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***Research Article***

# ESTIMATION OF LECITHIN CHOLESTROL ACYL TRANSFERASE ACTIVITY AND HIGH DENSITY LIPOPROTEIN CHOLESTROL IN TYPE 2 DIABETES MELLITUS

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## ABSTRACT
An important enzyme in modulating plasma HDL levels is lecithin cholesterol acyl transferase (LCAT) which is responsible for the formation of most of the cholesterol esters (CE) present in human plasma. This enzyme catalyses the transfer of fatty acid from the Sn-2 position of the lecithin to the free hydroxyl group of cholesterol. It utilizes linoleate for the esterification of cholesterol in preference to the other fatty acids. Thus the enrichment of the linoleate content of plasma lecithin which accompanies ingestion of a poly unsaturated fat diet leads to an increase in the proportion of cholesterol linoleate in plasma. The physiological substrate for LCAT is probably nascent HDL. The enzyme activity is dependent on apoA-1 and is inhibited by apoA-2. Thus the maturing HDL particles contain mainly esterified cholesterol which having been rendered non diffusible are trapped in the HDL and then are transferred to the liver to undergo catabolism.
Present studies have been carried out on diabetic patients with good glycaemic control and diabetics with poor glycaemic control and compared with normal control group. HDL cholesterol levels in diabetic patients with good and poor glycaemic control were significantly lower than the normal control group. LCAT in diabetic patients with good and poor glycaemic control were significantly lower than the normal control group. However HDL cholesterol level and LCAT activity in diabetic patients with poor glycaemic control was still lower this supports the observation that coronary artery disease (CAD) is 2-4 times higher in diabetes than in non diabetes.

***Key Words:*** *HDL (High Density Lipoprotein), LCAT (Lecithin Control Acyl Transferase), CAD (Coronary Artery Disease), NIDDM (Non-Insulin Dependent diabetes Mellitus)*

## INTRODUCTION
Accelerated coronary and peripheral vascular atherosclerosis is one of the most serious and chronic complication of long term diabetes. Coronary heart disease is 2-3 times higher in diabetes than in non-diabetes.
Mammilton (1972) has shown that HDL is synthesized in the liver and in its nascent form appears as bi-layered discs on electron microscopy. These nascent particles consist mainly of apoE, apoC, phospholipid and free cholesterol. Subsequently the apoE is largely replaced by apoA-1 and most of the cholesterol becomes esterified as a result of the action of the LCAT enzyme.
Reichi *et al.,* (1986) demonstrates that HDL/LCAT complex is able to act as acceptor of cellular free cholesterol, which is then esterified by LCAT and transferred to core of the particle to become $HDL_2$ the cholesterol ester can then be transferred to other lipoproteins of lower density by a lipid transfer protein and reach the liver for excretion.
Glomset (1968) reveals that LCAT is synthesized in liver and circulates in plasma associated with HDL. In disorders that obstruct flow of bile, concentration of un-esterified cholesterol and lecithin in plasma are increased. Esterification of cholesterol was decreased in many patients with impaired liver function.
Curtiss (1985) has shown that chronic hyperglycaemia in diabetic patient's leads to non-enzymatic glycation of proteins including apoA-1.
Gugliucci (1991) demonstrates that this modification of apoA-1 results in a decrease of LCAT activity.

## MATERIALS AND METHODS

### *Selection of Subjects*

The study is conducted in three groups of subjects selected from out patients as well as in patients from the Department of Medicine at Owaisi Hospital & Research Centre and Princess Esra Hospital, Hyderabad.

**Group 1**

Consists of (10) normal adult males and females between the age group of 30 – 50 years selected as control group with no history of diabetes mellitus.

**Group 2**

Consists of (15) subjects of similar age group with good glycaemic control of Diabetes Mellitus.

**Group 3**

Consists of (15) subjects of similar age group with poor glycaemic control of diabetes mellitus.

The following parameters were studied on the fasting blood sample:

1. Estimation of total cholesterol
2. Estimation of Free and Ester cholesterol
3. Estimation of HDL cholesterol

Fasting venous samples are collected in sterile clean and dry bottles. Serum was separated and assays are performed immediately.

### *Estimation of Total Cholesterol*

Method: Colourimetric Method using salkowski reaction (Zlatkis, 1953; and Varley, 1969).

Equipment: Bausch & Lomb photo colourimeter (Spectronic 20).

Principle: Proteins in serum are precipitated by ferric chloride. The cholesterol present in protein free filtrate is oxidized and dehydrated with ferric chloride, acetic acid and sulfuric acid to a red coloured compound. A measure of the intensity of the colour indicates the concentration of cholesterol in the serum.

### *Reagents*

1. Acetic Acid: (Analytical grade, aldehyde free).
2. Ferric chloride – (10%) 10 grams. Of Feb 13, $6H_2O$ dissolved in 100 ml of acetic acid.
3. Colour Reagent: 2.5 ml of 10% $FeCl_3$ was added to 500ml of aldehyde free acetic acid.
4. Sulfuric acid (Analar grade)
5. Stock cholesterol standard – 100 mg of cholesterol was added to 100ml of purified acetic acid.
6. Cholesterol working standard solution. The stock cholesterol standard was diluted one ml to 25 ml with colour reagent.

**Procedure:** In a centrifuge tube take 9.9 ml colour reagent and add 0.1 ml serum mix well and allowed to stand for 10 – 15 minutes at room temperature and centrifuged.

II. Take 3 test tubes and label as 'B' (blank) T (Test) and S (standard) and pipette the following reagents in the respective tubes.

| S. No. | Reagents | B (in ml) | T (in ml) | S (in ml) |
|---|---|---|---|---|
| 01 | Supernatant fluid | - | 5 | - |
| 02 | Colour Reagent | 5 | - | - |
| 03 | Working cholesterol std. | - | - | 5 |
| 04 | Concentrated Sulfuric acid | 3 | 3 | 3 |

Mix well and keep for 30 minutes at room temperature.

Test and standard are read at 560 nm against blank.

Calculations:
Mg of cholesterol / 100 ml of serum

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times \frac{0.2}{0.05} \times 100 \times \frac{\text{Reading of T}}{\text{Reading of S}} \times 400$$

***2. Estimation of Free and Ester Cholesterol***

Method: Digitonin precipitation (Sperry and Webb, 1950).

Principle: Digitonin forms a highly insoluble 1:1 complex with free cholesterol and thus, free cholesterol gets precipitated. The free cholesterol is then determined colourimetrically using Zlatkis, Zas and Boyle method.

***Reagents***

1. Ethanol- Ether, 3 volumes ethanol (950ml/L with water) 1 volume Ether.
2. Digitonin solution, 5g/L in ethanol (950ml/L with water)
3. Light Petroleum, B.P. 30-60 degree centrigade.
4. Cholesterol standard, 4mmol/L (193mg/ 100ml) in light petroleum. Dilute 1 to 10 with light petroleum for use.
5. Colour reagent 0.005% Ferric chloride in acetic acid (Aldehyde free)
6. Sulphuric acid- Analar grade.

***Procedure***

Take 5ml ethanol-ether mixture in a centrifuge tube and add 0.4ml serum and keep it in a water bath between 60-70 degrees centigrade for 10 minutes, cool and make up to 10ml with ethanol ether and centrifuge. Take 2.5ml supernatant in a centrifuge tube with a stopper and add 0.4ml digitonin solution. Mix and keep for 10 minutes, evaporate to dryness in a water bath and add 60-70degree centigrade, add 5ml light petroleum, bring to boil in water bath, cool stopper and centrifuge, decant the supernatant and repeat the extraction. Evaporate the combined extracts to dryness at 60-80 degree centigrade. As a blank, evaporate 10ml light petroleum and for standard evaporate 1ml of diluted standard (0.5umol).

To all the three tubes (T, B & S) add 5ml colour reagent and place in water bath between 60-80 degree centigrade for 10 minutes. Cool and add 3ml sulphuric acid. Mix well and keep for 30minutes. Read T & S against B at 560nm.

***Calculations***

$$\text{Serum ester cholesterol} = \frac{T}{S} \times 193 \text{ mg\%}$$

Serum free cholesterol= Total – Ester mg%

**Determination of LCAT (Glomset 1969):** After determination of total, free and ester cholesterol, the serum sample was incubated at 37degree centigrade for 24hrs and again total, free and ester cholesterol were estimated as above. The fall in free cholesterol was determined after incubation of serum at 37degree centigrade for 24hrs. LCAT activity is expressed as degrees in free cholesterol, expressed as nm/ml/hr.

$$\text{LCAT (nmoles/ml/hr)} = \frac{\text{F (in mg\%), Free cholesterol Before incubation} - \text{F (in mg\%), Free cholesterol Before incubation}}{100 \times 24 \times 386} \times 1000 \times 1000$$

= Decrease in free cholesterol (in mg%) X 1.08 nmoles/ml/hr.

Glomset (1968): Plasma lecithin cholesterol acyl transferase reaction.

***Estimation of HDL Cholesterol***

***Phosphotungstate Method***

***Intended Use***

This reagent kit is intended form vitro quantitative determination of HDL – Cholesterol (High Density Lipoprotein-Cholesterol) in serum or plasma.

***Clinical Significance***

HDL – Cholesterol and coronary heart disease are inversely related. Low concentrations of HDL – Cholesterol are associated with higher risk of coronary heart disease. Thus HDL-Cholesterol in combination with total cholesterol determination is a good index of the risk of coronary heart disease. Coronary Heart Disease (CHD) risk factor can be calculated using total lipid profile, as suggested by Castelli *et al.*,. The risk factor gives the most accurate and definite assessment of heart disease risk.

The factors are calculated by the ratio of Total Cholesterol to HDL – Cholesterol and by the ratio of LDL – Cholesterol (Low Density Lipoprotein-Cholesterol to HDL – Cholesterol).

| **Risk** | **Ratio: Total/HDL-Cholesterol** | | **Ratio : LDL/HDL – Cholesterol** | |
|---|---|---|---|---|
| | **Men** | **Women** | **Men** | **Women** |
| ½ Avg. | 3.43 | 3.27 | 1.00 | 1.47 |
| Avg. | 4.97 | 4.44 | 3.55 | 3.22 |
| 2 x Avg. | 9.55 | 7.05 | 6.25 | 5.03 |
| 3 x Avg. | 23.99 | 11.04 | 7.99 | 6.14 |

The value of LDL – Cholesterol can be calculated as follows. If the value of the Triglycerides is known, LDL-Cholesterol can be calculated based on Friedewald's equation.

$$\text{LDL CHOL mg/dl} = \text{Total CHOL} - \frac{\text{Triglycerides}}{5} + \text{HDL CHOL}$$

***Principle***

Chylomicrons, VLDL (Very Low Density Lipoproteins) and LDL fractions in serum or plasma are separated from HDL by precipitating with Phosphotungstic Acid and Magnesium Chloride. After centrifugation, the cholesterol in the HDL fraction which remains in the supernatant is assayed with enzymatic cholesterol method, using Cholesterol Esterase, Cholesterol Oxidase Peroxidase and the chromogen 4-Aminoantipyrine/Phenol.

***Sample Collection, Storage & Stability***

Serum is preferred. EDTA or heparinised plasma can also be used.

As far as possible, use samples on the same day. Samples are stable for a week when stored tightly capped at $2^{o}C/8^{o}C$.

Do not use hemolysed or grossly contaminated samples.

***Reagents:***

| | |
|---|---|
| Reagent 1 (Enzymes / Chromogen): | |
| Cholesterol Esterase | $\geq$ 200 U/l |
| Cholesterol Oxidase | $\geq$ 200 U/l |
| Peroxidase | $\geq$ 1000 U/l |
| 4-Aminoantipyrine | 0.5nmol / L |
| Reagent 1A (Buffer): | |
| Pipes buffer, pH 6.90 | 50nmol / L |
| Phenol | 24nmol / L |
| Sodium Cholate | 0.5nmol / L |
| Reagent 2 (Precipitating Reagent): | |
| Phosphotungstic Acid | 2.4nmol / L |

Magnesium Chloride 39nmol / L
STANDARD (HDL Cholesterol 50 mg/dL):
Cholesterol 0.5 g/L
Precaution:
Good laboratory safety practices should be followed when handling any laboratory reagent.

***Storage & Stability of the Reagents***

When stored at $2^oC/8^oC$ and protected from light, the reagents are stable until the expiry dates stated on the labels.

***Reagent Reconstitution***

Allow the reagents to attain room temperature. Dissolve the contents of one bottle of reagent 1 into one bottle of reagent 1A. Mix by gentle swirling till completely dissolved.

***Reagent Reconstitution***

Allow the reagents to attain room temperature. Dissolve the contents of one bottle of reagent 1 into one bottle of reagent 1A. Mix by gentle swirling till completely dissolved.
Wait for 5 minutes before using.

***Reconstituted Reagent Storage & Stability***

The reconstituted reagent is stable for 3 months when stored at $2^oC$ - $8^oC$.

***Procedure***

The samples, the precipitating reagent 2 and the reconstituted reagent should be brought to room temperature prior to use.

1 PRECIPITATION: Dispense into Centrifuge Tube:

| | **Test** |
|---|---|
| Sample | 0.20mL (200uL) |
| Precipitating Reagent 2 | 0.20mL (200uL) |

Mix well.
IV11X WC11.
Centrifuge at 1500 g or 3500-4000 rpm for 10 minutes. Separate the clear supernatant immediately and determine the cholesterol content as given in the Section II.

***Note***

The 50 mg/dL HDL-Cholesterol standard should not be subjected to the precipitation step.

II CHOLESTEROL ASSAY

The following general system parameters are to be used with this kit:

Reaction Type: End point
Reaction Slope: Increasing
Wavelength: 500nm (4952- 500nm)
Flowcell Temp.: $30^oC$
Incubation: 5 minutes $37^oC$
Sample Vol. (Supernatant): 20uL
Reagent Volume: 1.0mL
Standard Connection: 100 mg/dL (The standard of 50mg/dL is to be fed as 100mg/dL to account for the dilution of sample in the precipitation step)
Zero setting: Reagent Blank
Set the instrument using above system parameters.

Dispense into test tubes:

| | **Blank** | **Standard** | **Test** |
|---|---|---|---|
| Reconstituted Reagent | 1mL | 1mL | 1mL |
| Standard | - | 20uL | - |
| Supernatant | - | - | 20uL |

Incubate for 5 minutes at 37$^{o}$C Mix and read.
Application Sheets for most of the commonly used Chemistry Analyzers are available on request.

***Quality Control***

To ensure adequate quality control, the use of commercial reference control serum is recommended with each assay batch. It should be realized that the use of quality control material checks both, the instrument and the reagent functions together.

**Table 1: Total Cholesterol (mg%)**

| **S.No.** | **Group I Normal Control** | **Group II DM with Good Glycemic Control** | **Group III DM With Poor Glycemic Control** |
|---|---|---|---|
| 1. | 171 | 221 | 264 |
| 2. | 169 | 231 | 328 |
| 3. | 186 | 204 | 280 |
| 4. | 179 | 234 | 280 |
| 5. | 184 | 197 | 280 |
| 6. | 210 | 239 | 256 |
| 7. | 188 | 211 | 276 |
| 8. | 196 | 196 | 336 |
| 9. | 179 | 224 | 264 |
| 10. | 180 | 273 | 256 |
| 11. | | 255 | 296 |
| 12. | | 260 | 288 |
| 13. | | 275 | 320 |
| 14. | | 207 | 301 |
| 15. | | 242 | 294 |
| Mean | 184.20 | 231.27 | 289.00 |
| $\pm$ SD | 12.01 | 26.11 | 24.95 |
| $\pm$SE | 3.80 | 6.74 | 6.44 |

***Reference Values***

It is recommended that each laboratory establish its own reference values. The following values may be used a guideline (Lopes *et al.*, 1977; Allain et al., 1974; Richmond, 1973; and Miller *et al.*, 1977):
HDL – Cholesterol:
Serum – Plasma: 30 – 70 mg/dL

ANOVA:

**Table 1: Total Cholesterol**

| Source | DF | Sum of Squares | Mean Squares | F | Significance |
|---|---|---|---|---|---|
| Between Groups | 2 | 68242.56 | 34121.28 | 84.53 | P < 0.01 |
| Within Groups | 37 | 19562.53 | 528.71 | | |
| Total | 39 | 87805.10 | | | |

**Table 2: LCAT ACTIVITY (nmoles/ml/hr)**

| S.No. | Group I Normal Control | Group II DM with Good Glycemic Control | Group III DM With Poor Glycemic Control |
|---|---|---|---|
| 1. | 49.68 | 30.24 | 24.84 |
| 2. | 50.76 | 29.16 | 16.2 |
| 3. | 44.28 | 34.56 | 19.44 |
| 4. | 48.6 | 28.08 | 22.68 |
| 5. | 45.36 | 37.8 | 22.68 |
| 6. | 37.8 | 28.08 | 25.92 |
| 7. | 43.2 | 32.4 | 23.76 |
| 8. | 39.96 | 37.8 | 14.04 |
| 9. | 48.6 | 30.24 | 24.84 |
| 10. | 47.5 | 23.76 | 25.92 |
| 11. | | 25.92 | 19.44 |
| 12. | | 24.84 | 21.6 |
| 13. | | 23.76 | 17.28 |
| 14. | | 34.56 | 18.36 |
| 15. | | 27 | 22.68 |
| Mean | 45.57 | 29.88 | 21.31 |
| ± SD | 4.28 | 4.68 | 3.68 |
| ±SE | 1.35 | 1.20 | 0.95 |

ANOVA:

**Table 2: LCAT ACTIVITY (nmoles/ml/hr)**

| Source | DF | Sum of Squares | Mean Squares | F | Significance |
|---|---|---|---|---|---|
| Between Groups | 2 | 3543.98 | 1771.99 | 98.99 | P < 0.01 |
| Within Groups | 37 | 662.28 | 17.90 | | |
| Total | 39 | 4206.26 | | | |

**Table 3: HDL – CHOLESTEROL (mg%)**

| S.No. | Group I Normal Control | Group II DM with Good Glycemic Control | Group III DM With Poor Glycemic Control |
|---|---|---|---|
| 1. | 58 | 39 | 44 |
| 2. | 49 | 46 | 36 |
| 3. | 64 | 52 | 30 |
| 4. | 66 | 44 | 30 |
| 5. | 71 | 38 | 28 |
| 6. | 41 | 49 | 32 |
| 7. | 69 | 36 | 26 |
| 8. | 74 | 51 | 30 |
| 9. | 62 | 44 | 24 |
| 10. | 54 | 41 | 30 |
| 11. | | 54 | 32 |
| 12. | | 37 | 28 |
| 13. | | 34 | 24 |
| 14. | | 46 | 26 |
| 15. | | 50 | 24 |
| Mean | 60.80 | 44.07 | 29.60 |
| ± SD | 10.37 | 6.35 | 5.24 |
| ±SE | 3.28 | 1.64 | 1.35 |

ANOVA:

**Table 3: HDL – CHOLESTEROL**

| Source | DF | Sum of Squares | Mean Squares | F | Significance |
|---|---|---|---|---|---|
| Between Groups | 2 | 5877.64 | 2938.82 | 56.63 | P < 0.01 |
| Within Groups | 37 | 1920.13 | 51.89 | | |
| Total | 39 | 7797.77 | | | |

## RESULTS AND DISCUSSION

Results of the study indicate derangement of lipoprotein metabolism in patients with NIDDM. There was decrease in HDL cholesterol level and LCAT activity in patients with type 2 diabetes mellitus.

HDL Cholesterol levels in normal control are 60.80 $\pm$ 10.37. In diabetic patients with good glycemic control it is decreased (44.07 $\pm$ 6.35). It is further decreased in diabetic patients with poor control the levels being 29.60 $\pm$ 5.24 (P<0.01 (Table III).

Lecithin cholesterol acyl transferase activity in normal control is 45.57 $\pm$ 4.28

In diabetic patients with good glycaemic control, it is decreased 29.88 $\pm$ 4.68

It is further decreased in diabetic patients with poor glycaemic control, the levels being 21.31$\pm$ 3.68

Miller *et al.*, (1977) has shown that HDL cholesterol concentrations are strongly independently related to coronary heart disease, low HDL cholesterol levels are important predictor of coronary artery disease.

Brown (1994) has shown that low levels of HDL may be associated with coronary artery disease risk even when serum cholesterol and LDL levels are normal.

Lusitupa *et al.*, (1986) has shown that lowered HDL levels were reported in diabetes. An inverse relationship has been described between plasma insulin and HDL levels.

Nikhila *et al.*, (1969) showed that HDL concentration particularly HDL 2 have been reported to be lower in NIDDM but normal in IDDM.

Dunn (1988) showed that lowered HDL cholesterol levels in NIDDM have been reported to increase on diabetic treatment but still is lower than normal.

Luoma *et al.*, (1985) demonstrated that the decrease in LCAT may be due to alteration in hepatic microsomal activity. An altered hepato cellular structure and decreased microsomal enzyme activity was observed in NIDDM enhancement of hepatic microsomal function improves both cholesterol distribution and glycaemic control in diabetes thus the risk of coronary artery disease in diabetes as evaluated by HDL and blood glucose levels seems to be related to activity of liver microsomal enzyme system.

The present study also supports the observation by other workers that in NIDDM, HDL cholesterol levels are lowered. There is also associated lowering of LCAT activity which may further be related to derangement in lipoprotein metabolism in diabetes thus these patients are more prone to develop CAD. However HDL cholesterol levels and LCAT activity in diabetic patients with poor control was still lower supporting the observation that CAD is 2-4 times higher in diabetes than in non diabetes.

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