**Subjects:** A total of 600 subjects, 200 rural, 200 urban and 200 suburban population of apparently normal healthy subjects of Dehradun (uttarakhand) of various age groups of either sex were selected. The Manduwala, Bhauwala, Pitambarand Kheli village were selected as rural area. The population of this region was engaged in active physical work and mostly vegetarian in nature. The subjects selected for urban region were residing with in area from Premnagar to I.I.P. Dehradun. The population was mostly service class, business man and omnivorous in nature. The population selected for suburban region is the people living in Jajra and suddowala region. They were mostly farmers, commuters and shopkeepers. The diet is mostly omnivorous in nature. The Myocardial Infarction patients samples were collected with in three days of admission with Central Pathology laboratory of CMI.Dehradun. The diagnosis of MI was established by clinical E.C.G. and serum cardiac enzymes. None of the patients had thyroid dysfunction, liver or kidney disease.

All the subjects gave their informed consent, information regarding occupation, medical history, smoking, drinking habits and use of medication was obtained from all subjects. Height and weight were measured for the subjects and BMI was calculated. Obesity was defined as BMI greater than 27kg/m$^2$ and over weight as BMI greater than 25kg/m$^2$. Blood pressure was recorded in sitting position with mercury sphygmomanometer; hypertension was diagnosed when systolic blood pressure was 140mmHg or more and diastolic blood pressure was 90mmHg or more as per guidelines of United States National Health and Nutritional Assessment Survey. Diabetes mellitus was diagnosed if fasting blood glucose was more than 140mg/dL and post prandial level above 180mg/dl.. In India cigarettes, biddies, Indian pipes, raw tobacco and chewing tobacco were commonly consumed and people used tobacco in more than one form. We therefore characterized user of any form of tobacco as
smokers as was done in other studies. Subjects who admitted to drinking alcohol more than once a week were classified as drinkers. Physical activity was assessed by ascertaining both occupational and recreational activities according to Paffenberger et al., 1993 a person lives a sedentary life style if they walk less than 14.5km a week, climb fewer than 20 flights of stairs a week or perform no moderately vigorous physical activity on any 5 days a week. Subjects with high and low TSH levels were neglected. The Coronary heart disease was diagnosed, if there is documented history of chest pain suggestive of Angina or Infarction and/or previously diagnosed as coronary artery disease. If the subject has ancestral history of Myocardial Infarction or suspected of Coronary heart disease the electrocardiograph studies were done and interpreted with the help of cardiologist. Dietary intake was assessed as per information provided by subjects. In all subjects blood samples were collected in a sitting position using a tourniquet after an overnight or 12 hour fast. Serum was separated and all the serum samples were analyzed within 6 hours of collection.

3.1. Estimation of Cholesterol in serum by the formation of quinoneimine, using kit (Bayer Diagnostics India Ltd)(Allian et al., 1974)

**Principle:** The estimation of cholesterol involves the following enzyme catalyzed reaction.

\[
\begin{align*}
\text{CE} & : \text{Cholesterol ester} \quad \rightarrow \quad \text{Cholesterol + Fatty acid} \\
\text{CHOD} & : \text{Cholesterol + O}_2 \quad \rightarrow \quad \text{Cholest-4-en-3-one + H}_2\text{O}_2 \\
\text{POD} & : 2\text{H}_2\text{O}_2 + 4 -\text{Aminoantipyrine} + \text{Phenol} \quad \rightarrow \quad 4\text{H}_2\text{O}_2 + \text{Quinoneimine}
\end{align*}
\]

CE = Cholesterol Esterase, CHOD = Cholesterol Oxidase, POD = Phenol oxidase

The concentration of Cholesterol in the sample is directly proportional to the intensity of the red complex (Red Quinone) which is measured at 500nm.
Reagents:

<table>
<thead>
<tr>
<th>Reagent 1(Enzyme /Chromogen)</th>
<th>Reagent 1A(Buffer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol esterase ≥200 u/L</td>
<td>Pipes buffer pH 6.90 50mmol/L</td>
</tr>
<tr>
<td>Cholesterol Oxidase ≥200 u/L</td>
<td>Phenol 24mmol/L</td>
</tr>
<tr>
<td>Peroxidase ≥1000u/l</td>
<td>Sodium Cholate 0.5mmol/L</td>
</tr>
<tr>
<td>4-Aminoantipyrine</td>
<td>0.5mmol/L</td>
</tr>
</tbody>
</table>

**Standard(Cholesterol 200mg/dl)**

**Reagent Preparation:** The reagents were allowed to attain room temperature. Reagent 1 and reagent 1A are mixed by gentle swirling.

**Procedure:** The samples and the reconstituted reagent brought to room temperature prior to use. The regents were added as mentioned in the following table.

<table>
<thead>
<tr>
<th>Pipette into tubes Marked</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Reagent</td>
<td>1000µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Distill water</td>
<td>20 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>20 µl</td>
<td>-</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

All the content were mixed well with the help of cyclomixture and incubated for 37°C for 10 min. The absorbance was measured in semi automated analyzer SPAN AUTO CAM 2011. Absorbance of Blank, standard and sample tube was read against water at 505 nm.

**Calculation:**

\[
\text{Cholesterol (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of Standard}} \times \text{concentration of standard}
\]

Concentration. Of Standard=200mg/dl
**Comment:** This method has linearity of 500 mg/dl; the advantage of using this method is that it is specific for cholesterol and cholesterol esters and does not use corrosive reagents such as acetic anhydride and concentrated sulphuric acid as apposed to Watson method.

3.2. **Estimation of triglyceride in serum by the formation of Quinineimine or by calculating the intensity of chromogen Using kit (Bayer Diagnostics India Ltd)**(Buccolo and David ,1973; Werner et al.,1981)

**Principle:** The estimation of triglycerides involves the following enzyme catalyzed reaction.

\[
\text{LpL} \quad \text{Triglycerides} + \text{H}_2\text{O} \xrightarrow{\text{LpL}} \text{Glycerol} + \text{Fatty Acid}
\]

\[
\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-Phosphate} + \text{ADP}
\]

\[
2\text{H}_2\text{O}_2 + 4\text{Aminoantipyrine} + \text{ADPS} \xrightarrow{\text{Peroxidase}} \text{Red Quinone} + 4\text{H}_2\text{O}
\]

\[
\text{LpL} = \text{Lipoprotein Lipase}, \text{GK} = \text{Glycerol Kinase}, \text{GPO} = \text{Glycerol-3-Phosphate Oxidase}, \text{ADPS} = \text{N-Ethyl-N-Sulfopropyl-n-anisidine}
\]

The intensity of purple colored complex formed during the reaction is directly proportional to the triglycerides concentration in the sample and is measured at 546 nm.
Material and Methods

Reagents:

<table>
<thead>
<tr>
<th>Reagent 1(Enzyme /Chromogen)</th>
<th>Reagent 1A(Buffer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipoprotein Lipase</td>
<td>≥1100 u/L</td>
</tr>
<tr>
<td>Glycerol Kinase</td>
<td>≥800 u/L</td>
</tr>
<tr>
<td>Glycerol-3-Phosphate Oxidase</td>
<td>≥5000 u/l</td>
</tr>
<tr>
<td>Aminoantipyrine</td>
<td>0.7 mmol/L</td>
</tr>
<tr>
<td>ATP</td>
<td>0.3 mmol/L</td>
</tr>
</tbody>
</table>

Standard(Triglycerides 200mg/dl):

Glycerol 2gm/dl

Reagent Preparation: The reagents are allowed to attain room temperature reagent 1 and reagent 1A are mixed by gentle swirling

Procedure:

<table>
<thead>
<tr>
<th>Pipette into tubes</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marked</td>
<td>1000µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Working Reagent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distill water</td>
<td>10 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>10 µl</td>
<td>-</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

All the content were mixed well with the help of cyclomixer and incubated for 37°C for 10 min. The absorbance was measured in semi automated analyzer SPAN AUTO CAM 2011. Absorbance of Blank, standard and sample tube was read against water at 546 nm.

Calculation:  
Triglycerides (mg/dl) = \[\frac{\text{Absorbance of test}}{\text{Absorbance of Standard}} \times \text{conc of standard (mg/dl)}\]

Concentration. Of Standard=200mg/dl
3.3. Estimation of HDL-Cholesterol in serum by Phosphotungstate method using kit (Bayer Diagnostics India Ltd) (Burnstein et al., 1973)

**Principle:** Chylomicrons, VLDL (Very Low Density Lipoprotein) and LDL (Low Density Lipoprotein) fractions in serum or plasma are separated from HDL by precipitating with Phosphotungstic Acid and Magnesium Chloride. The HDL-Cholesterol remains unaffected in the supernatant.

\[
\text{Serum + Phosphotungstate} \rightarrow \text{HDL (Supernatant) + LDL, VLDL, Chylomicron (Precipitate)}
\]

**Reagents:**

<table>
<thead>
<tr>
<th>Reagent 1 (Enzyme/Chromogen)</th>
<th>Reagent 1A (Buffer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol esterase ≥ 200 u/L</td>
<td>Pipes buffer pH 6.90 50mmol/L</td>
</tr>
<tr>
<td>Cholesterol Oxidase ≥ 200 u/L</td>
<td>Phenol 24mmol/L</td>
</tr>
<tr>
<td>Peroxidase ≥ 1000 u/L</td>
<td>Sodium Cholate 0.5mmol/L</td>
</tr>
<tr>
<td>4-Aminoantipyrine 0.5mmol/L</td>
<td></td>
</tr>
</tbody>
</table>

**Standard (Cholesterol 50 mg/dl)**

**Precipitating Reagent:**

- Phosphotungstic acid 2.4 mmol/L
- Magnesium Chloride: 39 mmol/L

**Working Reagent Preparation:** The reagents were allowed to attain room temperature. Reagent 1 and Reagent 1A are mixed by gentle swirling.

**Procedure**

Precipitation of LDL, VLDL and Chylomicrons.

1. 125 µl sample was pipetted and 250 µl precipitant reagent was added in it.
2. It was mixed well and allowed the reaction mixture to stand for 10 min at room temperature.

3. It was Centrifuged at 4000rpm for 10 min to obtain a clear suspension.

4. Supernatant was added to determine the concentration of HDL-cholesterol in the sample by following procedure.

<table>
<thead>
<tr>
<th>Pipette into tubes Marked</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol Working reagent</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>50 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HDL Standard</td>
<td>-</td>
<td>50 µl</td>
<td>-</td>
</tr>
<tr>
<td>Supernatant</td>
<td>-</td>
<td>-</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

All the content were mixed well with the help of cyclomixture and incubated for 37°C for 10 min. The absorbance was measured in semi automated analyzer SPAN AUTO CAM 2011. Absorbance of Blank, standard and sample tube was read against water at 505 nm.

**Calculation:**

HDL Cholesterol (mg/dl) = \( \frac{\text{Absorbance of test}}{\text{Absorbance of Standard}} \times \text{conc. of standard (mg/dl)} \)

Conc. of Standard=100mg/dl

**3.4. Calculation of VLDL (Very Low Density Lipoprotein cholesterol) by Friedwald equation (Friedwald et al.,1972)**

\[
\text{VLDL (mg/dl)} = \frac{\text{Triglyceride}}{5}
\]
3.5. Calculation of LDL-Cholesterol (Low Density Lipoprotein cholesterol) by Friedwald equation (Friedwald et al., 1972)

\[ \text{LDL (mg/dl)} = \text{Total Cholesterol} - (\text{HDL} + \text{Triglycerides}/5) \]

3.6. Estimation of Phospholipids in serum by precipitation (Gomorri et al., 1942)

**Principle:** The phospholipids are precipitated by using trichloro acetic acid and estimated by using molybedate reagent and metol which forms coloured complex with phospholipids the intensity of which can be read at 660 nm.

**Preparation of Reagents:**

**Trichloro acetic acid reagent (10gm/dl):** It was prepared by dissolving 12gm trichloro acetic acid in 100 ml of water

**Molybdate reagent:** It was prepared by dissolving 1.0 gm of metol in 100 ml of 3g/dl Sodium Meta bisulphite solution.

**Phosphorous Standard :( 250mg/dl) It was prepared by dissolving 10.98 gm in one liter of glass distilled water.**

**Procedure:**

In a centrifuge tube following were pipetted

Sample, ml : 0.1

TCA reagent, ml : 2.0

It was mixed well and centrifuge for 10 minutes.

The following requirements were pipetted in the test tubes labeled as blank and standard and in centrifuge tube, labeled as test:

<table>
<thead>
<tr>
<th>Pipette into tubes</th>
<th>Marked</th>
<th>Blank</th>
<th>Std.</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perchloric acid: 70%, ml</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Phosphorous std: 5.0 mg/dl, ml</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Hydrogen Peroxide, ml</td>
<td></td>
<td>0.2</td>
<td>0.2</td>
<td></td>
</tr>
</tbody>
</table>
The contents were mixed well with the help of cyclomixer place into water bath at 180°-200°C for 15 minutes. The tubes were cooled under the running water and the following requirements were pipetted in these tubes:

<table>
<thead>
<tr>
<th>Pipette into tubes</th>
<th>Marked</th>
<th>Blank</th>
<th>Std.</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass distilled water, ml</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Molybdate reagent, ml</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Colour reagent, ml</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
</tr>
</tbody>
</table>

The contents were mixed well and after 10 minutes the absorbance were read at 660 nm.

**Calculation:**

\[
\text{Phospholipids (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of Standard}} \times (250 \text{ mg/dl})
\]

3.7. Calculation of Total Lipids (Sharma et al., 2004):

Total Lipids = Total Cholesterol + Triglycerides


**Principle:** Antiserum to Apo A-1 is diluted in buffer and added to an aliquot of patient of serum. The light scattering caused by antigen–antibody complexes is measured after incubation. The resulting light scattering is directly proportional to the apo A-1 concentration in the sample. Precalibration of the curve is defined by the batch specific parameters on a magnetic card. The single point calibration to check the curve during the assay is made with the calibrator solution found in the kit. The results are expressed as concentration units (g/l)
<table>
<thead>
<tr>
<th>S.No.</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Apo A-1 Buffer</td>
</tr>
<tr>
<td>2.</td>
<td>Apo A-1 Blank Buffer</td>
</tr>
<tr>
<td>3.</td>
<td>Apo A-1 Antiserum Reagent (Swine)</td>
</tr>
<tr>
<td>4.</td>
<td>Calibrator (Lyophilised)</td>
</tr>
<tr>
<td>5.</td>
<td>Magnetic Card</td>
</tr>
</tbody>
</table>

**Preparation of Reagents:**

**Antiserum Buffer solution:** 500 µl Antiserum reagent was pipetted into the reagent buffer (30ml). It was mixed gently. Solution is stable for three months when stored at 2-8°C.

**Blank Buffer:** It was ready to use.

**Calibrator:** It was reconstituted with 1.0 ml deionized water. The reconstituted calibrator is stable for three months when stored at 2-8°C. It was diluted to 1:51 (1+50) with 0.9% NaCl.

**Assay Procedure:**

1. Separate sample blank for each sample as well as a calibrator blank for the calibrator was prepared.

2. The calibrator were prepared as a duplicate

3. The following requirements were pipetted into cuvettes (µl)

<table>
<thead>
<tr>
<th></th>
<th>Calibrator</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blank</td>
<td>test</td>
</tr>
<tr>
<td></td>
<td>Blank</td>
<td>Test</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>30µl</td>
</tr>
<tr>
<td></td>
<td>30µl</td>
<td>30µl</td>
</tr>
<tr>
<td>Calibrator</td>
<td>30µl</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>30µl</td>
<td>-</td>
</tr>
<tr>
<td>Blank Buffer</td>
<td>500µl</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500µl</td>
</tr>
<tr>
<td>Antiserum Dilution</td>
<td>-</td>
<td>500µl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500µl</td>
</tr>
</tbody>
</table>
Material and Methods

Biochemical characterization of lipoprotein fraction of major segment of population in Dehradun region

4. It was mixed by shaking gently
5. It was allowed to stand for 30±5 minutes at room temperature

Measurement:
1. Each cuvette was mixed gently before measuring
2. The calibrators were measured according to the instructions of the instruments
3. The cuvettes were read in the following order:
   - Calibrator blank
   - Calibrator test 1
   - Calibrator test 2
   - Sample 1 blank
   - Sample 1 test
   - Sample 2 blank
   - Sample 2 Test
4. END. is pressed after the last cuvette was measured press
5. The calibrator concentration of 100mg/dl was used.

3.9. Estimation of apolipoprotein B by Nephelometric Method (Orion Diagnostica) (Jungner et al., 1998)

Principle: Antiserum to apo B is diluted in buffer and added to an aliquot of patient of serum. The light scattering caused by antigen–antibody complexes is measured after incubation. The resulting light scattering is directly proportional to the Apo A-1 concentration in the sample. Precalibration of the curve is defined by the batch specific parameters on a magnetic card. The Single point calibration to check the curve during the assay is made with the calibrator solution found in the kit. The results are expressed as concentration units (g/l)
## Material and Methods

### Biochemical characterization of lipoprotein fraction of major segment of population in Dehradun region

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Apo B Buffer</td>
</tr>
<tr>
<td>2.</td>
<td>Apo B Blank Buffer</td>
</tr>
<tr>
<td>3.</td>
<td>Apo B Antiserum Reagent (Swine)</td>
</tr>
<tr>
<td>4.</td>
<td>Calibrator (Lyophilised)</td>
</tr>
<tr>
<td>5.</td>
<td>Magnetic Card</td>
</tr>
</tbody>
</table>

### Preparation of Reagents:

**Antiserum Buffer solution:** 500 µl Antiserum reagent was pipetted into the reagent buffer (30 ml). It was mixed gently. Solution is stable for three months when stored at 2-8°C.

**Blank Buffer:** It was ready to use.

**Calibrator:** It was reconstituted with 1.0 ml deionized water. The reconstituted calibrator is stable for three months when stored at 2-8°C. It was diluted to 1:51 (1+50) with 0.9% NaCl.

### Assay Procedure:

1. Separate sample blank for each sample as well as a calibrator blank for the calibrator was prepared.

2. The calibrator were prepared as a duplicate.

3. The following requirement were pipetted into cuvettes (µl)

<table>
<thead>
<tr>
<th></th>
<th>Calibrator</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blank</td>
<td>test</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Calibrator</td>
<td>30 µl</td>
<td>30 µl</td>
</tr>
<tr>
<td>Blank Buffer</td>
<td>500 µl</td>
<td>-</td>
</tr>
<tr>
<td>Anti Serum Dilution</td>
<td>-</td>
<td>500 µl</td>
</tr>
</tbody>
</table>
4. It was mixed by shaking gently
5. It was allowed to stand for 30±5 minutes at room temperature

**Measurement:**

1. Each cuvette was mixed gently before measuring
2. The calibrators were measured according to the instructions of the instruments
3. The cuvettes were read in the following order:
   - Calibrator blank
   - Calibrator test 1
   - Calibrator test 2
   - Sample 1 blank
   - Sample 1 test
   - Sample 2 blank
   - Sample 2 test
4. END is pressed after the last cuvette was measured press
5. The calibrator concentration of 100mg/dl was used.

3.10 Apolipoprotein A1 (apo A1) determination by ELISA (Human Diagnostics) (Labeur et al., 1990)

**Principle of Procedure:** Solid phase capture sandwich ELISA assay using a microwell format. The polystyrene microplate strips have been coated Anti-Human apo A1, which constitutes the solid-phase antibody. The test sample is incubated in such well; Apo A1, if present in the sample or standard solution, will bind to the solid-phase antibody. Unbound substances are removed by washing the plate. Subsequently a sheep goat anti-apo A1, which has been labeled with the enzyme horse-radish peroxidase (HRP), is added. This labeled antibody binds to any solid-phase antibody/ apo A1 complex previously formed. Incubation with enzyme...
substrate produces a blue colour in the test well, which turns into yellow when the reaction is stopped with sulphuric acid. The amount of color produced in the wells is proportional to the amount of apo A1 originally present in the sample or standard solution.

**Reagents:**

1. Anti-Human Apo A1 coated microwell strips 12 x 8 with plastic frame
2. HRP conjugated affinity purified goat anti-Apo A1 - 12mL
3. Apo A1 standard (pre-diluted 1:10,000) - 1 ml
4. TMB/peroxide substrate color developer - 12mL
5. Sulphuric acid termination reagent (0.5N) - 12mL
6. 15 X Wash buffer concentrate - 2x60mL

**Reagent Preparation:**

**Wash Buffer Preparation:** The 15X wash buffer was diluted 1:15 using one part wash buffer concentrate and 14 parts reagent grade water.

**Patient Dilutions:** Each serum specimen to be tested was diluted 1:10,000 with diluted wash buffer to form a final 1:10,000 dilution.

**Standard Dilutions** serial two fold dilutions of the human apo A1 standard were prepared using diluted wash buffer as follows:
Material and Methods

Biochemical characterization of lipoprotein fraction of major segment of population in Dehradun region

<table>
<thead>
<tr>
<th>S.No</th>
<th>Standard Point</th>
<th>Dilution</th>
<th>[Apo A1] (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>P1</td>
<td>1 part Standard</td>
<td>320.0</td>
</tr>
<tr>
<td>2.</td>
<td>P2</td>
<td>1 part P1 + 1 part diluted wash buffer</td>
<td>160.0</td>
</tr>
<tr>
<td>3.</td>
<td>P3</td>
<td>1 part P2 + 1 part diluted wash buffer</td>
<td>80.0</td>
</tr>
<tr>
<td>4.</td>
<td>P4</td>
<td>1 part P3 + 1 part diluted wash buffer</td>
<td>40.0</td>
</tr>
<tr>
<td>5.</td>
<td>P5</td>
<td>1 part P4 + 1 part diluted wash buffer</td>
<td>20.0</td>
</tr>
<tr>
<td>6.</td>
<td>P6</td>
<td>1 part P5 + 1 part diluted wash buffer</td>
<td>10.0</td>
</tr>
<tr>
<td>7.</td>
<td>P7</td>
<td>1 part P6 + 1 part diluted wash buffer</td>
<td>5.0</td>
</tr>
<tr>
<td>8.</td>
<td>P8</td>
<td>1 part diluted wash buffer</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Assay Procedure:

1. 100uL of diluted specimen or standard to each microwell was added.
2. The well was incubated at room temperature for 2 hours.
3. Decanted and each microwell was washed four times with diluted wash buffer.
4. 100uL of HRP conjugated goat anti-apo A1 to each well was added
5. It was incubated at room temperature for 2 hours.
6. The step 3 was repeated again.
7. The 100uL of TMB/peroxide substrate was added and incubated at room temperature for 30 minutes.
8. The reaction was terminated with 100uL of 0.18N sulfuric acid.
9. The absorbance was measured with the help of ELISA reader at 450 nm.
10. A standard curve using the absorbance values obtained for each of the standards was constructed.
11. The unknowns from the standard curve were calculated.

3.11 Apolipoprotein B (apo B) determination using ELISA (Human Diagnostics) (Labeur et al., 1990)

**Principle of Procedure:** Solid phase capture sandwich ELISA assay using a microwell format. The polystyrene microplate strips have been coated Anti-Human apo B, which constitutes the solid-phase antibody. The test sample is incubated in such well; Apo B, if present in the sample or standard solution, will bind to the solid-phase antibody. Unbound substances are removed by washing the plate. Subsequently
a sheep goat anti-apo B, which has been labeled with the enzyme horse-radish peroxidase (HRP), is added. This labeled antibody binds to any solid-phase antibody/apo B complex previously formed. Incubation with enzyme substrate produces a blue colour in the test well, which turns into yellow when the reaction is stopped with sulphuric acid. The amount of color produced in the wells is proportional to the amount of apo B originally present in the sample or standard solution.

**Reagents:**

1. Anti-Human apo B coated microwell strips 12 x 8 with plastic frame
2. HRP conjugated affinity purified goat anti-apo B - 12mL
3. Apo B standard (pre-diluted 1:10,000) - 1 ml
4. TMB/peroxide substrate color developer - 12mL
5. Sulfuric acid termination reagent (0.5N) - 12mL
6. 15 X Wash buffer concentrate - 2x60mL
7. Apo B specimen diluent - 60mL

**Reagent Preparation:**

**Wash Buffer Preparation:** The 15X wash buffer was diluted 1:15 using one part wash buffer concentrate and 14 parts reagent grade water

**Patient Dilutions:** Dilute each serum to be tested was diluted 1:1,000 by first diluting 1:100 in PBS and then making a subsequent 1:10 dilution with the apo B specimen diluent provided to form a final dilution of 1:1,000.

**Standard Dilutions:** The serial two fold dilutions of the human apo B standard was prepared with the specimen diluent provided as given below.
Material and Methods

Biochemical characterization of lipoprotein fraction of major segment of population in Dehradun region

The specimen diluent alone as the blank control well was used.

**Assay Procedure:**

1. The 100uL of diluted specimen or standard was added to each microwell
2. The well was incubated at room temperature for 45 minutes
3. Well were decanted and washing was done five times with diluted wash buffer
4. The 100uL of HRP conjugated goat anti-Apo B to each well was added.
5. The plate was incubated at room temperature for 45 minutes
6. Step 3 was repeated.
7. The 100uL of TMB/peroxide substrate was added and incubate at room temperature for 15 minutes
8. Terminate The reaction was terminated with 100uL of 0.18N sulfuric acid
9. The absorbance was measured with the help of ELISA reader at 450 nm
10. A standard curve using the O.D. values obtained for each of the standards was constructed.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Standard Point</th>
<th>Dilution</th>
<th>[Apo B1] (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>P1</td>
<td>1 part Standard</td>
<td>260.0</td>
</tr>
<tr>
<td>2</td>
<td>P2</td>
<td>1 part P1 + 1 part Specimen diluent</td>
<td>130.0</td>
</tr>
<tr>
<td>3</td>
<td>P3</td>
<td>1 part P2 + 1 part Specimen diluent</td>
<td>65.0</td>
</tr>
<tr>
<td>4</td>
<td>P4</td>
<td>1 part P3 + 1 part Specimen diluent</td>
<td>32.5</td>
</tr>
<tr>
<td>5</td>
<td>P5</td>
<td>1 part P4 + 1 part Specimen diluent</td>
<td>16.25</td>
</tr>
<tr>
<td>6</td>
<td>P6</td>
<td>1 part P5 + 1 part Specimen diluent</td>
<td>8.12</td>
</tr>
<tr>
<td>8</td>
<td>P7</td>
<td>1 part Specimen diluent</td>
<td>0.00</td>
</tr>
</tbody>
</table>
11. The unknowns from the standard curve were calculated

3.12 Fractionation of Lipoprotein by Agarose Gel Electrophoresis (Noble.,1968)

**Principle:** Lipoproteins are colloidal in nature. Lipoproteins are composed of proteins and lipid molecules. The protein residues when subjected to pH 8.0 and the electric current all the lipoproteins behave like anions and moves towards the anode. The rate of migration depends upon their different molecular weights and sizes. Thus, high density lipoprotein (HDL) is the fastest moving component, followed by alpha (α), beta(β) and pre-β-lipoprotein. LDL migrates with the β globulins, VLDL and LP[a] between the α and β globulins.

**Reagents:**

1. **Phosphate buffer: pH 8.6(0.075 M)**

   - Monobasic sodium phosphate (x): It was prepared by mixing 2.92 gm of mono basic sodium phosphate in 250 ml of distilled water.
> Dibasic sodium phosphate (y) It was prepared by mixing 3.33 gm of Di
basic sodium phosphate in 250 ml of distilled water
> The above solution were mixed in a ratio 5.3(x) and 94.6(y): the final
volume was made to 200 ml after maintaining the pH to 8.6

2. **Stain (Sudan Black 1.0 gm/dl in 60% (v/v) ethanol):** 60%(v/v) ethanol was
prepared by adding 40 ml water to 60 ml ethanol and 1 gm sudan black wad
added to it.

3. **Destaining solution (50% (v/v) ethanol):** It was prepared by adding 50 ml
water to 50 ml ethanol.

4. **Fixative:** It was prepared by mixing 70 parts of Isoproponol, 25 parts of
distilled water and 5 parts of Glacial acetic acid.

5. **Dehydrating solution:** 90 parts of acetone and 10 parts of distill water were
mixed to get this solution.

6. **Sodium hydroxide(40 gm/dl):** It was prepared by dissolving 40 gm of NaOH
in 100 ml of water.

**Procedure:**

1. A clean dry gel casting plate was taken and a gel mould was prepared using
an adhesive tape along the sides of the plate to prevent running of the material
poured on the plate

2. The gel was prepared by dissolving 200 mg or 0.2gm Agarose in 20 ml
phosphate buffer (pH 8.6, 0.075 M).Added 20 ml of phosphate buffer (pH 8.0)
in 0.2 gm of agarose in conical flask and boiled for 2-3 min and kept at 50\(^{\circ}\)C
and then poured on to the gel casting plate. Immediately the supplied comb
was placed about 1 c.m. from one end of the plate in the gel ensuring the teeth
of the comb do not touch the glass plate
3. The comb and the tape surrounding the plate were removed carefully and the
gel was transferred to the electrophoretic tank such that wells were towards the
cathode.
4. Phosphate buffer was poured into the tank until the gel was completely
submerged.
5. Sample loading: 8-10 µl of the serum was applied by means of micropipette.
6. Amido Black stain (1gm/dl) was loaded in one of the wells as tracking dye.
7. The power supply was turned on and runned at 100 V (10-15 mA).
8. The gel was run for 2 hour at room temperature at constant voltage supply of
50 volt.
9. Turned off the power supply when the tracking dye had reached near the
opposite edge of the gel.
10. The gel was from transferred from electrophoretic tank in the fixative for 30
min.
11. The gel was dipped in the dehydrating solution for about 4 hrs.
12. The gel was dried in the incubator at 37°C.
13. Gel was dipped in the stain for 1 hr (added 1 drop of 40 gm/dl of sodium
hydroxide in the stain just before use).
14. The gel was dipped in the destaining solution for over night and afterwards in
the tap water till the background of the gel was clear. Gel was observed for the
presence of lipoprotein bands.

3.13. Estimation of LP[a] by Nephelometry using kit (Diachi Diagnostics)
(Berg., 1963)

**Principle:** LP[a] in serum reacts with antihuman LP[a] mouse monoclonal antibody
coated latex and agglutination occurs. LP[a] concentration is determined by
measurement of the change in turbidity that result from the agglutination reaction of Lp (a) with the antibody coated Latex. The reaction sequences of the assay are shown below.

**Reaction:** LP[a]+Antihuman monoclonal antibody coated latex

\[ \text{Immune complexes} \rightarrow \text{absorbance measurement} \]

**Procedure**

<table>
<thead>
<tr>
<th>Sample + Reagent 1 (5 µl)</th>
<th>37°C 5min</th>
<th>Regent2 (100 µl)</th>
<th>37°C 1min</th>
<th>Measurement (Abs.I)</th>
</tr>
</thead>
</table>

\[ \text{Abs I, II: Absorbance at 600nm} \]

**Calculation:**

\[ \text{Lp (a) (mg/dl) = } \frac{\text{Absorbance of test}}{\text{Absorbance of Standard}} x (12.5 \text{ mg/dl}) \]

**3.14 LP[a] Estimation by ELISA using kit (Bio Med Diagnostics) (Wang et al., 1992):**

**Principle:** The polystyrene microplate strips have been coated with a mouse monoclonal anti-LP[a] [antibody to LP[a]], which constitutes the solid-phase antibody. The test sample is incubated in such well; LP[a], if present in the sample or standard solution, will bind to the solid-phase antibody. Unbound substances are removed by washing the plate. Subsequently a sheep anti-apo B polyclonal antibody, which has been labeled with the enzyme horse-radish peroxidase (HRP), is added. This labeled antibody binds to any solid-phase antibody/LP[a] complex previously formed, because it can bind the apo B moiety of the LP[a] complex. Incubation with enzyme substrate produces a blue colour in the test well, which turns into yellow when the reaction is stopped with sulphuric acid. The amount of color produced in the
wells is proportional to the amount of LP[a] originally present in the sample or standard solution.

**Reagents :**

1. 1 sachet containing a strip-holder with 12x8 anti-LP[a] (mouse monoclonal) coated test wells and a silica gel bag as drying agent.
2. 1 vial containing 0.250 ml of a prediluted LP[a] standard labeled as 100 mg/dl (Phosphate buffer with stabilizing proteins, containing 0.05% Kathon CG as preservative).
3. 1 vial containing 0.25 ml of lyophilized control serum Level I (5.0 mg/dl)
4. 1 vial containing 0.25 ml of lyophilized control serum Level II (25.0 mg/dl)
5. 2 vials containing 25 ml of concentrated sample diluent (phosphate buffer with stabilizing proteins, containing 0.05% Kathon CG as preservative), to be diluted 10 x before use.
6. 1 vial containing 0.4 ml of concentrated conjugate (sheep anti-apo B polyclonal antibody labeled with HRP, containing 0.05% kathon CG as preservative), to be diluted 100 x (procedure A) or 40 x (procedure B) before use.
7. 1 vial containing 20 ml of conjugate diluent (phosphate buffer with stabilizing Proteins, containing 0.05% Kathon CG as preservative).
8. 1 vial containing 0.3 ml of concentrated TMB substrate solution (tetramethylbenzidine dissolved in dimethyl sulfoxide), to be diluted 100 x before use.
9. 1 vial containing 20 ml of substrate buffer (phosphate citrate buffer containing 0.006% hydrogen peroxide); It was ready to use.
10. 2 vials containing 30 ml of concentrated wash solution (phosphate buffer containing Detergent and 0.17% Kathon CG as preservative) to be diluted 25 x before use.

11. Sulphuric acid of analytical grade in the range of 1 to 2 mol/liter

**Instruments used:**

1. Precision pipettes with disposable tips to deliver 10 or 100 µl.
2. Timer.
3. Water bath set at 37°C with direct warming (i.e. the bottom of the wells must be in Contact with the water)
4. Photometric reading: microplate reader (Span Auto chem.), equipped with a 450 nm filter and also with a 620 nm or 690 nm filters.

**Storage and stability:**

1. If kept at 2° to 8°C, all test reagents, including the coated test wells, are stable until the expiration date given on the pack.
2. All reagents and the sachet containing the test wells must be brought to room temperature (20-25°C) approximately 30 minutes before use and must be returned to the refrigerator immediately after use.
3. Unused test wells, stored at 2-8°C, are stable for 8 weeks if stored in the plastic minigrip bag with silicagel.
4. Diluted wash solution is stable for 2 weeks, if kept at 2-8°C.
5. Diluted conjugate is stable for 8 hours at room temperature (20-25°C) if kept in the dark.
6. Diluted substrate is stable for 1 hour at room temperature (20–25°C) if kept in the dark.
7. After using some of the contents of vials containing standard solutions, sample diluent, concentrated conjugate, conjugate diluent, concentrated substrate, substrate buffer, and concentrated wash solution, the contents are stable until the expiration date if kept at 2-8°C and stored in the closed original

**Preparation and manipulation procedures:**

**Preparations:**

1. Washing solution was prepared by diluting concentrated wash solution (10) 25 x with distilled or deionized water. Wash solution must be at room temperature (20-25°C) when used.

2. The sample diluent was diluted 1/10 with distilled water.

3. The serial 2-fold dilutions out of the LP[a] stock solution were prepared using sample diluent into plastic tubes e.g. 100 µl LP[a] standard stock solution with 100 µl diluent and homogenize; 100 µl of this 2-fold dilution with 100 µl of diluents and homogenize etc. The serial dilutions of the standard were labeled as: 100 mg/dl (undiluted), 50 mg/dl, 25mg/dl, 12.5 mg/dl, 6.3 mg/dl, 3.1 mg/dl. The diluted standards were stable for 4 h at 25 °C.

4. The lyophilized controls were reconstituted using 250 µl distilled water these were Diluted 1:200 in sample diluent this dilution were vortex for 30 seconds. After reconstitution these controls are stable for 4 weeks when stored at 2-8°C.

5. Conjugate was prepared by diluting concentrated conjugate (6) to 100x.

6. Substrate was prepared by diluting concentrated TMB substrate (8) 100 x with Substrate buffer (9).

7. Samples were diluted 1/200 by adding 10 µl sample to 2 ml diluted sample diluent.
Test procedure:

1. The temperature of the water bath or the incubator was adjusted to 37°C.

2. The strip-holder with the required number of strips was taken considering that for each test run 6 standards and one blank (sample diluent) should be included. During the test-run the strips stay in the strip-holder and can be marked on one edge.

3. The 10 µl of the appropriate specimen (1:200 diluted, cfr. preparations 7), standard (100, 50, 25, 12.5, 6.3, 3.1 mg/dl, cfr. preparations 3) or controls (1:200 diluted, cfr Preparations 4) were added to each well.

4. The 100 µl of sample diluent to each test well reserved for specimen, standards and Controls was added. The 100 µl of sample diluent was added to one test well reserved as blank.

5. The strips were covered with an adhesive sealer.

6. Strips were incubated for 120 minutes at 37°C.

7. Each well was washed for 4 times.

8. 100 µl prepared conjugate solution was added to each well, the strip-holder was tapped carefully to mix.

9. The strips were covered with a new adhesive sealer.

10. Strips were Incubated for 60 minutes at 37°C.

11. Each well was washed 4 times.

12. 100 µl prepared substrate solution was added to each well, the strip-holder was tapped carefully to eliminate air bubbles that may occur.

13. Strips were incubated for 30 minutes at 20-25°C.

14. 100 µl sulphuric acid was to each well to stop the reaction in the same sequence and at the same time intervals as the substrate solution.
15. The reader was blanked with air and the absorbance of the solution in the wells was measured at 450 nm using the second filter of 620 nm.

**Calculation of the results:**

The standard curve was constructed by plotting the mean absorbance values obtained for each of the LP[a] standard solutions on the vertical (Y) axis versus the corresponding LP[a] Concentrations (100, 50, 25, 12.5, 6.3, 3.1 mg/dl) on the horizontal (X) axis, using rectilinear graph paper. The best fitting curve was drawn through these points.

![Standard Curve for Lp[a]](image)

Using the mean absorbance value for each sample to be tested, corresponding concentration of LP[a] in mg/dl was determined from the standard curve.

**3.15. Isolation and purification of LP[a] and LDL (Nerukar and Taskar, 1985)**

**Reagents**

1. **Tris HCl buffer pH 7.0 (0.05 M) in 0.15 M NaCl:** The 0.15 M NaCl was prepared by taking 2.19 gm NaCL in volumetric flask and making the final volume to 250 ml mark. The 1.97 gm of Tris HCL was weighed and taken in 250
ml of volumetric flask and volume was made up to mark with 0.15 M NaCl. The pH was adjusted to 7.0 using 0.1N NaOH and 0.1 N HCL.

2. **Saturated ammonium sulphate Solution:** It was prepared by dissolving the ammonium sulphate in 100 ml of water till the saturated solution was obtained.

3. **Sodium phosphotungstate solution (4% w/v):** It was prepared by dissolving 4gm of Sodium phosphotungstate in 100 ml of distilled water.

4. **Magnesium chloride (2 M) \([\text{MgCl}_2\cdot6\text{H}_2\text{O}]\):** It was prepared by weighing 101.65 gm of Magnesium chloride and dissolving in small amount of water and making the final volume to 250 ml with volumetric flask.

5. **Sodium Chloride (0.15 M):** The 0.15 M NaCl was prepared by taking 2.19 gm NaCL in volumetric flask and making the final volume to 250 ml mark.

6. **Sodium Carbonate (10% w/v):** It was prepared by dissolving 10 gm of Sodium carbonate in 100 ml of water.

**Procedure**

1. 3 ml of plasma was diluted to 6ml with Tris HCl buffer. The sample was centrifuged for 1 hr at 20,000 g. The supernatant 1 ml was removed for chylomicrons. Subnatent 4 ml was taken for separation of VLDL, LP[a], LDL and HDL.

2. Concentration of 4 ml of subnatant obtained from step I was raised to 20% saturation of ammonium sulphate by adding 1 ml of saturated ammonium sulphate. The precipitate was separated by centrifugation at 10,000 g for 30 min. The saturation of 4 ml of supernatant was adjusted to 35% by addition of 0.92 ml of ammonium sulphate. The precipitate of LP[a] was separated by centrifugation and dissolved in 2ml of tris buffer.
3. The 4 ml supernatant from step II was adjusted to 50% saturation by adding 1.2 ml of saturated ammonium sulphate. The precipitate of crude VLDL was separated by centrifugation and dissolved in 2ml of tris buffer.

4. The 4 ml supernatant from step III was adjusted to 60% saturation by adding 1.0 ml of saturated ammonium sulphate solution and crude LDL fraction was obtained and dissolved in 2ml of tris buffer.

5. Crude HDL fraction was obtained by raising the saturation to 70% of 4 ml of supernatant from step IV by adding 1.32 ml of saturated ammonium sulphate.

6. All the lipoproteins are checked by agarose gel electrophoresis to confirm that there is no contamination with albumin.

From the crude LP[a],VLDL, and LDL fractions, the purified lipoproteins were obtained by the precipitation using 0.2 ml of sodium phosphotungstate and 0.05 ml of magnesium chloride solution. The precipitate obtained was dissolved in 0.15ml sodium chloride solution and made alkaline with sodium carbonate (10%w/v) to make final volume to 1ml. All the LP[a] fractions were confirmed for their purity by electrophoresis on 3.8% polyacrylamide gel and further subjected to protein and cholesterol analysis by Lowry method described below and cholesterol oxidase method described above.

3.16. Estimation of Protein in LP[a] (Lowry et al., 1951)

**Principle:** It is the most commonly used method for determination of proteins in cell free extracts because of its high sensitivity. The –Co-NH– (Peptide bonds) in polypeptide chain react with copper sulphate in an alkaline medium to give a blue coloured complex. In addition, tyrosine and tryptophan residues of proteins cause reduction of the phosphomolybdate and phosphotungstate components of the Cio
calteau reagent to give bluish products which contribute towards enhancing the sensitivity.

**Reagents**

1. **Alkaline Sodium Carbonate reagent:** Dissolve 2.0 gm Sodium Carbonate was dissolved in 0.1 N Sodium Hydroxide and the volume was made to 100 ml with 0.1N Sodium Hydroxide.

2. **Copper sulphate reagent 0.5% CuSO$_4$.5H$_2$O in 1% sodium potassium tartarate:** It was prepared by dissolving 0.5 gm of CuSO$_4$.5H$_2$O in 1% sodium potassium tartarate solution, which is prepared by adding 1 gm of sodium potassium tartarate in 100 ml of water.

3. **Alkaline copper sulphate reagent:** 1 ml of reagent 2 was added to 50 ml of reagent 1. This reagent should be prepared fresh.

4. **Folin’s reagent:** Dilute The readymade reagent was appropriately diluted so that it is 1 N in respect of its acid content.

5. **0.1 N Sodium Hydroxide:** It was prepared by dissolving 0.4 gm in small amount of water and finally volume was made to 100 ml with distilled water.

6. **Bovine serum albumin (BSA)Standard (100 µg / ml):** Firstly the stock solution was prepared by dissolving 0.1gm in 100 ml of distilled water(1mg/ml) and diluting it to 10 times with distilled water.

**Procedure**

100 µl of the purified LP[a] sample was taken and made to 1.0 ml with the distilled water and to it 5 ml of freshly prepared alkaline copper sulphate reagent was added. Contents were mixed properly and after 10 min 0.5 ml of Folin’s reagent was added. Contents were mixed instantaneously. Color was allowed to develop for 30 min. Absorbance was recorded at 660 nm after setting the instrument with reagent blank.
which contained 1 ml of 0.1N NaOH instead of sample. In another set of tubes suitable aliquots of BSA solution (in a range of 0-100µg) was taken and the total volume was made to 1 ml with 0.1N NaOH and the colour was developed as mentioned previously and absorbance was recorded at 660nm. A standard graph of Abs. versus conc. of BSA in µg was plotted. From this standard curve amount of protein was determined in the purified LP[a] sample and this was multiplied by 200 to obtain the protein 100 ml.

3.17 Characterization of LP[a] on the basis of lysine binding heterogeneity(Xia et al.,2000)

**Reagents:**

**Buffer A:** Tris HCL (20mM; pH 7.4 containing 150 mM NaCl, 0.01M Sodium azide) The 150 mM NaCl was prepared by taking 2.19 gm NaCl in volumetric flask and making the final volume to 250 ml mark. The 0.876 gm of Tris HCL was weighed
and taken in 250 ml of volumetric flask and volume was made upto mark with 0.15 M NaCl. 0.1625 gm of Sodium azide was added to it. The pH was adjusted to 7.4 using 0.1N NaOH and 0.1 N HCL

**Lysine Sephrose 4B:** It is the L-Lysine immobilized to Sepharose 4B by the cyanogen bromide method. L-Lysine is coupled via its α–amino group, leaving both the ε amino α –carboxyl group free to interact with sample substances during chromatography. It is designated for the purification of molecules with biospecific or charge dependent affinity for L-Lysine. Lysine Sephrose 4B is a group specific adsorbant and has been used for the isolation of plasminogen and plasminogen activator, separation of (rRNA) and purification of double stranded DNA

**Characteristics of Lysine Sephrose CL-4B:**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding Capacity</td>
<td>0.6mg human plasminogen/ml drained medium</td>
</tr>
<tr>
<td></td>
<td>0.6-0.7 mg r RNA /ml drained medium</td>
</tr>
<tr>
<td>Bead Structure:</td>
<td>4% agarose</td>
</tr>
<tr>
<td>Mean particle size</td>
<td>90µm</td>
</tr>
<tr>
<td>Bead Size range:</td>
<td>45-165µm</td>
</tr>
<tr>
<td>Maximum linear flow rate</td>
<td>75Cm/h at 25°C ,HR16/10 column ,5CM Bed height</td>
</tr>
<tr>
<td>P_H Stability</td>
<td>Long term 2-11, Short Term 2-11</td>
</tr>
<tr>
<td>Chemically Stability</td>
<td>Stable to all commonly used aqueous buffers</td>
</tr>
<tr>
<td>Physical Stability</td>
<td>Negligible volume variation due to changes in P_H or ionic strength</td>
</tr>
</tbody>
</table>

Biochemical characterization of lipoprotein fraction of major segment of population in Dehradun region
**Preparation of the medium:**

1. Lysine Sepharose 4B was supplied freeze–dried in the presence of additives. This additive was washed away at neutral pH.

2. The required amount of freeze dried powder (1gm gives about 4ml final volume) was suspended in the distilled water.

3. The medium swells immediately and washed for 15 minutes with distilled water on a sintered glass filter. Approximately 200 ml distilled water per gram freeze dried powder was added in several aliquots.

**Procedure:**

1. In all cases, 20µg of LP[a] (as determined by previous procedures) was diluted to 7ml in buffer A.

2. The Samples were applied to 2 ml of lysine sephrose CL -4B(Phamacia) in a 1.1x10 Cm column. The column was sealed on both ends and rock gently for 2 hours at 4°C to allow the batch adsorption of LP[a] on the resin .

3. The slurry was allowed to packed in the column and flow through(A) was collected Column was then washed with 25 ml of buffer A(.The resultant flow through (A) was then collected and then combined with flow through (A).The combined fractions were designated as unbound or LP[a] Lys⁻ fraction.

4. Specifically bound LP[a] was then eluted with 12.5 ml of buffer A containing 0.2molar ε Aminocarpoic Acid .The eluted material was designated as LP[a] Lys⁺ fraction

5. The LP[a] content in both Lys⁻ and Lys⁺ fraction was determined by previous procedures and expressed in terms of percentage of LP[a] that was capable of binding to lysine sephrose column.
3.18 Fibrinogen Estimation by Kjeldahl Nessrization Method (Godkar and Godkar ,2003)

**Principle:** Fibrinogen is converted to fibrin clot with the addition of calcium ions. The fibrin clot is digested by using 50% (v/v) H₂SO₄ containing selenium dioxide as oxidizing agent. A drop of H₂O₂ helps the quick digestion of clot. The end product of the digestion is ammonium sulphate. Concentration of ammonium sulphate is determined by using Nessler’s reagent. This reagent react with ammonium sulphate (NH₄)₂SO₄ and forms yellow coloured complex which is proportional to fibrinogen concentration in plasma.

**Reagents:**

1. **Calcium chloride (2.5gm/dl):** It was prepared by dissolving 2.5 gm of calcium chloride in 100 ml of water.

2. **Digestion mixture (Selenium oxide (1gm/dl)in 50% (v/v) Sulphuric Acid):** The sulphuric acid was diluted by adding 50 ml of sulphuric acid in 50 ml of water slowly with continuous stirring.

3. **Hydrogen Peroxide30% (w/v):** Readymade to the regent was used

4. **Stock Nessler’s Reagent:**

   **Solution A:** Pottasium Iodide (150 gm) and Mercuric Iodide (200 gm) were dissolved in about one litre water; filtered and final volume was made upto 2 liters.

   **Solution B (10 gm/dl Sodium Hydroxide):** It was prepared by dissolving 10 gm sodium hydroxide in 100 ml of distilled water.

5. **Working Nessler’s Reagent:** It was prepared by mixing the solution A (15 ml), solution B and distilled water (15 ml)
6. **Stock Standard**: (0.3277 gm/dl Ammonium sulphate: 0.3277 gm of ammonium sulphate was weighed and poured in 100 ml of volumetric flask the water was added to make up the final volume to 100 ml mark.

7. **Working Standard**: Dilute 5ml of stock Standard was diluted to 100 ml by using distilled water This solution contains 4 mg/dl nitrogen.

**Procedure:**

1. 0.5 ml plasma, 9 ml distilled water and 0.5 ml of calcium chloride was mixed in a beaker.A fine glass rod was placed in the beaker and kept at 37°C ( water bath) until a clot was formed (generally clot was formed after 30 minutes but sometimes it took overnight for the formation of clot).

2. Rod was rotated to collect the clot. Clot was placed on a filter paper and dried by gentle tapping.

3. This clot was placed in the digestion tube and 1 ml of digestion mixture was added. The tube was heated carefully by using a microburner. A drop of hydrogen peroxide was added. The mixture first darkened. Heating was continued until the mixture turned colourless.

4. Tube was cooled and distilled water added upto 10 ml mark and mixed thoroughly.

5. 2.0 ml of this mixture was pipetted in a test tube. Cold distilled water (5.0ml) was added. 3.0 ml of cold working Nessler’s reagent was added and mixed thoroughly.

6. Absorbance was read at 440 nm immediately.

7. Standard was put up by pipetting 1.0 ml of working Std. in a test tube. 6.0 ml of cold distilled water was added. After this 3.0 ml of cold working Nessler’s reagent was added and mixed.
8. Absorbance was read at 440 nm immediately.

**Calculation**

\[
\frac{\text{Absorbance of test}}{\text{Absorbance of Std.}} \times 0.04 \times 100 \times 6.25
\]

\[
\frac{\text{Absorbance of test}}{\text{Absorbance of Std.}} \times 250 = \text{Fibrinogen in plasma (mg/dl)}
\]

**Note:**

- 0.04 mg/ml = Concentration of Standard
- 0.5 ml of plasma was used in the determination but only 1/5 of the digestion mixture is used in the determination. Hence actual or quantity of specimen used = 0.1 ml
- 6.25 factor is used to convert gm/dl protein nitrogen into gm/dl fibrinogen (as protein).

**3.19 Fibronectin Estimation by Kit (Bender Med systems)(Romberger.,197)**

**Assay principle:** Fibronectin Antigen is a one-step sandwich ELISA designed with a highly purified rabbit polyclonal antibody specific for fibronectin. First, the immunoconjugate, which is a polyclonal antibody specific for fibronectin coupled to Horse Radish Peroxidase (HRP), is introduced into the microwells. Then, the diluted tested sample is immediately introduced, and the immunological reaction starts. When present, Fibronectin binds onto the polyclonal antibody coated solid phase, and fixes the antibody coupled to HRP through free epitopes. Following a washing step, the peroxidase substrate, 3,3′,5,5′ – Tetramethylbenzidine (TMB), is introduced and a blue colour develops. When the reaction is stopped with Sulfuric Acid, a yellow colour is obtained. The amount of colour developed is directly proportional to the concentration of Fibronectin in the tested sample.
Material and Methods

Test sample: Trisodium Citrate or Sodium EDTA anticoagulated human plasma.

Reagents:

1. **Coat:** Micro ELISA plate, containing 12 strips of 8 wells, coated with a highly purified rabbit polyclonal antibody specific for Fibronectin, then stabilised; the late was packed in an aluminium pouch hermetically sealed in presence of a desiccant.

2. **SD:** 2 vials containing 50ml of Fibronectin Sample Diluent. It was ready to use.

3. **Cal:** 3 vials of Fibronectin Calibrator, lyophilised. When restored with 2.5 ml of Fibronectin Sample Diluent, a solution containing 50 ng/ml of human Fibronectin was obtained.

4. **CI:** 1 vial of Fibronectin Control I (High), lyophilised.

5. **CII:** 1 vial of Fibronectin Control II (Low), lyophilised.

6. **IC:** 3 vials of Anti-Fibronectin-HRP immunoconjugate, a polyclonal antibody coupled to HRP, lyophilised.

7. **CD:** 1 vial of 25 ml of Fibronectin Conjugate Diluent. It was ready to use.

8. **WS:** 1 vial of 50 ml of 20 fold concentrated Wash Solution.

9. **TMB:** 1 vial of 25 ml peroxidase substrate: 3,3’,5,5’ – Tetramethylbenzidine containing hydrogen peroxide. It was ready to use.

10. **SA:** 1 vial of 6 ml of 0.45M Sulfuric acid (Stop solution). It was ready to use.

Equipment used:

1. 8-channel or repeating pipette allowing dispensing 50-300 µl.

2. 1-channel pipettes at variable volumes from 0 to 20 µl, 20 to 200 µl and 200 to

3. Micro ELISA plate washing equipment and shaker.

4. Micro ELISA plate reader with a wavelength set up at 450 nm.
Reagents preparation, storage and stability:

1. **Micro ELISA plate**: When out of the pouch, the strips must be used within 30 minutes. Unused strips can be stored at 2-8°C for 4 weeks in their original aluminium pouch, in presence of the desiccant, hermetically closed and protected from any moisture, and stored in the provided microplate storage bag (minigrip).

2. **Fibronectin Sample Diluent**: It was ready to use. When open, it can be used for 4 weeks, stored at 2-8 °C, and provided that any bacterial contamination is avoided during use.

3. **Fibronectin Calibrator**: Each vial was restored with 2.5 ml of fibronectin Sample Diluent in order to obtain a solution containing 50 ng/ml. This solution was stable for at least 8 hours at room temperature.

4. **Fibronectin Control I (high)**: Each vial was restored with 1 ml fibronectin Sample Diluent.

5. **Fibrinogen Control II (low)**: Each vial was restored with 1 ml Fibrinogen Sample Diluent. When restored, controls were stable for 8 hours at room temperature, 24 hours at 2-8°C or 2 months frozen at –20°C or below.

6. **Anti-Fibronectin-HRP immunoconjugate**: each vial was restored with 4 ml of fibronectin Conjugate diluent. Shaked the vial gently in order to homogeneize the content. The restored conjugate was stable for at least 24 hours at room temperature or for at least 4 weeks at 2-8°C.

7. **Fibronectin Conjugate Diluent**: It was ready to use. When open, it was stable at 4 weeks, stored at 2-8 °C, and provided that any bacterial contamination is avoided during use.

8. **Wash Solution**: The vial was incubated for 15-30 minutes in a water bath at 37°C until complete dissolution of solids. The vial was shaked and the amount required
was diluted with 1:20 in distilled water. The Wash Solution was be stored at 2-8°C in its original vial and used within 4 weeks following opening. The diluted Wash Solution was used within 7 days, when protected from any contamination and stored at 2-8°C.

9. **TMB substrate:** It was ready to use. When open, it can be used for 4 weeks, stored 2-8°C, and provided that any bacterial contamination was avoided during use.

10. **Stop solution:** It was ready to use.

**Procedure:**

**Tested plasma or sample:** The sample tested was diluted in order to obtain a concentration < 50ng/ml in the fibronectin sample diluent. For example, human plasma was tested at 1:8000, or 1:16000, or more in the fibronectin sample diluent. Wherever high dilutions were required serial dilutions (i.e. 1:10, then 1:10, etc...) were done.

**Controls I and II,** restored with 1ml of fibronectin sample diluent, were tested “undiluted”.

**Calibrations:** The 50 ng/ml fibronectin Calibrator was used to prepare the following dilutions:

<table>
<thead>
<tr>
<th>Fibronectin Cal.of 50ng/ml</th>
<th>50 ng/ml</th>
<th>25ng/ml</th>
<th>10ng/ml</th>
<th>5ng/ml</th>
<th>2.5ng/ml</th>
<th>0ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vol. of Fibronectin</td>
<td>1 ml</td>
<td>0.5 ml</td>
<td>0.2 ml</td>
<td>0.1 ml</td>
<td>0.05 ml</td>
<td>0 ml</td>
</tr>
<tr>
<td>Sample Diluent</td>
<td>0 ml</td>
<td>0.5 ml</td>
<td>0.8 ml</td>
<td>0.9 ml</td>
<td>0.95 ml</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

It was mixed gently for a complete homogenization. The standard dilutions were stable for at least 8 hours at room temperature.
Material and Methods

Assay procedure:

The required number of strips from the aluminium pouch were removed for the series of measures to be performed. The strips were loaded in the frame provided. Following assay steps were performed as indicated below:

1. The 100 µl of Anti-fibronectin- HRP immunoconjugate was introduced in the micro ELISA plate wells
2. 100 µl of fibronectin calibrator or controls or tested sample or fibronectin sample diluent (blank) was introduced immediately in the corresponding micro ELISA plate well
3. It was mixed gently on a plate shaker or manually and incubated for 1 hour at room temperature
4. The plates were washed four times with diluted washing solution using the standard washing protocol
5. The 200 µl substrate was introduced into the wells immediately after washing. The substrate distribution, was done row by row, at exact time intervals
6. It was incubated for exactly 5 minutes at room temperature (18-25 °C) Following exactly after the same time intervals than for the addition of substrate, The color development was stopped by introducing the 0.45M sulfuric acid.
7. The absorbance was measured exactly after 10 minutes of the addition of stop regent at 405 nm.

Results:

On a linear graph paper the fibronectin concentrations, in ng/ml, on abscissae and the corresponding absorbances ($A_{450}$) on ordinates was Plotted as shown below:
For obtaining the fibronectin concentration in the tested sample, this multiplied by
the dilution factor for controls I and II, the concentrations measured were directly
deduced from the curve.

3.20: Plasminogen Estimation by ELISA kit (Max Human Diagnostics)
(Carmeliet Colen ,1996 )

Principal of the Assay: The Assay Max Human Plasminogen ELISA kit is designed
for detection of human plasminogen in plasma and cell culture supernatants. This
assay employs a quantitative sandwich enzyme immunoassay technique that measures
plasminogen in 2.5 hours. A murine antibody specific for plasminogen has been pre-
coated onto a 96-well microplate with removable strips. Plasminogen in standards and
samples is sandwiched by the immobilized antibody and the biotinylated polyclonal
antibody specific for plasminogen, which is recognized by a streptavidin-peroxidase
conjugate. All unbound material is then washed away and a peroxidase enzyme
substrate is added. The color development is stopped and the intensity of the color is
measured.
Material and Methods

**Reagents:**

1. **Plasminogen Microplate:** A 96-well polystyrene microplate (12 strips of 8 wells) coated with a murine antibody against human plasminogen.

2. **Sealing Tapes:** Each kit contains 3 pre-cut, pressure-sensitive sealing tapes that was cut to fit the format of the individual assay.

3. **Plasminogen Standard:** Human plasminogen in a buffered protein base (200 ng, lyophiliized).

4. **Biotinylated Plasminogen Antibody (100x):** A 100-fold biotinylated polyclonal antibody against human plasminogen (80 µl).

5. **Streptavidin-Peroxidase Conjugate (SP Conjugate):** A 100-fold concentrate (120 µl).

6. **EIA Diluent Concentrate (10x):** A 10-fold concentrated buffered protein base (30 ml).

7. **Wash Buffer Concentrate (10x):** A 10-fold concentrated buffered surfactant (2 x 30 ml).

8. **Chromogen Substrate:** It was ready-to-use stabilized peroxidase chromogen substrate tetramethylbenzidine (8 ml).

9. **Stop Solution:** A 0.5 N hydroxychloric acid (12 ml) to stop the chromogen substrate reaction.

**Storage Condition:**

1. Unopened kit was Stored at 2-80°C up to expiration date.

2. Opened reagents were stored for one month at 2-80°C. The reconstituted standard was Stored at-200°C or below.

**Instruments:**

1. Microplate reader used for measuring absorbance at 450 nm
2. Pipettes (1-20 µl, 20-200 µl, and multiple channel)

**Sample Collection, Preparation and Storage:**

**Plasma:** The plasma was collected using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Samples were centrifuged at 2,000x g for 10 minutes and assay. The samples were diluted 1:10,000 with EIA Diluent as follows: The 5 µl of sample was added to 495 µl of EIA Diluent (1:100) to make Solution A; then 5 µl of Solution A was added to 495 µl of EIA Diluent (1:100) to make a final working solution (1:10,000). The undiluted samples were to be stored at -20°C or below for up to 3 months.

**Reagent Preparation**

1. **EIA Diluent Concentrate (10x):** The EIA Diluent 1:10 was diluted with reagent grade water.

2. **Biotinylated Plasminogen Antibody (100 xs):** The antibody was Spin down briefly and the desired amount of the antibody was diluted 1:100 with EIA Diluent.

3. **Wash Buffer Concentrate (10x):** The Wash Buffer Concentrate was diluted 1:10 with reagent grade water.

4. **SP Conjugate (100x):** The SP Conjugate was Spin down briefly and dilute the desired amount of the conjugate was diluted 1:100 with EIA Diluent.

**Standard Curve:** The 200 ng of human Plasminogen Standard was reconstituted with 1.25 ml of EIA Diluent to produce a 160 ng/ml of solution. The triplicate standard points were prepared by serially diluting the standard solution (160 ng/ml) twofold with equal volume of EIA Diluent to produce 80, 40, 20, 10, 5, and 2.5 ng/ml of solutions. EIA Diluent serves as the zero standards (0 ng/ml).
**Assay Procedure**

1. The assay was performed at room temperature (20-30°C).

2. Add 50 µl of Standard or sample per well was added. Cover the wells were covered and incubated for one hour.

3. Washing was done five times with wash Buffer. Invert the plate was inverted and decant the contents were decanted and plate was hit 4-5 times on absorbent paper towel to complete remove liquid at each step.

4. The 50 µl of biotinylated Plasminogen Antibody was added to each well and incubated for 30 minutes.

5. Washing was again done five times with 200 µl of Wash Buffer as above.

6. The 50 µl of Streptavidin-Peroxidase conjugate per well was added and incubated for 30 minutes.

7. Washing was again done five times with 200 µl of wash Buffer as above.

8. The 50 µl of Chromogen Substrate per well and incubated for approximately 7 to 10 minutes or till the optimal blue color density develops. Gently the plate was tapped gently to ensure thorough mixing and break the bubbles in the well with pipette tip.

9. The 50 µl of Stop Solution was added to each well. The color changed from blue to yellow.

10. The absorbance on a micro plate reader was read at a wavelength of 450 nm immediately.

**Data Analysis:** The standard curve was generated by plotting the graph using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance on the y-axis. The best-fit line was determined. The standard curve was shown below.
The unknown sample concentration from the Standard Curve was determined and multiplied by the dilution factor value by the dilution factor.

**Standard Curve**

![Standard Curve for Plasminogen](image)

3.20 **Estimation of LDL apo B:** LDL was isolated as method previously described in 3.15 and from the isolated LDL the apo B was estimated by method 3.11 as for total apo B.

3.21 **Statistical Analysis:** The data was collected using stratified sampling technique was analyzed using Genstat 32 for factorial experiments.