exactly 1.5 min extinction test and standard was read against the blank at 520 nm.

Calculations

Serum tocopherol was calculated in mg/l by following formula-

\[
\text{Serum tocopherol (mg/l)} = \frac{(\text{Extinction of unknown at 520nm} - \text{Extinction at 460nm} \times 0.29)}{\text{Extinction of standard at 520nm} \times 10}
\]
Estimation of ascorbic acid (Method – Ayekyaw 1978)

The estimation of plasma L-ascorbic acid was done by colorimetric method.

Principle

Ascorbic acid reduces phosphotungstic acid in acidic medium to blue phosphotungstate chromogen, which has absorption maximum at 700 nm.

Reagents.

1. Phosphotungstic acid - Colour developing solution.

Solution A – 20gms of Sodium Tungstate Na$_2$WO$_4$: 2H$_2$O, Disodium Hydrogen Phosphate Na$_2$HPO$_4$ :2H$_2$O – 10 gm taken in 300ml distilled water and warmed to dissolve.

Solution B – 15ml Distilled water and 5ml of H$_2$SO$_4$ solution B was poured in warm solution A and the content was boiled gently for minimum 2 hours under reflux. The resulting solution was then cooled to room temperature by allowing it to stand on the table and mixture was diluted to 500 ml with distilled water.

2. 0.5% Oxalic acid solution – 0.5 gms of Oxalic acid dissolved in distilled water and volume made upto 100ml.

3. Stock Std. ascorbic acid – This solution was prepared by dissolving 50 mg of L-ascorbic acid in 100ml of 0.5% (W/V) Oxalic acid solution.

4. Working standard solution (1mg/100ml) – Dilute stock standard solution 50 times with 0.5% Oxalic acid.
**Procedure** - 1ml of plasma sample was taken in a test tube marked test ‘T’ and 2ml of distilled water in a test tube marked blank ‘B’.

<table>
<thead>
<tr>
<th></th>
<th>T</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1)</td>
<td>Plasma</td>
<td>1.0ml</td>
</tr>
<tr>
<td>2)</td>
<td>Distilled Water</td>
<td>-</td>
</tr>
<tr>
<td>3)</td>
<td>Coloured reagent</td>
<td>2.0ml</td>
</tr>
</tbody>
</table>

Mix thoroughly and allow standing for 30 minutes at room temperature. Then tubes were centrifuged at 3000 RPM for 10 minutes. The clear supernatent was taken in cuvette without disturbing precipitate and absorbance was measured at 700nm against blank.

**Standardization :-**

5 ml stock solution was diluted to 100 ml with 0.5% oxalic acid. So the concentration of this standard solution is 2.5 mg/100 ml. This standard and serial dilutions of the resulting standards were treated with reagents as shown in the table –

<table>
<thead>
<tr>
<th></th>
<th>B</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Std. S (ml)</td>
<td>0.0</td>
<td>0.2</td>
<td>0.4</td>
<td>0.6</td>
<td>0.8</td>
<td>1.0</td>
<td>1.2</td>
<td>1.4</td>
<td>1.6</td>
<td>1.8</td>
<td>2.0</td>
</tr>
<tr>
<td>Dist. Water (ml)</td>
<td>2.0</td>
<td>1.9</td>
<td>1.6</td>
<td>1.4</td>
<td>1.2</td>
<td>1.0</td>
<td>0.8</td>
<td>0.6</td>
<td>0.4</td>
<td>0.2</td>
<td>0.0</td>
</tr>
<tr>
<td>Color reagent (ml)</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Conc. Of Std. mg/l</td>
<td>2.0</td>
<td>.025</td>
<td>0.50</td>
<td>0.75</td>
<td>1.0</td>
<td>1.25</td>
<td>1.5</td>
<td>1.75</td>
<td>2.0</td>
<td>2.25</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Mixed thoroughly and allowed to stand for 30 minutes at room temperature.

After this the blue colour developed was read at 700 nm. Graph plotted using absorbance (optical density) against concentration.
Calculation -

Serum/Plasma ascorbic acid in mg/dl = \frac{A_{\text{test}} - A_{\text{blank}}}{A_{\text{standard}} - A_{\text{blank}}} \times 1
DETREMINATION OF SERUM COPPER, ZINC AND SELENIUM

Estimation of serum copper, zinc, selenium was done by Atomic Absorption Spectrophotometer (AAS), Parkin Elmer model-3030 at the USIC Department Shivaji University Kolhapur. AAS is an accurate and sensitive analytical method. The advantage of this method was interference by other elements was greatly reduced even when in relatively low amounts.

Principle

Vapourised atoms in the ground state absorb light at very narrowly defined wavelengths. If these atoms in the vapour state are excited they can return in the ground state by emitting light of the same discrete wavelengths as the line spectrum.

In AAS the ionic form of the element is dissociated from its chemical bond and by attesting free electrons produced by the consumption process is placed in the atomic ground state. In this form, it is capable of absorbing light at the specific wavelength of its line spectrum.

\[ A^{++} + 2^- \rightarrow A^0 \]

Ionized metal + Electron \( \rightarrow \) Ground state metal atom

\[ A^0 + h\nu \rightarrow A \]

Ground state metal ion + light \( \rightarrow \) Excited atom

\[ A \rightarrow A^0 + h\nu \]

Excited atom \( \rightarrow \) Atom with ground state energy level + photon

In AAS a beam of radiant energy containing the line spectrums of the element to be measured is passed through a flame containing vaporized metal to be determined. The source of radiant energy is a hollow cathode lamp. The wavelength of absorbed radiant energy is the same as that which would be
emitted if the element were excited. With the aid of monochrometer a measurement is made of the attenuation of one of the wavelengths of the incident light. This attenuation is caused by photons interacting with ground state atoms in the flame. The absorbance of light and concentration of atoms in the flame are related by Beerlambert’s law, only a small percentage of the atoms are in a form capable of absorbing radiant energy emitted by hollow cathode lamp.

**Technique**

The serum samples were approximately diluted with deioned water before aspiration into the burner of AAS. The instrument was equipped with a high solid burner head to avoid clogging due to proteins. The wavelengths selected were 324nm for copper, 213.9nm for zinc and 195 nm for selenium. Commercially available standard solutions were used for calibration and standardization of the instruments.

- For Copper: 4 ppm standard solution
- For zinc: 1 ppm
- For selenium: 30 ppm
CHOLESTEROL

PRINCIPLE OF THE METHOD
The cholesterol present in the sample originates a coloured complex, according to the following reactions:

- Cholesterol esters + H₂O $\xrightarrow{CHE}$ Cholesterol + fatty acids
- Cholesterol + $\overrightarrow{O_2}$ $\xrightarrow{CHOD}$ 4 - Cholesterol + H₂O₂
- 2H₂O₂ +Phenol + 4-AP $\xrightarrow{POD}$ Quinonimine + 4H₂O

The intensity of the color former is proportional to the cholesterol concentration in the sample.

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

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

Clinical diagnosis should not be made on a single test result, it should integrate clinical and other laboratory data.
Oxidative stress, lipoproteins in cardiovascular dysfunctions

R

<table>
<thead>
<tr>
<th>Component</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 6.9</td>
<td>90 mmol/L</td>
</tr>
<tr>
<td>Phenol</td>
<td>26 mmol/L</td>
</tr>
<tr>
<td>Cholesterol esterase (CHE)</td>
<td>1000 U/L</td>
</tr>
<tr>
<td>Cholesterol Oxidase (CHOD)</td>
<td>300 U/L</td>
</tr>
<tr>
<td>Peroxidase (POD)</td>
<td>650 U/L</td>
</tr>
<tr>
<td>4 – Aminophenazone (4-AP)</td>
<td>0.4 mmol/L</td>
</tr>
</tbody>
</table>

CHOLESTROL CAL Cholestrol aqueous primary standard 200 mg/dt.

PREPARATION

All the reagents are ready to use.

STORAGE AND STABILITY

All the components of the kit are stable until the expiration date on the label when stored tightly closed at 2-8°C, protected from light and contaminations prevented during their use.

Do not use reagents over the expiration date.

CHOLESTEROL CAL.

Once open is stable up to 1 month when stored tightly closed at 2-8°C, protected from light and contaminations prevented during their use.

Signs of reagent deterioration:

- Presence of particles and turbidity.
- Blank absorbance (A) at 505 nm \( \geq 0.26 \).

ADDITIONAL EQUIPMENT

- Spectrophotometer or colorimeter measuring at 505 nm.
- Matched cuvettes 1.0 cm light path.
- General laboratory equipment.
SAMPLES
Serum or plasma. Stability of the sample for 7 days at 2.8°C or freezing at -20°C will keep samples stable for a few months.

PROCEDURE
1. Assay conditions:
   - Wavelength: 505 nm (500-550)
   - Cuvette: 1 cm light path
   - Temperature: 37°C / 15-25°C
2. Adjust the instrument to zero with distilled water.
3. Pipette into a cuvette:

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>R (mL)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Standard (uL)</td>
<td>--</td>
<td>10</td>
<td>--</td>
</tr>
<tr>
<td>Sample (uL)</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
</tbody>
</table>
4. Mix and incubate for 5 min at 37°C or 10 min at 15-25°C.
5. Read the absorbance (A) of the samples and calibrator, against the Blank.
   The colour is stable for at least 60 minutes.

CALCULATIONS
(A) Sample x 200 (Standard conc.) = mg/dL. Cholesterol in the sample
(A) Standard
TRIGLYCERIDES

PRINCIPLE OF THE METHOD

Sample triglycerides incubated lipoprotein lipase (L.P.L.), liberate glycerol and free fatty acids. Glycerol is converted to glycerol-3 phosphate (G3P) and adenosine-5-diphosphate (ADP) by glycerol kinase and ATP. Glycerol-3-phosphate (G3P) is then converted by glycerol phosphate dehydrogenase (GPO) to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H$_2$O$_2$).

In the last reaction hydrogen peroxide (H$_2$O$_2$) reacts with 4 aminophenazone (4-AP) and P-chlorophenol in presence of peroxidase (POD) to give a red colored dye:

\[
\text{Triglycerides} + H_2O \xrightarrow{LPL} \text{Glycerol} + \text{free fatty acids} \\
\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol kinase}} \text{Glycerol} + \text{free fatty acids} \\
G3P + \overline{O}_2 \xrightarrow{\text{GPO}} \text{DAP} + H_2O_2 \\
H_2O_2 + 4 – \text{AP} + p-\text{Chlorophenol} \xrightarrow{\text{POD}} \text{Quinone} + H_2O
\]

The intensity of the color formed is proportional to the triglycerides concentration in the sample.

CLINICAL SIGNIFICANCE

Triglycerides are fats that provide energy for the cell.

Like cholesterol, they are delivered to the body’s cells by lipoproteins in the blood. A diet with a lot of saturated fats or carbohydrates will raise the triglyceride levels. The increases in serum triglycerides are relatively non-specific. For example liver dysfunction resulting from hepatitis, extra hepatic
biliary obstruction or cirrhosis, diabetes mellitus is associated with the increase.

Clinical diagnosis should not be made on a single test result: it should integrate clinical and other laboratory data.

**REAGENTS**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOOD pH 7.5</td>
<td>50 mmol/L</td>
</tr>
<tr>
<td>p-Chlorophenol</td>
<td>2 mmol/L</td>
</tr>
<tr>
<td>Lipoprotein lipase (LPL)</td>
<td>150000 U/L</td>
</tr>
<tr>
<td>Glycerol kinase (GK)</td>
<td>500 U/L</td>
</tr>
<tr>
<td>Glycerol-3-oxidase (GPO)</td>
<td>3500 U/L</td>
</tr>
<tr>
<td>4 – Aminophenazone (4-AP)</td>
<td>0.1 mmol/L</td>
</tr>
<tr>
<td>ATP</td>
<td>0.1 mmol/L</td>
</tr>
</tbody>
</table>

**PREPARATION**

Reagent and calibrator provided are ready to use.

**STORAGE AND STABILITY**

All the components of the kit are stable until the expiration date on the label when stored tightly closed at 2-8°C, protected from light and contaminations prevented during their use. Do not use reagents over the expiration date.

TRIGLYCERIDES CAL Once open is stable up to 1 month when stored tightly closed at 2-8°C, protected from light and contaminations prevented during their use.
Signs of reagent deterioration:
- Presence of particles and turbidity.
- Blank absorbance (A) at 505 nm \( \geq 0.40 \).

ADDITIONAL EQUIPMENT
- Spectrophotometer or colorimeter measuring at 505 nm.
- Matched cuvettes 1.0 cm light path.
- General laboratory equipment.

SAMPLES
Serum of plasma
Stability of the sample: Triglycerides are stable for 5 days at 2-8°C

PROCEDURE
1. Assay conditions.
   - Wavelength ......................... 505 nm (490-550)
   - Cuvette ................................. 1 cm light path
   - Temperature ........................... 37°C / 15.25°C
2. Adjust the instrument to zero with distilled water.
3. Pipette into a cuvette.

<table>
<thead>
<tr>
<th>R (mL)</th>
<th>Blank</th>
<th>Standard</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Standard (uL)</td>
<td>--</td>
<td>10</td>
<td>--</td>
</tr>
<tr>
<td>Sample (uL)</td>
<td>--</td>
<td>--</td>
<td>10</td>
</tr>
</tbody>
</table>

4. Mix and incubate for 5 min at 37°C or 10 min at 15.25°C
5. Read the absorbance (A) of the samples and calibrator, against the Blank. The colour is stable for at least 30 minutes.

CALCULATIONS
\[
\frac{(A)_{Sample}}{(A)_{Standard}} = 200(S \tan dardconc.) = \text{mg/dL triglycerides in the sample.}
\]

Conversion factor: mg/dL x 0.0113 = mmol/L.
HDL - CHOLESTEROL

PRINCIPLE OF METHOD

Directly determination of serum HDLc (high-density lipoprotein cholesterol) levels without the need for any pre-treatment or centrifugation of the sample.
The method depends on the properties of a detergent which solubilizes only the HDL, so that the HDL-c is released to react with the cholesterol esterase, cholesterol oxidase and chromogens to give colour. The non HDL lipoproteins LDL, VLDL and chylomicrons are inhibited from reacting with the enzymes due to absorption of the detergents on their surfaces.
The intensity of the color formed is proportional to the HDLc concentration in the sample.

CLINICAL SIGNIFICANCE

HDL particles serve to transport lipoproteins in the blood-stream.
HDL is known as “good cholesterol” because high levels are thought to lower the risk of heart disease and coronary artery disease.
A low HDL cholesterol levels, is considered a greater heart disease risk.\textsuperscript{1,5,6}
Clinical diagnosis should not be made on a single test result: it should integrate clinical and other laboratory data.

REAGENTS

| R 1 | Good pH 7.0                              | 1000 U/L |
|     | Cholesterol oxidase                     |         |
|     | Peroxidase                              | 1300 U/L |
|     | DSBmT                                   | 1 mM    |
| R 2 | Good pH 7.0                              |         |
|     | Cholesterol esterase                    | 1500 U/L |
|     | 4 – Aminoantiphrine (4-AP)              | 1 mM    |
|     | Detergent                               | 2%      |
|     | Ascorbic oxidase                        |         |
| HDLc / LDLc CAL | Calibrator, Lyophilized human serum |         |
PRECAUTION

Components from human origin have been tested and found to be negative for the presence of HBsAg, HCV, and antibody to HIV ('1/2). However handle cautiously as potentially infectious.

PREPARATION

- R1 and R2: Are ready to use.
- HDLc / LDLc CAL: Dissolve the contents with 1 mL of distilled water. Cap vial and mix gently to dissolve contents.

STORAGE AND STABILITY

All the components of the kit are stable until the expiration date on the label when stored tightly closed at 2-8°C and contaminations are prevented during their use. Do not freeze the reagents.
- R1 and R2: Once opened is stable 8 weeks at 2-8°C.
- HDLc / LDLc CAL: Once reconstitute 1 week at 2-8°C or 5 weeks at –20°C.

Do not use reagents over the expiration date.

- Presence of particles and turbidity.

ADDITIONAL EQUIPMENT

- Spectrophotometer or colorimeter measuring at 600 mm.
- Matched cuvettes 1.0 cm light path.
- General laboratory equipment.
SAMPLES
Serum or heparinized plasma, free of hemolysis\(^1\): Anticoagulants containing citrate should not be use.
Removed from the blood clot as soon as possible.
Stability of the sample: 7 days at 2-8\(^\circ\)C.

PROCEDURE
1. Assay conditions:
   - Wavelength: 600-700 nm
   - Cuvette: 1 cm light path
   - Temperature: 37\(^\circ\)C

2. Adjust the instrument to zero with distilled water.
3. Pipette into a cuvette.

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Calibrator</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>R 1 (uL)</td>
<td>300</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>Calibrator (uL)</td>
<td>---</td>
<td>3</td>
<td>---</td>
</tr>
<tr>
<td>Sample (uL)</td>
<td>---</td>
<td>----</td>
<td>3</td>
</tr>
</tbody>
</table>

4. Mix and incubate for 5 min at 37\(^\circ\)C.
5. Read the absorbance \(A_1\) of the samples and calibrator.
6. Add:

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Calibrator</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>R 2 (uL)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

7. Mix and incubate for 5 min at 37\(^\circ\)C.
8. Read the absorbance \(A_2\) of the samples and calibrator, against against the Blank.
9. Calculate the increase of the absorbance \(\Delta A = A_2 - A_1\)
LDL- cholesterol

Calculation of LDL- cholesterol

According to the friedewaid Formula:

\[
LDL \text{ cholesterol} = \text{Total cholesterol} - \frac{\text{Triglycerides}}{5} - \text{HDL cholesterol}
\]