MATERIAL AND METHODS
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Case material.

The clinical material of present study constituted by the healthy volunteers and patients admitted in medical wards. The healthy volunteers having the evidence of coronary artery disease, cerebrovascular accidents, peripheral atherosclerotic diseases, diabetes, nephrotic syndrome, myxoedema and other diseases affecting the lipid metabolism were excluded from the present study. Patients of those diseases were selected which were usually associated with hyperlipoproteinaemia. All of them were submitted to thorough clinical examination, investigations and were also enquired about their detailed dietary history to assess the amount of cholesterol/fat consumed.
Other particulars of healthy and diseased cases were collected according to proforma (Appendix - C).

On the basis of plasma lipid and lipoproteins they were grouped into (1) Normolipoproteinaemia, (2) Hyperlipoproteinaemia and its different types.

Methodology.

Cholesterol/fat ingestion (Test meal):

Amount:

Cholesterol/fat load 550 mg. cholesterol approx.
P/S ratio 0.4 for egg 36.5 gram fat content total.
0.1 for milk, butter 18.4 gram fat being saturated of total.

Composition:

egg yolk of whole eggs - 2 or
0.5 gram crystalline cholesterol.
milk - whole (4% fat) 200 ml.
butter - 20 gram.
Bread slice 2.

Technique: - Patients/subjects were asked to consume low fat, low cholesterol diet for 2 weeks preceding the test. Sampling was deferred for two weeks after a minor illness, or for two to three months after myocardial infarction in patients. The weight of subjects remained steady for last 2 weeks and during the test period. They were asked to take their usual dinner at 6.00 P.M. on day preceding the test.
Fasting blood samples were collected at 8.00 A.M. (14 hours fast) without producing venous stasis in recumbent posture (Koerselman, Lewis and Pilkington, 1961). Plasma was preferred for lipoprotein analysis since it could be kept constantly at 0°C-4°C, and analysed on the same day or within 7 days at the least (Beaumont, 1970).

After the collection of fasting samples, these subjects were asked to take the above mentioned cholesterol/fat diet.

Subsequent blood samples were collected at 0 hour/8.00 AM. 4 hours/12 Noon 8 hours/4 PM. 8 A.M. 8 A.M
Fasting sample 1st day 1st day 1st day. 3rd 5th
1st day

During the collection of blood samples individuals were allowed to take usual diet after 4.00 P.M. on 1st day.

Following tests performed.

1. Refrigerator test (Observation of standing plasma):

If the chylomicrons or VLDL are present in sufficient concentration (usually presenting a triglyceride concentration of 300 mg/100 ml. or greater), they will scatter light and impart turbidity or lectescence to the sample (lipaemia). When plasma stands in a tube at 0°C-4°C (without freezing) for 18-24 hours chylomicrons will rise to the top of the tube and form a visible layer of "cream". Diffuse turbidity indicates an increase in
SHOWING FASTING PLASMA 24 HOURS AFTER TEST MEAL.

Test tube No. 1 - Clear plasma.
Test tube No. 2 - Clear plasma with "cream" layer of chylomicrons.
Test tube No. 3 - "Cream" layer of chylomicrons with infranatant turbidity.
Test tube No. 4 - Only infranatant turbidity.
VLDL concentration. But minimal elevation in triglyceride and VLDL may be present in the absence of turbidity (Beaumont et al, 1970).


PROCEDURE:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Pipette into three test tubes labelled (T), (S) &amp; (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Test(T)</td>
</tr>
<tr>
<td>Cholesterol Reagent (1)</td>
<td>5.0 ml.</td>
</tr>
<tr>
<td>Cholesterol Standard(2)</td>
<td>-</td>
</tr>
<tr>
<td>Serum</td>
<td>0.05 ml.</td>
</tr>
</tbody>
</table>

Mix well and immediately keep the test tubes in a boiling water bath exactly for one and a half minute. Cool them immediately under running tap water. Measure the optical density of the Test (T) and Standard (S) at 560 nm or using yellow green filter against Blank (B) to set zero.

CALCULATION:

Serum cholesterol in mg/100 ml = \( \frac{(T)}{(S)} \) x 200

NORMAL VALUES

Normal values for different age groups has been shown in Table No. 3 (Fredrickson et al, 1968).
3. Thin layer chromatography - Quantitative analysis of plasma triglyceride and free plasma cholesterol (Gloster and Fletcher, 1966).

Chemical methods for the analysis of all lipids are often unsuitable for the routine use and ultracentrifuge separation is too costly. Thin layer chromatography of lipids, however, uses the small samples, gives good separation and requires only small amount of solvents to quantitate the separated lipids. Thin layer chromatography gives a rapid method which is suitable for routine use and requires no expensive equipment (Gree and Saukkonen, 1964).

PROCEDURE:

Serum extract was prepared by mixing 0.2 ml. of plasma with 4.0 ml. of chloroform: Methanol mixture 2:1 by volume. The extract was dissolved in 50 microlitre of chloroform and separation of lipids was done with solvent containing mixture of 85 ml. petroleum ether, 15 ml. diethyl ether and 2 ml. of glacial acetic acid. In present study square glass jar with silica gel slide was used for thin layer chromatography. Spots were detected with most simplest and sensitive method of iodine vapour.

Lipids containing zones are removed from the plate as soon as possible by careful scraping with a scalpel blade and placed in glass stoppered centrifuged tubes. Unstained areas of similar size were removed to
Showing spot localization of lipid fractions by thin layer chromatography.
act as blanks. Normal lipids were eluted from the jel
by shaking with two successive 5 ml. portion of diethyl
ether, centrifuging and decanting the supernatant.
Lipids were taken to dryness under air stream. To free
plasma cholesterol 6.0 ml. glacial acetic acid was added
and warmed gently for 5 minutes to ensure solution, before
free plasma cholesterol was estimated (Macintyre and Ralston
1954). The triglyceride was dissolved in ethanol : ether
3 : 1 and estimated by modified hydroxyline method
(Morgan and Kingsbury, 1959).

4. Paper electrophoresis - for lipoprotein analysis
(Lees and Hatch, 1963).

Fredrickson in 1977 at recent seminar on the
"Disorders of Lipid Metabolism" held at New Delhi has
emphasized that with modern paper electrophoresis
technique, it is possible to identify practically all the
lipoprotein families without ultracentrifugation.

PROCEDURE :

(i) Electrophoresis

(ii) Staining and Densitometry

Electrophoresis

This was performed at room temperature in
barbitone buffer of ionic strength, 0.5 and pH 8.6.
0.02 ml of plasma was applied with micropipette in a streak
SHOWING EQUIPMENTS OF ELECTROPHORESIS.
on a whatman paper No. 3 near the negative pole of cell.
Electrophoresis was carried out for 16 hours (overnight run)
at constant voltage (110 V.) with a current of 0.1 milli-
amp./cm. width of the strip. Optimal voltage gives a beta
lipoprotein migration of at least 1.5 cm. and also causes
the best resolution of beta and pre-beta fractions. The
polarity of the electrodes was reversed with each
successive run to minimise pH changes and buffer
crystallisation on the electrode wire.

Staining and densitometry:

Strips were dried either in air or an oven and
stained with sudan black-B (a saturated solution of dye
in ethanol) for 30 minutes. Strips were washed with a
quick rinse in 50% ethanol and thoroughly in 40% ethanol
until most of black ground clears. These were scanned
using an alab densitometer and coloured filter of 530 nm.

In paper electrophoresis study, the number of
cases showing dense band were reported rather than
percentage of different lipoproteins. It was done so
because change in amount of one lipoprotein cause change
in relative percentage of other and confound interpretation.

Criteria for typing hyperlipoproteinaemia:

After estimation of total plasma cholesterol,
plasma triglyceride and study of lipoprotein pattern,
cases were grouped into normolipoproteinaemia,
hyperlipoproteinaemia and its different types (Beaumont,
1970).

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