The focus of the present study is on the fishermen families residing in a coastal village "Ponnappudi Pattapu Palem" of Nellore district of Andhra Pradesh. The present investigation was taken up with the following objectives.

1. To estimate haemoglobin, packed cell volume, serum iron, total iron binding capacity, per cent transferrin saturation and serum ferritin levels in order to assess the iron nutritional status and to estimate serum total cholesterol, HDL-cholesterol and triglyceride levels (LDL-cholesterol and VLDL cholesterol levels by calculation), to assess the lipid profiles and also to record anthropometric data (Body Mass Index, Waist/Hip ratio), blood pressure readings and other relevant information in a sample (n=500) of persons of different age groups (i.e. 21-70 years) and sex groups from the fishing communities of Nellore district.

2. To conduct a diet survey among the subjects of the sample in order to record the dietary patterns and food habits, to calculate the mean nutrient composition of the diets consumed by each subject of the sample, and

3. To study the inter-relationship and associations among the parameters of iron nutritional status, lipid profiles, diet and other relevant data (Anthropometric data and blood pressure readings) in the study sample, by subjecting the data to appropriate statistical tests.
Selection of the Village and Fishing Hamlets

The fishing villages of Nellore district were identified and 'Ponnapudi Pattapu Palem' of Vidavalur Mandal, Nellore district was selected for the present study. This village was selected because it has no migrated population and does not receive any financial assistance from the Government. It was previously covered by the investigator to assess the general nutritional status of the mothers and children and was found that most of the women folk suffered from anaemia. Ponnapudi Pattapu Palem and other hamlets near the sea shore were separated from the main village Ramatheeretham. These hamlets are situated between Buckingham canal and the sea shore. These hamlets are in north eastern direction and 38.2 km away from Nellore, a big town in coastal Andhra Pradesh. There are seven fishing hamlets occupying 100 to 150 acres of land between Buckingham canal and the sea shore. The distance between any two hamlets ranges from 0.5 to 2 kms. Previously all the families lived close to each other near the sea in a place called Ponnapudi Pattapu Palem. During high tide and cyclones the sea water inundates these hamlets. So, to avoid this problem the fishermen families moved towards Buckingham canal. The area was divided into six hamlets namely Ponnapudi Peddapalem, Kothuru, Laxmipuram, Venkatanarayanapuram, Ponnapudi and Ramachandrapuram. Few people were converted as Christians and a separate seventh hamlet namely Prasanthagiri was formed twenty years back. The location of the main village Ramatheeretham and Ponnapudi Pattapu Palem (study area) is given in Fig.13.
Fig. 13: Location of the study Area
Preliminary Survey

A preliminary survey was conducted to identify the members of different age groups. General information with regard to their family size and age was collected from all the families of 7 hamlets. The total number of families in 7 hamlets was 1395, with an estimated population of 2394 in the age group of 21 to 70 years. Among these 1183 are males and 1211 are females.

Selection of the Sample for Intensive Survey

Subjects from fishermen families in the five age groups from 21 to 70 years (i.e. 21-30 y, 31-40 y, 41-50 y, 51-60 y and 61-70 y) were selected for the detailed survey. Out of a total of 1395 families in 7 hamlets, subjects of these age groups were found in all families except a few. From the total sample of 1183 males, 250 males (21.1%) and from the 1211 females, 250 females (20.6%) were selected as stated below. The 1183 males and 1211 females were categorised age group wise, and a sample of 500 (250 males and 250 females) was drawn using stratified random sampling method. In each age group, 50 males and 50 females were drawn using tables of random number procedure (Tippett, 1959). The experimental design is given in fig. 14.

To assess the nutritional status of these subjects from the fishermen community, various methods have been employed. Collection of the data on the nutritional adequacy of the diets consumed, anthropometric measurements, clinical examination to ascertain the prevalence of nutritional deficiency symptoms and biochemical tests for iron and lipid status assessment are the methods used to assess nutritional status. From the study sample of 500, a sub sample of 300 (30 men and 30 women in each age group) was drawn using random number procedure. In the sub-sample (n=300), anthropometric measurements, blood pressure readings, a three day dietary survey and biochemical analysis were carried out. The blood samples for bio-chemical tests were drawn on the day following the diet survey.
Fig. 14: The Experimental Design of the Study
Preparation of the Schedule

A schedule was prepared keeping in view, the objectives of the study. The schedule was divided into 12 sections. The various sections covered in the schedule were as follows: General information, food habits, foods consumed regularly, frequency of food consumption, dietary weighment schedule, data regarding female, male, anthropometry, clinical examination, biochemical assessment, and other information. Under each section various aspects were covered. The schedule used for the survey is given in Appendix-i.

Pretesting of the Schedule

To standardize the schedule and biochemical methods, pre-testing was done among 50 members (25 males and 25 females). During the pre-test rapport was established with the subjects of fishing communities. Depending upon the responses given by the subjects during pretest, the schedule was modified wherever necessary. The finalised schedule was then administered to the people of families selected for the final study.

Dietary Survey

Dietary assessment is the process of evaluating what people eat. Specifically, it is the indicator of dietary status, which is one of the several methods used to identify the possible occurrence, nature and extent of poor diet or impaired nutritional status (Dwyer, 1998).

A diet survey was conducted to record the information about the food habits, regular meal patterns, the frequency of consumption of different food items, the food choices, etc, by the fishermen families. The quantity of foods
consumed was assessed in a three day weighment of cooked foods among 300 members of the sample. For the weighment method, thirty members in each age group were selected. The three day dietary survey was carried out among the subjects who were willing to extend their fullest cooperation by participating and maintaining food consumption records for three consecutive days (n=300).

Three Day Weighment

Standardized steel cups were given to the subjects. The quantity of food, in terms of the given cups were noted and calculated. From these data the raw weight equivalents of the recipes were determined in the laboratory and nutrient composition i.e. total calories, proteins, fats, SFA, PUFA, MUFA, P/S ratio, carbohydrates, calcium, iron, β-carotene, vitamin C, and fibre was calculated (Gopalan, 2000). To calculate the n-6 and n-3 PUFA [ALNA, EPA and DHA] the information given by Ghafoorunissa et. al., (2000), Gopakumar and Nair (1972, 1975 and 1977) was used (vide Review of Literature page 118).

Clinical Examination

Clinical examination is an important method among the various methods used for assessing the nutritional status of a community. This method is based on an examination for changes, believed to be related to inadequate nutrition, that can be seen or felt in superficial epithelial tissues, especially the skin, eyes, hair and buccal mucosa or in organs near the surface of the body such as parotids and thyroid glands.
All the subjects of the sample of fishermen community (n=500) were examined for signs of deficiency diseases and general diseases. The classified list of the signs for various deficiencies given by Jelliffe (1966) was adopted to identify the nutritional deficiencies in the subjects.

**Anthropometry**

Nutritional anthropometry is concerned with the measurement of the variations of the physical dimensions and the gross composition of the human body at different age levels and degree of nutrition (Jelliffe, 1966). Certainly the physical dimensions of the body are much influenced by nutrition at any age. Selected body measurements give valuable information. The body measurements selected for the present study were height, weight, BMI and waist to hip ratio for all the subjects surveyed. Weight is a sensitive index to health and nutritional status of the individual than height. Anthropometric measurements indirectly indicate present or past nutrition and may be markers of full health.

**Height**

Height is a measure of linear growth of the body - the degree of skeletal development. Height was measured to the nearest 0.05 cms with an anthropometric rod. The subjects were made to stand on the platform of the measuring rod which was placed on the flat ground. The feet were kept parallel and the heels, buttocks, shoulders and back of the head was kept in an upright position touching the rod. The head was kept comfortably erect and the arms were made to hang at the sides in natural manner. The head piece, a steel plank, was gently lowered crushing the hair and making contact
with the top of the head. The height was measured at the point at which the steel plank stood firmly above the head.

Weight

Bathroom scale/platform scale was used for weighing. The subject was made to stand on the centre of the platform of the weighing machine without touching any other object. The weight was recorded to the nearest 0.5 kg.

The weights of both men and women of the selected groups were recorded three hours after their noon meal. To maintain uniformity in the time of weighing, all the subjects were weighed at a specific time.

Body Mass Index

The standard height and weight of the subjects were taken using standard equipments. The BMI was calculated using formula Wt. (kg) / Ht. (mts)$^2$ given by Deorenberg et. al., (1989). It is also known as Quetlet’s index. On the basis of the index, the subjects would be graded as follows:

<table>
<thead>
<tr>
<th>Grade</th>
<th>Index (Kg/m$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 0 or normal</td>
<td>20-24.9</td>
</tr>
<tr>
<td>Grade I</td>
<td>25-29.9</td>
</tr>
<tr>
<td>Grade II</td>
<td>30-39.9</td>
</tr>
<tr>
<td>Grade III</td>
<td>&gt; 40</td>
</tr>
</tbody>
</table>

 Waist

Subject was asked to stand erect, abdomen relaxed, arms at the sides and feet together and with weight equally divided on both legs. The subjects
were asked to breathe out gently at the time of measurement to prevent them from contracting their muscles or from holding their breathes. The tape was placed horizontally mid-way between the lowest rib margin and iliac crest and measurement was taken in centimetres.

Hip

The subject was asked to stand erect with arms at the sides and the feet together. The measurement was taken at the point yielding the maximum circumference over the buttocks, the tape was then placed in horizontal plane touching the skin but not indenting the soft tissue.

Blood Pressure

Blood pressure was measured using sphygmomanometer with normal cuff size (23 x 12 cms). Half an hour before the measurements, the subjects were advised to rest quietly for five minutes. The subject sat relaxed in chair. The right arm was positioned and supported at the heart level. The bladder was placed over the branchial artery and the cuff was inflated far above the pulsed systolic pressure. Then the bladder was slowly deflated. The first appearance of sound was recorded as ‘systolic blood pressure’ and pressure was allowed to fall preferably about 2-3 mm of Hg/second. The disappearance of sound (phase-V) was recorded as ‘diastolic pressure’. Care was taken to record the blood pressure correctly.

Standards for Anthropometry

The results of anthropometric survey should preferably be expressed in relation to local standards that have been constructed from measurement of apparently healthy subjects of the same ethnic groups.
In order to compare the anthropometric data collected for the present study, standard heights and weights of the men and women of their age were referred as per the Indian standards. BMI was calculated from the weights and heights for the assessment of obesity in the individuals.

**Bio-Chemical Measurements**

Biochemical measurements represent the most objective assessment of the nutritional status of an individual, frequently providing clinical and sub-clinical information. The levels of the nutrients or their products present in the blood or urine do indicate the dietary intake to some extent. To assess the iron nutritional status and lipid profiles, blood samples were collected from each subject of the sample.

To assess the iron nutritional status, parameters selected were per cent haemoglobin, packed cell volume, serum iron, total iron binding capacity (TIBC) and per cent transferrin saturation.

To assess the lipid profile the parameters selected were total cholesterol (TC), triglycerides (TG), High Density Lipo Proteins Cholesterol (HDL-C), Low Density Lipo Protein Cholesterol (LDL-C) and Very Low Density Lipo Proteins (VLDL).

**Selection of the subjects for serum ferritin analysis**

The sample was screened for serum ferritin analysis. The serum iron levels of the subjects of the sample were lower than the median standard reference value i.e. 95 μg/dl. Based only on their serum iron levels the subjects were selected for the estimation of serum ferritin levels. Following cut off points were used to select the subjects in different age and sex groups. The cut off points for serum iron values for young male and female subjects (21-40y) were > 70 μg/dl and > 65 μg/dl respectively. For middle aged male and female (41-60y) subjects it was > 65μg/dl and >55 μg/dl respectively. For
the elderly males and females (61-70y) it was > 55 µg/dl. The corresponding serum cholesterol levels of these subjects were noted. For the younger male and female subjects the range of serum cholesterol levels were 190-227 mg/dl and 150-203 mg/dl respectively; for middle aged males and females the levels were 160-213 mg/dl and 150-207 mg/dl respectively; and for the elderly males and females the levels were 160-205 mg/dl and 175-218 mg/dl respectively. It was observed that those subjects with the higher serum iron levels (within the range of minimum & maximum estimated values) also showed higher serum total cholesterol levels (in the range of minimum and maximum estimated values for the same group). Thus, for the analysis of serum ferritin levels a sub sample of 100 subjects (50 males and 50 females) was selected as stated above.

Collection of the Blood Sample

The subjects were intimated the exact date and time of collection of blood samples. Disposable syringes and needles were used to draw the blood samples. About ten ml of the venous blood was drawn from each subject, with the use of tourniquet. The samples were transferred to labelled sterile glass vials and allowed to clot for two to three hours. All precautions were taken in collection, preparation and storage of the blood samples and care was also taken to prevent haemolysis.

Serum Preparation

The collected blood samples were allowed to stand at room temperature for two to three hours. After the clot was formed and the serum separated, the serum was transferred into clean dry centrifuge tubes and was centrifuged for twenty minutes at 3000 rpm. The clear serum was transferred into clean sterile dry bottles. The serum was stored under refrigeration for subsequent estimation of various parameters of iron status and lipid profiles. All the estimations were carried out within 24-48 hours.
Iron Status Assessment

Haemoglobin

Cyanomethemoglobin method was used to assess the haemoglobin levels of the subjects (Dacie and Lewis, 1984).

Principle

In the presence of potassium ferricyanide, at alkaline pH, haemoglobin and its derivatives (except sulmethemoglobin) are oxidised to methaemoglobin. Methaemoglobin so formed reacts with potassium cyanide to form cyanmethemoglobin, a red coloured complex, which is measured colorimetrically. The colour intensity is proportional to the haemoglobin concentration of the blood sample.

Reagents: 1. Drabkin's solution, 2. Cyanmethemoglobin standard

Procedure

0.02 ml of blood was taken using Sahli's pipette and 6.0 ml Drabkin's working reagent was added to this. The contents were mixed well and allowed to stand at room temperature for five minutes. The optical density of test against Drabkin's solution was measured at 640 nm in Spectronic 21D UV.

Standard

The optical density of the cyanmethemoglobin standard was read directly against Drabkin's solution. The plotted standard curve is presented in fig. 15.

Calculation

\[
\text{OD test} = \frac{\text{Blood Hb g\%}}{\text{OD standard}} \times \text{concentration of standard in mg\%} \times 0.25
\]

Concentration g/dL

Standard Curve for the Estimation of Haemoglobin

Fig. 15. Standard Curve for the Estimation of Haemoglobin
**Determination of Packed Cell Volume (PCV) (or) Haematocrit**

Wintrobe method is used to estimate haematocrit (Wintrobe, 1981).

**Principle**

When anticoagulated blood is centrifuged at a standard speed, RBC which are heavier than WBC and platelets and plasma sediments at the bottom. This red cells column is called packed red cell volume which is expressed as a fraction of whole blood.

**Chemicals and Equipment Required**

1. Wintrobe haematocrit tube: It is 110 mm long narrow test tube with a 3 mm internal bore graduate from 0 to 10 cm (100 mm) with the graduations both in ascending and descending order on the two sides of the graduations. Thus the top '0' and 10 cm coincide. It holds about one ml of blood.

2. Transfer pipette - fine capillary pasteur pipette or special syringe.

3. Centrifuge

4. EDTA or heparin 1 mg/ml double oxalate 2 mg/ml (only potassium oxalate should not be used. It shrinks red cells).

**Procedure**

1. Carefully the blood specimen was mixed after collection with the anticoagulant.

2. The Wintrobe tube was filled to the 10 cm mark which represents 100%. Care was taken to see that air bubbles are not there.

3. For balance second tube was filled with water.

---

4. The two tubes were placed in the opposite directions in the centrifuge.
5. The tubes were centrifuged for 30 min. at 2500-3000 rpm.
6. After 30 min the packed red cell volume (PCV) read directly from the graduation marks noted on the tube. (The height of column of RBC).

**Calculation:** If the reading is 42 of the packed red cells, the result is expressed as 42%.

**Serum Iron**

Bathophenanthroline method (Raghuramulu et. al., 1983; NIN lab manual) was used to estimate the serum iron (Br.J. Haematology, 20, 451,1971).

**Principle**

Iron in serum is present as Fe³⁺ bound to transferrin. In estimations of serum iron, the iron is released by mild acid treatment, the proteins are precipitated and the released iron (Fe³⁺) is reduced to Fe²⁺ by reducing agents and then this Fe²⁺ is reacted with bathophenanthroline to form a pink colour complex, whose intensity can be measured at 540 nm.

**Reagents**

1. **Protein precipitant:** Aqueous solution made to contain 100 g TCA, 30 ml of thioglycolic acid and 2 ml HCL per litre. This should be stored in a dark brown bottle and is stable for at least two months.
2. **Chromogen solution:** Sodium acetate (2N) contains 250 mg bathophenanthroline sulfonate per litre.
3. **Iron standard solution**: Stock solution: 70.2 mg ferrous ammonium sulphate, FeSO₄(NH₄)₂SO₄·6H₂O dissolved in water containing 0.2 ml of 2NH₂SO₄ and made upto one litre. The iron concentration of the solution is 1mg/dl.

**Working standard**: 40 ml stock solution is diluted to 100 ml with glass distilled water. This working standard contains 400 μg/dl.

**Procedure**

To one ml of plasma, one ml of water and two ml of protein precipitant solution was added. It was mixed thoroughly and allowed to stand for five minutes and centrifuged to get an optically clear supernatant solution (2000 rpm for 15 minutes). Two ml of the clear supernatant was transferred to a clean tube and two ml of the chromogen solution was added. It was mixed and allowed to stand for atleast five minutes. The optical density of the colour developed was measured in a spectronic 21DUV.

A reagent blank was prepared by substituting water for serum. The optical density of the blank should not exceed 0.015 against distilled water in a one cm pathway. A standard curve was plotted using 0.1, 0.5, 1.0, 1.5 and 2.0 ml of working standard and treating them similar to the test sample and presented in fig. 16.

**Calculation**

X amount of iron was present in 0.5 ml plasma. This value when multiplied by 200 would give a plasma iron concentration in μg/dl serum.
Fig. 16. Standard Curve for the Estimation of Serum Iron
Total Iron Binding Capacity

Principle

Transferrin is saturated 100 per cent by adding iron from outside in Fe⁺⁺⁺ form. After chelating the iron not bound to transferrin, the transferrin iron is estimated as in the case of serum iron.

Transferrin normally is saturated to only 33 per cent by iron. Determination of transferrin saturation provides a good index of iron nutritional status (Advances in clinical chem Vol.1, Acad Press, p.17, 1958) (Raghuramulu et. al., 1983; NIN Lab Manual).

Reagents

1. Ferric chloride solution containing 5 μg iron/ml in 0.005 N HCl.
2. Reagents as for iron estimation by bathophenanthroline method.

Procedure

One ml of serum and two ml of ferric chloride solution was added in a test tube. It was shaken well and the mixture was allowed to stand for five minutes (This gives a total addition of 1000 μg of iron to each 100 ml serum while unsaturated iron binding capacity of the serum ranges from 0-500 μg/dl in normal and pathological states). To the above mixture 200 mg magnesium carbonate was added and the test tube agitated frequently and thoroughly for 45 minutes. The tube was centrifuged at 3000 rpm for ten minutes and the iron content of supernatant was determined using 1.5 ml aliquot.
Calculation

X μg amount iron is present in 0.25 ml of serum. This value when multiplied by 400, would give TIBC value in μg/dl of serum.

Per cent Transferrin Saturation

This was calculated by using the following formula.

\[
\text{Per cent transferrin saturation} = \frac{\text{Serum Iron}}{\text{TIBC}} \times 100
\]

Serum Ferritin

To assess the serum ferritin levels, the method used was enzyme immunoassay (EIA) - a kit provided by Merck Company (White et. al., 1986).³

Principle

The ferritin quantitative EIA test is based on a solid phase enzyme-linked immunosorbant assay (ELISA). The assay system utilizes one rabbit anti-ferritin antibody for solid phase (microtiter wells) immobilization and a mouse monoclonal antiferritin antibody in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test sample is allowed to react simultaneously with the antibodies, resulting in the ferritin molecules being sandwiched between the solid phase and enzyme linked antibodies. After a 45 minute incubation at room temperature, the wells are washed with water.

to remove unbound labelled antibodies. TMB reagent is added and incubated at room temperature for twenty minutes, resulting in the development of a blue colour. The colour development is stopped with the addition of 1N HCl, and the colour is changed to yellow and measured spectrophotometrically at 450 nm. The concentration of ferritin is directly proportional to the colour intensity of the test sample.

Reagents

1. Antibody coated microtiter plate with wells.
2. Enzyme conjugate reagent
3. Ferritin reference standards, containing 0, 15, 80, 250, 500 and 1000 ng/ml (NIBSC-WHO 80/602 human liver standard) liquid, ready to use.
4. TMB reagent
5. Stop solution (1N HCl).

Reagent Preparation

All reagents should be allowed to reach room temperature (18° to 25°C) before use.

Procedure

The desired number of coated wells were secured in the holder. Into appropriate wells 20 µl of standard, specimens and controls were dispensed. Into each well 100 µl of enzyme conjugate reagent was dispensed and mixed
gently for thirty seconds (Complete mixing is very important in this step). The mixture was incubated at room temperature for 45 minutes. The incubated mixture was removed by flicking plate contents into sink. The microtiter wells were rinsed with distilled or deionized water for five times and flicked (tap water should not be used). The wells were sharply struck on to absorbent paper or paper towels to remove all residual water droplets. To each well, 100 μl TMB reagent added and gently mixed for five seconds. The mixture was incubated at room temperature in the dark for 20 minutes. By adding 100 μl of stop solution to each well the reaction was stopped. This solution was mixed for 30 seconds. It was ensured that all the blue colour changed to yellow colour completely. The optical density was read at 450 nm with a microfilter plate reader within 15 minutes.

**Calculation of Results**

The average absorbency values (A450) for each set of reference standards, control and samples were calculated. A standard curve was constructed by plotting the mean absorbence obtained from each reference standard against its concentration in ng/ml on linear graph paper with absorbence values on the y-axis and concentration on the x-axis and presented in fig.17.

Using the mean absorbence value of each sample, the corresponding concentration of ferritin in ng/ml from the standard curve was determined.
Fig. 17. Standard Curve for the Estimation of Serum Ferritin
Assessment of Lipid Profiles

Estimation of Serum Total Cholesterol

Cholesterol estimation was done using Parekh and Jung (1970) method in which ferric chloride is converted to ferric acetate by the addition of ammonia and dissolved in glacial acetic acid. This reagent while functioning as cholesterol precipitate, rules out the production of HCl and elimination of interfering chromogen is further aided by the addition of uranium acetate and develops a very stable purple colour reaction in the presence of sulphuric acid -ferrous sulphate reagent, the intensity of which is proportional to the concentration of the substance and read against the blank at 540 nm wavelength in spectronic 21D UV.

Reagents

1. Ferric acetate - Uranium acetate reagent: To 500 mg of FeCl₃, 10 ml of distilled water was added and dissolved well. 3 ml of ammonia was added to it and shaken well and centrifuged at 3000 rpm for 15 min. Then the precipitate was washed with glacial acetic acid and finally dissolved in glacial acetic acid, forming ferric acetate in acetic acid to 1 litre volume. Then 100 g of powdered uranium acetate was added and shaken well before and after standing overnight. The reagents were stored in a brown bottle which is stable for at least 6 months.

2. Ferrous sulphate - sulphuric acid reagent: 50 ml of glacial acetic acid was poured slowly into a pyrex flask containing 450 ml of H₂SO₄ slowly. The contents were mixed well by gentle rotation. To this 50 mg of ferrous sulphate was added and stirred well and stored in brown bottle.

3. Cholesterol standard: 200 g of recrystallized cholesterol was dissolved in 100 ml of chloroform and mixed well.

Procedure

In a series of clean, labelled test tubes (2 Nos.), 0.05 ml of serum and standard were added respectively. To this, 10 ml of ferric - acetate uranium acetate reagent was added. It was shaken well and left to stand for 20 min. Then the contents were transferred into centrifuge tubes and centrifuged for 20 min at 5000 rpm. After this 3 ml aliquot of the supernatant was transferred from each tube into appropriately labelled test tubes. To this 2 ml of ferrous sulphate - sulphuric acid reagent was added. The contents were shaken well and left for 20 min. Blank was prepared with 3 ml of reagent 1 and 2 ml of reagent 2. The readings were taken after 20 min, against the blank at 560 nm wavelength.

Calculation

\[
\text{OD of unknown Cholesterol (mg/dl)} = \frac{\text{OD of unknown}}{\text{OD of standard}} \times 200
\]

The standard curve was plotted with concentrations ranging from 15 µg cholesterol standard and the curve is given in fig.18.
Fig. 18. Standard Curve for the Estimation of Total Cholesterol
Estimation of HDL-C

Precipitation and Quantification of HDL-C

Reagents for HDL-C

1. 10,000 units of Heparin (Sigma Chem. Co., USA) is dissolved in 5 ml of double distilled water to as to make the concentration 20,000 units per ml.

2. Heparin manganese chloride: 1.584 of MnCl₂ is added to a graduated tube. Then 0.5 ml of heparin (20,000 units) is added and made upto 4 ml with double distilled water. This concentration has 2,500 units/ml of Heparin. This reagent is stored under refrigeration.

3. 0.89% NaCl₃ saline solution using double distilled water.

4. Cholesterol standard: 2 mg/ml of working standard solution was prepared.

Heparin manganese precipitation procedure was followed as given by Warnick (1978) and Borstein (1970).

Lipoproteins form insoluble complexes with sulphated polysaccharide and divalent cations. The larger the sulphated polysaccharide or lipoprotein, the greater the density for complex formation. A common approach to HDL precipitation uses Heparin and Mn₂⁺ (Warnick and Albers, 1978; Borstein et al., 1970).


**Procedure**

To a series of 5 ml capacity propylene tubes (3 nos.), one ml of distilled water, one ml of standard and one ml of serum respectively were added. One ml of saline was added to each of the tubes and shaken well and kept in an ice bath. Then 0.18 ml of heparin - MnCl₂ was added and the tubes were kept in an ice bath and kept at 4°C for 30 min. Then the tubes were centrifuged at 10,000 rpm for 30 min. From the supernatant, 0.1 ml was taken for quantification of cholesterol, by the same method used for total cholesterol estimation.

**Calculation**

\[
\text{HDL-C (mg/dl)} = \frac{\text{Reading of Test solution}}{\text{Reading of Standard}} \times 100
\]

**Estimation of Serum Triglycerides**

**Principle**

Glycerol moiety is oxidised to formaldehyde and the latter condensed with ammonia and 2,4-pcnenedine (acetyl acetone) to produce 3,5-diacetyl 1,4-dihydrotoludine, which is yellow in colour and has absorption at 405 nm (Fletcher method modified by Foster and Dunn, 1973)⁷.

---

Reagents

1. Alumina: The alumina was washed with water until all the fine particles are removed and dried in an oven at 100-110°C overnight. It was cooled and stored in a desiccator. This solution was stable for six months.

2. Saponification reagent: Five grams of KOH was dissolved in 60 ml of distilled water and 40 ml isopropanol was added to it.

3. Sodium metaperiodate reagent: In 700 ml distilled water, 77 g of anhydrous ammonium acetate, 60 ml glacial acetic acid and 650 mg of sodium metaperiodate was dissolved and diluted to 1:1 with distilled water.

4. Acetyl Acetone reagent: To 20 ml of isopropanol, 0.75 ml of acetyl acetone was added and mixed well. To this 80 ml of distilled water was added and mixed well.

5. Stock standard solution: Triolein 1 g/dl (Sigma Chemical Co, USA) Working standard: 300 mg/dl stable for at least 6 months at 4°C in a tightly sealed container.

Procedure

Extraction: 0.1 ml of serum standards were taken in screw capped tubes. The volume was made up to four ml with isopropanol and mixed well. To this 0.4 g of washed alumina was added (a calibrate scoop may be used) to all the tubes and they were placed on mechanical shaker for fifteen minutes and centrifuged.
Two ml of supernatant fluid was transferred after centrifugation to appropriately marked tubes. 0.6 ml of saponification reagent was added to the tubes and incubated at 60-70°C for 150 minutes. After cooling, 1.0 ml of sodium metaperiodate solution was added and mixed well. Then 0.5 ml acetyl acetone reagent was added and mixed again. The tubes were incubated at 50°C for 30 minutes. After cooling, the absorbance was read at 405 nm in a spectronic 21D UV. A standard curve was plotted and presented in fig.19.

**Calculation**

\[
\text{mg Triglyceride/dl serum} = \frac{\text{OD of sample}}{\text{OD of standard}} \times 200 \times \frac{1}{1000} \times 100/0.1
\]

**Calculation of LDL-C**

LDL-C was calculated using the Friedwald formula:\(^8\)

\[
\text{LDL-C (mg/dl)} = \text{TC} - (\text{HDL} - \text{TG}/5)
\]

**Calculation of VLDL**

Cholesterol in VLDL lipoprotein fraction was calculated based on the above two estimations of HDL and LDL using the formula.

\[
\text{VLDL-C} = \text{TC} - (\text{LDL-C} + \text{HDL-C})
\]

---

Fig. 19. Standard Curve for the Estimation of Triglycerides
Statistical Analysis

All the data recorded was tabulated and subjected to ANOVA, Duncan's Multiple Range test, 't' test and Pearson's Correlation Coefficient as necessary and appropriate. The statistical analysis was carried out by using SPSS (11.5 version).