CHAPTER-4

MATERIALS AND METHODS

The present study was carried out at Maharishi Markandeshwar Institute of Medical Sciences and Research, Mullana, Ambala in the Department of Biochemistry in collaboration with the Department of Medicine. The study was undertaken after approval by Institutional Ethics Committee.

STUDY GROUPS

A total of one hundred fifty subjects in the age range of >30 years, irrespective of sex, constituted the material of the study. One hundred patients with metabolic syndrome attending the OPD and/or admitted in wards of Medicine Department of M.M Institute of Medical Sciences and Research, Mullana, Ambala and Fifty of normal subjects >30 years amongst the staff members, attendants of patients and volunteers were included as controls. The patients were diagnosed as metabolic syndrome by the Department of Medicine on the basis of NCEP ATP III criteria. The subjects indulged in the study were divided into two groups:

1. Group I: Patients with metabolic syndrome
2. Group II: Healthy controls

CRITERIA USED FOR DIAGNOSIS OF METABOLIC SYNDROME

Patients were diagnosed as ‘metabolic syndrome’ meeting three of these five criteria having the metabolic syndrome according to the following diagnostic criteria issued by NCEP-ATP-III.\textsuperscript{52}
SCREENING AND SELECTION OF PATIENTS

At first the fasting glucose level and lipid profile were estimated in Biochemistry laboratory to select the patients attending OPD/IPD of Medicine department.

Patients with one or more than one abnormal results among fasting Glucose level (>100mg/dl), TG (>150mg/dl) and HDL (<50 mg/dl in Female & HDL< 40 mg/dl) were selected. Further their Waist circumference and BP were also measured. Patients meeting less than three NCEP-ATP III criteria were excluded.

100 patients meeting 3 or more than 3 criteria of NCEP-ATP III were involved in the study and their history along with rest anthropometric measurements were taken. The rest biochemical tests were estimated in their sample.

A detailed history of the present as well as past illness was recorded and the clinical examination was done as per the proforma attached.

Informed consent, both in English as well as vernacular language, was taken from all the participants included in the study.

INCLUSION CRITERIA

1. Metabolic syndrome patients according to NCEP-ATP III criteria given below.

   Table 4.1: Criteria used for diagnosis of MetS in present study.52

   | 1. Central obesity | (Waist circumference) | i) Men: ≥102 cm  
   | 2. Hypertriglyceridemia | Fasting Triglycerides | >150mg/dl  
   | 3. Low HDL-cholesterol | Fasting HDL-cholesterol | i) Men: <50mg/dl  
   | 4. Glucose intolerance | Fasting glucose | ~100 mg/dl  
   | 5. Hypertension | Sitting blood pressure | ≥ 130/85 mmHg  

2. Obese and non-obese (meeting the other criteria of NCEP-ATP-III).

3. Age >30 years of either sex.
EXCLUSION CRITERIA

1. Patients taking anti-diabetic drugs and/or lipid lowering agents regularly.
2. Patients taking other hormonal therapy like steroids and thyroxine.
3. Patients with thyroid disorders.
5. Patients with hs-CRP ≥ 10 µg/ml were excluded from the study group.
6. Patients diagnosed with metabolic syndrome along with other diseases like acute bacterial infections, acute rheumatic fever, autoimmune diseases (Systemic lupus erythematosis, rheumatoid arthritis, reiter’s syndrome), bone diseases, known case of cardiovascular diseases, GIT diseases, polymyalgia rheumatic, dermomyositis, neoplastic diseases were also excluded from the study group as these are also associated with increased hs-CRP level.

ANTHROPOMETRIC MEASUREMENTS

Anthropometric indices including height, weight, waist circumference and hip circumference were measured. Blood Pressure was measured by using sphygmomanometer. Waist hip ratio (WHR) and BMI was calculated.

1. **Height:**
   
   Height of the subjects was measured in meter using manual stadiometer. Height of subjects was recorded with shoes removed and on light clothes. Subjects were asked to stand straight with their back to the wall and look straight ahead then the reading was recorded.  

   ![Figure 4.1: Taking height measurement using stadiometer.](image)
2. **Weight:**

A digital weight scale was used to measure the weight of the subjects in kilogram. Subjects were asked to take weight with shoes removed and on light clothes and instructed to stand in the center of the scale platform, hands at sides and looking straight ahead.\(^{272}\)

![Figure 4.2: Taking body weight in digital weight scale](image)

3. **Waist Circumference:**

Subjects were asked to stand with arms at the sides, feet positioned close together, and weight evenly distributed across the feet.

The measurement was made at the approximate midpoint between the lower margin of the last palpable rib and the top of the ilia crest with the stretch resistant tape parallel to the floor. Waist circumference was measured in centimeter (cm).\(^{273}\)
4. **Hip circumference:**

   Subjects were asked to stand with arms at the sides, feet positioned close together, and weight evenly distributed across the feet.

   Hip circumference was taken around the widest portion of the buttocks with the tape parallel to the floor. Hip circumference was measured in centimeter (cm).\(^{273}\)

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**Figure 4.3: Method of taking waist circumference.**

**Figure 4.4: Method of taking hip circumference.**
5. **Waist Hip Ratio (WHR):**

WC was divided by hip circumference to calculate WHR.\(^{273}\)

6. **BMI:**

Body Mass Index (BMI) was calculated by dividing body weight (in kilograms) by squared height (in square meters) according to WHO procedure.\(^{274}\)

**Table 4.2: Classification of weight status as per BMI.**\(^{275}\)

<table>
<thead>
<tr>
<th>Classes</th>
<th>BMI (kg/m(^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Underweight</td>
<td>&lt; 18.5</td>
</tr>
<tr>
<td>Healthy weight</td>
<td>18.5-21.9</td>
</tr>
<tr>
<td>Overweight</td>
<td>25.0-29.9</td>
</tr>
<tr>
<td>Obesity class</td>
<td>&gt;30</td>
</tr>
</tbody>
</table>

**CLINICAL EXAMINATION**

1. **Blood pressure:**

Blood Pressure was measured according to ‘American Heart Association Guidelines for in-clinic Blood Pressure Measurement’ by using mercury sphygmomanometer.\(^{276}\)

- Subjects were instructed to sit comfortably, with back supported, legs uncrossed and upper arm bared.
- Subjects arm was supported at heart level.
- Cuff bladder was encircled 80 percent or more of the subjects arm circumference.
- The 1\(^{st}\) and 2\(^{nd}\) audible sounds were recorded as systolic and diastolic blood pressure respectively.
Table 4.3: Classification of hypertension:

<table>
<thead>
<tr>
<th>Classification</th>
<th>Blood Pressure (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Systolic</td>
</tr>
<tr>
<td>Normal</td>
<td>90-119</td>
</tr>
<tr>
<td>Pre hypertensive</td>
<td>120-139</td>
</tr>
<tr>
<td>Stage 1 hypertensive</td>
<td>140-159</td>
</tr>
<tr>
<td>Stage 2 hypertensive</td>
<td>160 or higher</td>
</tr>
</tbody>
</table>

BIOCHEMICAL INVESTIGATIONS

Fasting blood sample was collected from patients for biochemical investigations. Under biochemical investigations, glucose and insulin were assessed in plasma whereas lipid profile and hs-CRP were assessed in serum as described below in detail.
Collection and processing of blood sample

Seven milliliters (7 ml) of venous blood sample was collected in dry disposable syringe under aseptic conditions from ante-cubital vein of the subjects after an overnight fasting of 10-12 hours. Four milliliters (4 ml) of blood was transferred to a sterile, dry and acid washed vial, allowed to stand for half an hour and after the clot formation, the supernatant fluid was centrifuged to perform the following biochemical investigations.

1. Fasting lipid profile (Total Cholesterol, Triglycerides, HDL-cholesterol and LDL-cholesterol levels)
2. High Sensitivity C-reactive protein (hs-CRP) test in serum

The remaining 3 ml of blood was transferred to sodium fluoride and potassium oxalate containing vial, and centrifuged to separate plasma which was then used for estimation of:

1. Fasting plasma glucose
2. Fasting plasma insulin

Insulin resistance was calculated by:

\[
\text{HOMA-IR} = \frac{\text{Fasting plasma insulin} \times \text{Fasting plasma glucose}}{405}
\]

DETAILS OF INVESTIGATIONS:

1. ESTIMATION OF PLASMA GLUCOSE
   
   Plasma Glucose was estimated by GOD-POD method\textsuperscript{278} using ERBA diagnostics fully automated analyzer kit.

Principle

The estimation of Glucose by GOD-POD method involves the following enzymatic reactions:
The absorbance of quinoeimine dye (pink color) thus formed is read between 500-540 nm which is directly proportional to the concentration of glucose in the sample.

**Reagents**

1. Glucose reagent consisted of the following:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose Oxidase</td>
<td>20000 IU/L</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>3250 IU/L</td>
</tr>
<tr>
<td>4- Aminoantipyrene</td>
<td>0.52 mmol/L</td>
</tr>
<tr>
<td>4-Hydroxybenzoic acid</td>
<td>10 mmol/L</td>
</tr>
<tr>
<td>Phosphate buffer</td>
<td>110 mmol/L</td>
</tr>
</tbody>
</table>

2. Glucose standard (100 mg/dl or 5.55 mmol/L)

3. Diluent for Erba Glucose with preservatives, stabilizer and lipid clearing agent (200ml)

**Reagent Preparation**

The vial containing Glucose and Glucose diluent were allowed to attain room temperature. Vials containing glucose reagent were dissolved using glucose diluent with special lipid clearing agent. Final volume of 200 ml was made and transferred into a clean dry amber colored bottle. This working reagent was stable at 2-8°C.

**Procedure**

A set of 3 test tubes were taken and marked as B, S and T for blank, standard and test respectively.
Mixed well and incubated at 37°C for 15 minutes. The absorbance readings from all the three test tubes were read at 505nm.

**Calculation of Results**

Concentration of Glucose (mg/dl) =

\[
\frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Concentration of standard (mg/dl)}
\]

**Reference Range**

Fasting levels = 60-100 mg/dl

2. **ESTIMATION OF PLASMA INSULIN**

Fasting plasma insulin levels were measured by Chemiluminescence immunoassay (CLIA) using CLIA VAST enabled kit (Monobind Inc. USA) supplied by Lilac Medicare Private Limited.

**Principle**

For this immunoenzymometric assay high affinity specific Ab is required. In this procedure, the opaque chemiluminescent reaction cell is used. Streptavidin is coated on the cell. The biotinylated monoclonal antibody coupled to insulin is added. The immobilization takes places during the
assay. The complex with test serum containing the native antigen forms a soluble sandwich complex. The interaction is illustrated by the following equations:

\[
\text{Enz Ab (P)} + \text{Ag_{ins}} + \text{Bn Ab (M)} \xrightleftharpoons[k_d]{k_a} \text{Enz Ab (P)} - \text{Ag_{ins}} - \text{Bn Ab (M)}
\]

Where \( \text{Enz Ab (P)} \) = enzyme labeled polyclonal antibody (excess quantity)
\( \text{Ag_{ins}} = \) native antigen (variable quantity)
\( \text{Bn Ab (M)} = \) biotinylated monoclonal antibody (excess quantity)
\( \text{Fnr Ab (P)} - \text{Ag_{ins}} - \text{Bn Ab (M)} = \) antigen-antibodies sandwich complex

\( k_a \) = rate constant of association
\( k_d \) = rate constant of dissociation

The complex gets deposited in the cell. This interaction is illustrated below:

\[
\text{Enz Ab (P)} - \text{Ag_{ins}} - \text{Bn Ab (M)} + \text{Streptavidin CW} \rightarrow \text{Immobilized complex}
\]

Where \( \text{Streptavidin CW} = \) Streptavidin immobilized on well
Immobilized complex = Sandwich complex bound to solid surface

After equilibrium, the bound-Ab fraction is separated from unbound antigen by decantation. The enzyme activity that generates light, in the antibody-bound fraction is directly proportional to the native antigen concentration.
**Materials Required and Used**

1. Micropipettes (5-100 µl and 200-1000 µl).
2. Lumax-A semi automated Chemiluminescence (CLIA) analyzer and Autoplex A processor for CLIA.
3. Test tubes for mixing of reagents.
4. Absorbent paper for blotting CLIA reaction cells.
5. Reaction cell cover for incubation steps.
6. Timer
7. Storage container for storage of wash buffer.
8. Distilled water.
9. Master CLIA VAST enabled kit (Monobind Inc. USA)

**Reagents**

1. **Insulin Calibrators (6 x 2 ml):** Six vials of calibrators (marked as A,B,C,D,E and F) containing insulin antigen at levels of 0(A), 5(B), 25(C), 50(D), 100(E) and 300(F) µIU/ml were reconstituted independently with 2 ml of distilled water. The reconstituted calibrators were stable for sixty days at 2-8 ºC.
2. **Insulin Tracer (1 x 13 ml):** One vial containing enzyme labeled, affinity purified, biotinylated monoclonal mouse anti-insulin IgG antibody in buffer, dye and preservative.
3. **Opaque streptavidin coated CLIA light reaction cells (pack size = 96 wells):** There are 96 CLIA light reaction cells coated with streptavidin and packaged in an aluminium bag with a drying agent.
4. **Wash solution Concentrate (2 x 60 ml):** contains a surfactant in buffered saline and a preservative.
5. **CLIA Signal Reagent A (1 x 30 ml):** contains luminol in buffer.
6. **CLIA Signal Reagent B (1 x 30 ml):** contains hydrogen peroxide in buffer.
All reagents were brought to room temperature (20-27 ºC) prior to use.

Reagent Preparation

1. **Wash Buffer**: It was prepared by diluting 60 ml of wash concentrate to 3000ml with distilled water in a suitable storage container and stored at room temperature of 20-27 ºC.

2. **Working Signal Reagent solution**: It was prepared according to the amount of reagent needed by mixing equal portions of signal reagent A and signal reagent B in a clean container. For example: 1ml of A and 1 ml of B were added per two (2) eight well strips. The unused portion was discarded, else it was stored at 2-8 ºC for 36 hours.

Sample Preparation

The samples were collected and stored at a temperature of -20 ºC. The stored samples were thawed and mixed thoroughly just prior to assay.

Test Procedure

1. 25 µl of calibrators and samples were pipetted into the appropriate reaction cells.
2. 50 µl of insulin tracer was added into each reaction cell.
3. The plate was swirled for 60 seconds and incubated at room temperature (22 – 26 ºC) for 60 minutes.
4. Reaction cells were then washed five times with 350 µl of wash buffer (0.35 ml per cell).
5. 50 µl of working signal reagent solution was added into each reaction cell.
6. The microplate was incubated for 5 mins at RT.
7. The relative light units (RLUs) were read in each well in a microplate strip luminometer for at least 0.2 seconds/well.

**Calculation of Results**

A dose response curve was constructed by plotting the mean relative light units or RLU's (Y) of calibrators against the known concentration (X) of standards with units on a linear graph paper. The best-fit curve was drawn through plotted points. The results were reported as the concentration of insulin (µIU/ml) in samples.

**Reference Range**

In fasting state, Adult (normal) = 0.7-9.0 µIU/ml  
Diabetic (Type 2) = 0.7-25.0 µIU/ml  
In well-fed state, normal insulin levels = up to 40 µIU/ml

**3. ESTIMATION OF SERUM hs-CRP.**

Serum CRP levels were measured by **Chemiluminescence immunoassay (CLIA)** \(^{279}\) using CLIA VAST enabled kit (Monobind Inc. USA) supplied by Lilac Medicare Private Limited.

**Principle**

For this immunoenzymometric assay high affinity specific Ab is required. In this procedure, the opaque chemiluminescent reaction cell is used. Streptavidin is coated on the cell. The biotinylated monoclonal antibody coupled to hs-CRP is added. The immobilization takes places during the assay. The complex with test serum containing the native antigen forms a
soluble sandwich complex. The interaction is illustrated by the following equations:

\[
\begin{align*}
\text{Enz Ab}_p + \text{Ag CRP} + \text{Btn Ab}_M & \quad \underset{k_a}{\rightarrow} \quad \text{Enz Ab}_p - \text{Ag CRP} - \text{Btn Ab}_M \\
\text{Enz Ab}_p - \text{Ag CRP} - \text{Btn Ab}_M & \quad \underset{k_a}{\rightarrow} \quad \text{Enz Ab}_p + \text{Ag CRP} + \text{Btn Ab}_M
\end{align*}
\]

Where \( \text{Enz Ab}_p \) = enzyme labeled polyclonal antibody (excess quantity)

\( \text{Ag CRP} \) = native antigen (variable quantity)

\( \text{Btn Ab}_M \) = biotinylated monoclonal antibody (excess quantity)

\( \text{Enz Ab}_p - \text{Ag CRP} - \text{Btn Ab}_M \) = antigen-antibodies sandwich complex

\( k_a \) = rate constant of association

\( k_a \) = rate constant of dissociation

The complex is deposited in the reaction cell through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated below:

\[
\text{Enz Ab}_p - \text{Ag CRP} - \text{Btn Ab}_M + \text{Streptavidin}_CW \quad \underset{\text{Immobilized complex}}{\text{Immobilized complex}}
\]

Where \( \text{Streptavidin}_CW \) = Streptavidin immobilized on well

Immobilized complex = Sandwich complex bound to solid surface
After equilibrium, the bound-Ab fraction is separated from unbound antigen by decantation. The enzyme activity that generates light, in the antibody-bound fraction is directly proportional to the native antigen concentration.

**Materials Required and Used**
1. Micropipettes (5-100 µl and 200-1000 µl).
2. Lumax-A semi automated Chemiluminescence (CLIA) analyzer and Autoplex A processor for CLIA.
3. Test tubes for mixing of reagents.
4. Absorbent paper for blotting CLIA reaction cells.
5. Reaction cell cover for incubation steps.
6. Timer.
7. Storage container for storage of wash buffer.
8. Distilled water.
9. Master CLIA VAST enabled kit (Monobind Inc. USA)

**Reagents**
1. **hs-CRP Calibrators (6 x 0.5 ml):** Six vials of calibrators ready to use (marked as A,B,C,D,E and F) containing insulin antigen at levels of 0(A), 0.5(B), 2(C), 5(D), 15(E) and 30(F) µg/ml were provided. Calibrators were stable for sixty days at 2-8 °C.
2. **Serum diluent (1 x 6 ml):** 6 ml of serum diluent was mixed with 54 ml of distilled water before use.
3. **hs-CRP Tracer (1 x 60 T):** One vial containing biotin labeled monoclonal mouse anti IgG and anti-CRP HRP in buffer, dye and preservative.
4. **Opaque streptavidin coated CLIA light reaction cells (pack size = 96 wells):** There are 96 CLIA light reaction cells coated with streptavidin and packaged in an aluminium bag with a drying agent.
5. **Wash solution Concentrate (2 x 60 ml):** contains a surfactant in buffered saline and a preservative.
6. **CLIA Signal Reagent A (1 x 30 ml):** contains luminol in buffer.
7. **CLIA Signal Reagent B (1 x 30 ml):** contains hydrogen peroxide in buffer.

All reagents were brought to room temperature (20-27 °C) prior to use.

**Reagent Preparation**

1. **Wash Buffer:** It was prepared by diluting 60 ml of wash concentrate to 3000ml with distilled water in a suitable storage container and stored at room temperature of 20-27 °C.
2. **Working Signal Reagent solution:** It was prepared according to the amount of reagent needed by mixing equal portions of signal reagent A and signal reagent B in a clean container. For example: 1ml of A and 1 ml of B were added per two (2) eight well strips. The unused portion was discarded, else it was stored at 2-8 °C for 36 hours.

**Sample Preparation**

The samples were collected and stored at a temperature of -20 °C. The stored samples were thawed and mixed thoroughly just prior to assay.

Sample dilution: 10 μl of sample was mixed in 2ml of diluent.

**Test Procedure**

1. 10 μl of sample was added in 2 ml diluent and mixed well.
2. 13 μl of calibrators and samples were pipetted into the appropriate reaction cells.
3. 50 μl of hs-CRP tracer was added into each reaction cell.
4. The plate was swirled for 60 seconds and incubated at RT for 15 minutes.
5. Reaction cells were then washed five times with 350 μl of wash buffer (0.35 ml per cell).
6. 50 µl of working signal reagent solution was added into each reaction cell.
7. The microplate was incubated for 5 mins at RT.
8. The relative light units (RLUs) were read in each well in a microplate strip luminometer for at least 0.2 seconds/well.

**Calculation of Results**

A dose response curve was constructed by plotting the mean relative light units or RLUs (Y) of calibrators against the known concentration (X) of standards with units on a linear graph paper. The best-fit curve was drawn through plotted points. The computer data reduction software designed for chemiluminescence assays was used for data reduction. The results were reported as the concentration of hs-CRP (mg/L) in samples.

**Reference Range**

- **Low risk**  < 1.0 mg/L (µg/ml)
- **Normal**  1 – 3 mg/L (µg/ml)
- **High risk**  > 3.0 mg/L (µg/ml)

**4. ESTIMATION OF SERUM TOTAL CHOLESTEROL**

Serum total cholesterol (TC) was determined by **Cholesterol Oxidase-Peroxidase in Aminoantipyrine (CHOD-PAP) method** using ERBA diagnostics fully automated analyzer kit.

**Principle**

The estimation of cholesterol by CHOD-PAP method involves the following enzymatic reactions:
The absorbance of quinoeimine thus formed is read which is directly proportional to the concentration of cholesterol in the sample.

### Reagents

I. Cholesterol reagent consisted of the following:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol esterase (pancreatic)</td>
<td>&gt;200 IU/L</td>
</tr>
<tr>
<td>Cholesterol oxidase (microbial)</td>
<td>&gt;150 IU/L</td>
</tr>
<tr>
<td>Peroxidase (Horseradish)</td>
<td>&gt;2000 IU/L</td>
</tr>
<tr>
<td>Sodium phenolate</td>
<td>20 mmol/L</td>
</tr>
<tr>
<td>4-Aminoantipyrene</td>
<td>0.5 mmol/L</td>
</tr>
<tr>
<td>Phosphate buffer (pH 6.5 ± 0.1)</td>
<td>68 mmol/L</td>
</tr>
<tr>
<td>Lipid clearing agent</td>
<td>-</td>
</tr>
</tbody>
</table>

II. Cholesterol standard (200 mg/dl or 5.14 mmol/L).

III. Aqua-4: double deionized, 0.2 micron, membrane filtered, particle-free water for reconstitution of cholesterol reagent.

### Reagent Preparation

The cholesterol and aqua-4 were allowed to attain room temperature. Working cholesterol reagent was made by adding 20 ml of aqua-4 to vial 1 of cholesterol...
reagent. The contents were dissolved by swirling and not by shaking. This working reagent was stable at 2-8ºC.

Procedure
A set of 3 test tubes were taken and marked as T, S and B for test, standard and blank respectively.

<table>
<thead>
<tr>
<th>S. No</th>
<th>REAGENT</th>
<th>T (µl)</th>
<th>S (µl)</th>
<th>B (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Working reagent</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>2</td>
<td>Distilled Water</td>
<td>-</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>Standard (200 mg/dl)</td>
<td>-</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Sample (serum)</td>
<td>20</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Mixed well and incubated at 37ºC for 10 minutes. The absorbance of all three test tubes was read at 505nm.

Calculation of Results

Concentration of cholesterol (mg/dl) =

\[
\frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Concentration of standard (mg/dl)}
\]

Reference Range

Fasting levels = 140-250 mg/dl
5. ESTIMATION OF SERUM TRIGLYCERIDES

Serum triglycerides (TG) were measured by GlycerolPhosphate Oxidase (GPO)-Trinder method using ERBA diagnostic kit for fully automated analyzer.

Principle

The determination of triglycerides involves the following enzymatic reactions:

1. Triglycerides are enzymatically hydrolyzed by lipase to free fatty acids and glycerol.
2. The glycerol is phosphorylated by adenosine triphosphate (ATP) with glycerol kinase (GK) to produce glycerol-3-phosphate and adenosine diphosphate (ADP).
3. Glycerol-3-phosphate is oxidized to dihydroxyacetone phosphate (DAP) by glycerol phosphate oxidase (GPO) producing H₂O₂.
4. In Trinder type of colour reaction catalyzed by peroxidase, the H₂O₂ reacts with 4-aminoantipyrine (4 AAP) and 3,5-dichloro 2-hydroxybenzene sulphonate (DHBS) to produce a red coloured quinoeimine dye.

The intensity of quinoeimine formed is proportional to triglycerides concentration in the sample when measured at 505 nm (500-540nm).
Reagents

1. Triglycerides (dynamic extended stability) reagent composed of the following:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>2.5 mmol/L</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>2.5 mmol/L</td>
</tr>
<tr>
<td>4-aminopyrinate</td>
<td>0.8 mmol/L</td>
</tr>
<tr>
<td>3,5 DHBS</td>
<td>1 mmol/L</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>More than 2000 U/L</td>
</tr>
<tr>
<td>Glycerol kinase</td>
<td>More than 550 U/L</td>
</tr>
<tr>
<td>Glycerol phosphate oxidase</td>
<td>More than 8000 U/L</td>
</tr>
<tr>
<td>Lipoprotein lipase</td>
<td>More than 3500 U/L</td>
</tr>
<tr>
<td>Buffer (pH 7.0 ± 0.1 at 20°C)</td>
<td>53 mmol/L</td>
</tr>
</tbody>
</table>

In addition, it also contains non-reactive fillers, stabilizers and surfactants.

2. Triglycerides standard (200 mg/dl or 2.3 mmol/L)
3. Aqua-4 is double deionized, 0.2 micron, membrane filtered, particle-free water for reconstitution of triglycerides reagent.

Reagent Preparation
The reagent bottle and aqua-4 were allowed to attain room temperature. Then 20 ml of aqua-4 was added into contents of reagent vial. The contents were dissolved by swirling and then allowed to stand for 10 minutes at room temperature.

Procedure
A set of 3 test tubes was taken and marked as T, S and B for test, standard and blank respectively and was proceed as follows:
<table>
<thead>
<tr>
<th>S.No.</th>
<th>REAGENT</th>
<th>T(µL)</th>
<th>S(µL)</th>
<th>B(µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Working triglyceride reagent</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>2.</td>
<td>Distilled Water</td>
<td>-</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>3.</td>
<td>Standard (200 mg/dl)</td>
<td>-</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>4.</td>
<td>Serum</td>
<td>10</td>
<td>-</td>
<td>10</td>
</tr>
</tbody>
</table>

Mixed all the tubes and incubated for 10 minutes at 37°C. The absorbance was read at 505nm (500-540nm).

**Calculation of Results**

Concentration of triglycerides (mg/dl) =
\[
\frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Concentration of standard (mg/dl)}
\]

**Reference Range**

Normal fasting levels = 25-160 mg/dl

**6. ESTIMATION OF HDL-CHOLESTEROL (HDL-C)**

Serum high density lipoprotein-cholesterol (HDL-C) levels were determined using **Precipitation method**\(^{282}\) using fully automated analyzer based ERBA diagnostics kit.

**Principle**

The assay is based on a modified polyvinyl sulfonic acid (PVS) and polyethylene-glycol-methyl ester (PEGME) coupled classic precipitation method with optimum quantities of PVS/PEGME and selected detergents. Low density lipoproteins(LDL), very low density lipoproteins(VLDL) and chylomicrons (CM) react with PVS and
PEGME and the reaction results in inaccessibility of LDL, VLDL and CM by cholesterol oxidase (CHOD) and cholesterol esterase (CHER). The enzymes selectively react with HDL to produce H₂O₂ which is detected through Trinder reaction.

\[
\text{HDL} + \text{LDL} + \text{VLDL} + \text{CM} \xrightarrow{\text{PVS/PEGME}} \text{HDL} + (\text{LDL} + \text{VLDL} + \text{CM})
\]

\[
\text{CHOD} \quad \text{HDL} \quad \text{CHER} \quad \text{Fatty acid} + \text{H}_2\text{O}_2
\]

\[
\text{peroxidase} \quad 2\text{H}_2\text{O}_2 + 4\text{-aminoantipyrine} + \text{TODB} \quad \text{Quinone} + 5\text{H}_2\text{O}
\]

The absorbance of quinine is then read at 600nm (primary wavelength) using 700nm as secondary wavelength.

**Reagents**

1. Reagent 1 contains the following:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>MES buffer (pH 6.5)</td>
<td>6.5 mmol</td>
</tr>
<tr>
<td>N,N-bis(4-sulfobutyl)-3-methylaniline (TODB)</td>
<td>3.0 mmol</td>
</tr>
<tr>
<td>Polyvinyl sulfonic acid</td>
<td>50 mg/L</td>
</tr>
<tr>
<td>Polyethylene glycol methyl ester</td>
<td>30 mL/L</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>2 mmol</td>
</tr>
<tr>
<td>EDTA</td>
<td></td>
</tr>
<tr>
<td>Detergent</td>
<td></td>
</tr>
</tbody>
</table>
2. Reagent 2 is composed of the following:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>MES buffer (pH 6.5)</td>
<td>50 mmol</td>
</tr>
<tr>
<td>Cholesterol esterase</td>
<td>5kU/L</td>
</tr>
<tr>
<td>Cholesterol oxidase</td>
<td>20kU/L</td>
</tr>
<tr>
<td>Peroxidase</td>
<td></td>
</tr>
<tr>
<td>4-aminoantipyrine</td>
<td>0.9g/L</td>
</tr>
<tr>
<td>Detergent</td>
<td>0.5%</td>
</tr>
</tbody>
</table>

3. Calibrator for HDL (12 mg/dl)

**Procedure**

A set of 3 test tubes was taken and marked as T, S and B for test, standard and blank respectively and proceed as follows:

<table>
<thead>
<tr>
<th>S. No.</th>
<th>REAGENT</th>
<th>T(µL)</th>
<th>C(µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Reagent 1</td>
<td>375</td>
<td>375</td>
</tr>
<tr>
<td>2</td>
<td>Calibrator</td>
<td>-</td>
<td>05</td>
</tr>
<tr>
<td>3</td>
<td>Serum</td>
<td>05</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mixed and incubated at 37°C for 5 minutes.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Reagent 2</td>
<td>125</td>
<td>125</td>
</tr>
</tbody>
</table>

Mixed and incubated at 37°C for 5 minutes. The absorbance was read at 600nm.

**Calculation of Results**

Concentration of HDL-C (mg/dl) =

\[
\frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \frac{\text{Concentration of calibrator (mg/dl)}}{\text{Concentration of calibrator (mg/dl)}}
\]
Reference Range
Males = 35.3 – 79.5 mg/dl;
Females = 42.0 – 88.0 mg/dl

7. ESTIMATION OF VLDL-CHOLESTEROL AND LDL-CHOLESTEROL
Low Density Lipoprotein-Cholesterol (LDL-C) was calculated using Friedwald formula, based on assumption that very low density lipoprotein (VLDL) is present in serum at a concentration equal to 1/5 th of triglyceride concentration.²⁸³

Hence, LDL-C = TC-[HDL-C + TG/5]

Where,
TC = total cholesterol
HDL-C = high density lipoprotein cholesterol
TG = triglycerides

The formula is only valid when all concentrations are given in mg/dl and at serum triglyceride concentration of less than 400 mg/dl. For higher triglyceride levels, sample was appropriately diluted.

8. DETERMINATION OF INSULIN RESISTANCE
IR was estimated using HOMA-IR. The fasting plasma glucose (FPG) X fasting plasma insulin (FPI) is index of hepatic insulin resistance²⁰⁸ and 405 is the product of normal fasting plasma ensulin and glucose of a normal healthy individual.⁸¹

HOMA-IR was calculated by the following formula:

\[
\text{HOMA-IR} = \frac{\text{Fasting plasma insulin(μIU/ml)} \times \text{Fasting plasma glucose (mg/dl)}}{405}
\]
‘STATISTICAL ANALYSIS

Statistical analyses were carried out using SPSS version 23.0 software. Descriptive statistics were calculated for different characteristics of the subjects. Student t-test and Pearson correlation used to compare the statistical differences between continuous variables.