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notes on methodology

An enzymatic method for the determination of the initial rate of cholesterol esterification in human plasma

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Summary A method is described for the determination of the initial rate of cholesterol esterification in human plasma, based on the enzymatic determination of free cholesterol in the plasma before and after incubation at 37°C. The cholesterol esterification rate was linear up to 40 minutes. In 18 normal male and 10 normal female subjects the cholesterol esterification rate was 91 ± 15 (mean ± SD) and 62 ± 12 nmoles/hr/ml of plasma, respectively.

Supplementary key words cholesterol esterification · LCAT · unesterified and total cholesterol

Free cholesterol in human plasma is esterified by the action of LCAT. This enzyme catalyzes the transfer of an acyl group from the 2-position of lecithin to the 3-hydroxyl group of cholesterol (1). Only the initial rate of cholesterol esterification (about 40 minutes incubation period for adult human plasma) reflects the enzyme level in the cholesterol esterifying system (2).

Chemical estimations of free cholesterol or phosphatidylcholine used so far are not sensitive enough for measurement of the initial rate of cholesterol esterification because the reaction is very slow (2, 3). The possible disadvantages of radioassays, which require equilibration of exogenous cholesterol in native plasma (4) or in heated plasma (5), have been discussed (2, 6). These disadvantages may be of significance under conditions in which an abnormal lipoprotein, such as lipoprotein-X, is present (2). In order to overcome these difficulties, a new method for LCAT determination based on a sensitive measurement of free cholesterol by gas–liquid chromatography (7) was recently presented.

We present here a method for the determination of the initial esterification rate of endogenous cholesterol in human plasma that is based on the measurement of free cholesterol by an enzymatic procedure.

Methods. Blood taken after an overnight fast was collected in plastic tubes containing heparin (7 U.S.P. units/ml of whole blood) and cooled in crushed ice. The plasma, obtained after low speed centrifugation at 4°C, was used immediately.

Determination of free and total cholesterol. In the presence of oxygen, free cholesterol is oxidized by cholesterol oxidase to Δ^4-cholestenone. The hydrogen peroxide that is formed then oxidizes methanol to formaldehyde in the presence of catalase. The formaldehyde reacts with ammonium ions and acetylacetone to form 3,5-diacetyl-1,4-dihydrolutidine whose extinction coefficient is then measured at 405 nm (8).

For the determination of total cholesterol, cholesterol esters are hydrolyzed by cholesterol esterase into free cholesterol and fatty acids. A detergent is included in the formula to aid in maintaining free cholesterol in solution (8).

All solvents used were reagent grade. The enzymes and other constituents of the solutions used were purchased from Boehringer Mannheim, Germany (Cat. No. 15738). The following solutions were used: (*1*) Preciset-cholesterol standard (300 mg/dl). (*2*) Solution A: 1.7M methanol, 0.02M acetylacetone, 0.1% hydroxypolyethoxydodecane, catalase (more than 670 mU/ml) in 0.57 M ammonium phosphate buffer (pH 7.0). For LCAT determination 1.5 mM iodoacetate was included. (*3*) Solution B: cholesterol esterase (26 mU/ml) in addition to the constituents of solution A. (*4*) Solution C: cholesterol oxidase, more than 4 U/ml.

For the determination of free cholesterol, 5 ml of solution A were carefully mixed with 0.2 ml of the plasma sample. Two ml of this mixture were pipetted into each of two plastic tubes, one containing 0.02 ml of buffer solution (for the sample blank) and one containing 0.02 ml of solution C (for the plasma sample). Using the standard cholesterol instead of plasma, the same procedure was followed to prepare a cholesterol standard and a standard blank. The contents of the four tubes (plasma sample, sample blank, cholesterol standard, and standard blank) were incubated in a water bath for 60 min at 37°C. Then the absorbance values were determined at 405 nm using an Eppendorf photometer (Type 1101 M Netheler and Hinz, Hamburg, Germany) in a 2-cm light path. The concentration of free cholesterol was calculated from the difference between readings for the plasma sample and the sample blank.

In normal plasma, readings for the plasma sample blank ranged between 0.060 and 0.124 after subtraction of the reagent blank value. Readings for the plasma sample blank and the reagent blank did not change during incubation. Absorbance values for the sample blank have been reported to be dependent on variable factors such as bilirubin concentration in the plasma sample (9).

For determination of total cholesterol, five ml portions of solution B were mixed with 0.05 ml portions of the plasma sample and cholesterol standard, respectively. The rest of the procedure was the same as for free cholesterol.

Color development was complete after 60 min and color intensity was stable for another 45 min (8). Dilutions of the cholesterol standard and the plasma were carried out using 0.05 M potassium phosphate buffer (pH 7.3).

To find out if all cholesterol in the lipoprotein would be accessible to the enzymes, comparison tests of the method used on plasma alone and on lipid extracted from the plasma were performed. Lipid was extracted by a modification (10) of the procedure of Folch, Lees, and Sloane Stanley (11). After the chloroform phase was evaporated under nitrogen, the residue was redissolved in isopropanol and used for meas-

Abbreviation: LCAT, lecithin:cholesterol acyltransferase

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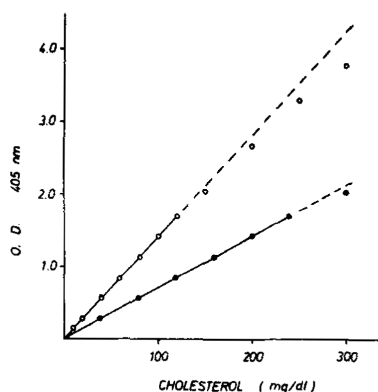

Fig. 1. Standard curve for determination of free cholesterol. Each point is the mean of six determinations. SD is negligible in this scale. Absorbance (OD) was determined using an Eppendorf-photometer in a 2-cm light path (circles) and in a 1-cm light path (points).

urement of free and total cholesterol. In these experiments cholesterol (U.S.P., cryst. puriss., Fluka AG, Buchs SG, Switzerland) was dissolved in isopropanol and used as the standard.

For comparison, determinations of free and total cholesterol were also carried out according to the procedure of Zak et al. (12).

Determination of initial esterification rate of endogenous cholesterol. Plasma cholesterol esterification was determined by measuring the decrease in concentration of unesterified cholesterol in the plasma before and after incubation. Plasma samples were incubated in stoppered plastic test tubes in a Dubnoff shaking incubator at 37°C. Incubation time was 40 min. The standard deviation (SD) of cholesterol esterification was calculated by the formula: $SD = (SD_0^2/m + SD_t^2/n)^{1/2}$, where SD_t and SD_0 are standard deviations at time t and time 0, respectively, and n and m are the numbers of determinations (six in our experiments).

For comparison, the initial cholesterol esterification rate was measured by the procedure of Stokke and Norum (4) using an incubation time of 40 min. [7(n)α-^{3}H]Cholesterol, 9.3 Ci/mmole, was purchased from the Radiochemical Center, Amersham, England (Code TRK, Batch 13).

Results. By diluting the standard with buffer, cholesterol concentrations of 10–300 mg/dl were used, and the response factor for each standard concentration was calculated as the mean absorbance of six determinations.

When a 2-cm light path was used, a linear response of the absorbance was observed up to a concentration of 120 mg/dl free cholesterol. Since the linear response increased to 240 mg/dl of free cholesterol when a 1-cm light path was used, it was concluded that the deviation of the linear relationship was caused by the high color intensity (**Fig. 1**). Therefore, we kept the concentration of free cholesterol in the samples below 120 mg/dl by diluting the samples of higher concentration with a buffer solution. The effect of different dilutions of a plasma sample containing more than 120 mg/dl free cholesterol is shown in **Table 1.**

TABLE 1. Effect of different dilutions of a plasma sample containing 161 mg/dl free cholesterol

Dilution[a]	Readings[b]
Undiluted	2.142 ± 0.016
1:2	1.132 ± 0.004
1:4	0.565 ± 0.004
1:8	0.284 ± 0.002
1:10	0.226 ± 0.001

[a] Dilution was carried out with 0.05 M potassium phosphate buffer (pH 7.3).

[b] The readings represent the mean difference between sample and sample blank of six determinations ± SD at 405 nm (2-cm light path).

When unesterified cholesterol was estimated in 100 different human plasma samples (concentration of unesterified cholesterol 10–120 mg/dl), the coefficient of variation (SD × 100/mean) of six determinations from each plasma was found to be 0.42% ± 0.24 (mean ± SD), range 0.07–1.00%. Reproducibility of this procedure was determined by measuring the free cholesterol concentration in one plasma (stored at −18°C) each day for 10 days. The mean concentration of free cholesterol was 66.6 mg/dl ± 0.4 (SD), range 65.9–67.2 mg/dl.

Free and total cholesterol were measured both on plasma and on lipid extracted from the same plasma. In these comparative studies the correlation coefficients (n = 17) were 0.982 and 0.979 for free and total cholesterol, respectively. The enzymatic method was also compared to the method of Zak et al. (12). For free cholesterol there was a correlation coefficient of 0.983 (n = 26); the total cholesterol coefficient was 0.989 (n = 31).

LCAT activity in the plasma before and after incubation was stopped by addition of iodoacetate, an inhibitor of the LCAT (13) in a final concentration of 1.5 mM. Measurement of free cholesterol in a standard solution (50 mg/dl) was carried out with and without iodoacetate. Since iodoacetate did not influence the readings for free cholesterol at this concentration, it was concluded that iodoacetate had no inhibiting effect on the enzymes used for determination of unesterified cholesterol.

The effect of iodoacetate on cholesterol esterification in human plasma was also studied. Free cholesterol was determined before and after incubation in a mixture of plasma (2.5 ml) and buffer (0.5 ml) with and without 9 mM iodoacetate. A complete inhibition of cholesterol esterification was achieved by iodoacetate (final concentration 1.5 mM), and therefore iodoacetate was always included in solution A.

In a study of the time course of cholesterol esterification in human plasma, linearity up to 40 min was observed (**Fig. 2**). Total cholesterol remained unchanged during incubation.

The initial cholesterol esterification rate was studied in 18 normal male and 10 female subjects (ages 20–50). In the male group, the mean initial rate was 91 ± 15 (SD), range 76–133 nmoles of cholesterol esterified/hr/ml of plasma. In the female group the values were 62 ± 12 nmoles of cholesterol esterified/hr/ml of plasma, range 44–77 nmoles. The fractional rate of esterification was 6.12% ± 0.8 cholesterol esterified/hr in the male group and 4.79% ± 1.11 in the fe-

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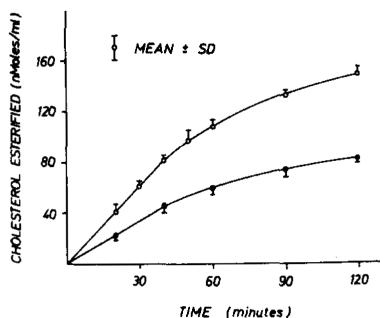

Fig. 2. Time curve of cholesterol esterification in the plasma of two normal subjects.

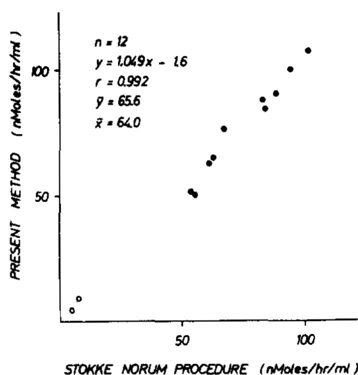

Fig. 3. Comparison of the present method with the procedure of Stokke and Norum (4) for the determination of the initial cholesterol esterification rate in human plasma. The open circles represent plasma samples stored for 4 days at room temperature. The closed circles represent plasma samples used immediately for LCAT determination.

male group. The sex dependent difference was significant ($P < 0.01$). The mean of the standard deviations of LCAT determinations (six in each plasma) expressed as percentages, considered to be the error of the method, yielded an average value of 7.20% ± 2.10 (SD) for assays carried out on the 28 subjects. The reproducibility of LCAT determination was tested by measuring the activity of the same plasma six times on the same day. The mean cholesterol esterification rate was 85.8 nmoles/hr/ml of plasma ± 3.5 (SD), range 82–90 nmoles.

A comparison of this method with the procedure of Stokke and Norum (4) was performed on 10 normal subjects. In order to compare plasmas having low cholesterol esterification rates, two plasma samples were stored for 4 days at room temperature (4) (**Fig. 3**).

Discussion. In the studies described in this report, we measured the initial esterification rate of endogenous cholesterol in human plasma. It should be stressed that the estimation of free cholesterol in the whole plasma before and after incubation does not actually measure the LCAT activity per se, but the esterification rate of cholesterol in the plasma, which might resemble the physiological conditions for plasma cholesterol esterification.

For measurement of free cholesterol, an enzymatic method was used. A similar enzymatic method for cholesterol determination has been previously described (14) and evaluated (15). The present method uses a different chromophor system that was found to exhibit adequate sensitivity (16). The accuracy of this method was reported to be satisfactory (8, 9). This was confirmed in our study by comparison with an established method (12). That all cholesterol in lipoproteins was accessible for determination was demonstrated by comparing use of the method on plasma and on lipid extracted from the plasma. The precision of the determination of free cholesterol was comparable with gas–liquid chromatographic results (7) and was higher than the precision of chemical measurement of free cholesterol (3, 17, 18). When free cholesterol in the sample was above 120 mg/dl, a dilution of the plasma was necessary. Thus the precision of LCAT determination might be diminished by dilution. Measurement of cholesterol was not influenced by increased concentration of bilirubin (up to 20 mg/dl), triglyceride (up to 1000 mg/dl), hemoglobin (up to 500 mg/dl), or creatinine (up to 20 mg/dl) (8).

The time necessary for determination of the cholesterol esterification rate in three different plasmas was about 3.5 hours, which is shorter than either the procedure of Stokke and Norum (4) or the methods based on gas–liquid chromatography. Although we used 2.5 ml of plasma for each assay, we feel that the method can be adapted for use with smaller amounts of plasma, if necessary.

Values for the cholesterol esterification rate in normal subjects are in agreement with other investigations that also used endogenous cholesterol for LCAT determination (4, 7, 19). We have no explanation for the considerably lower values found by other investigators (20, 21).

A sex-dependent difference, first described by Gjone, Blomhoff, and Wiencke (20), and confirmed by other authors (7), was evident in this study. No difference in LCAT activity was found by Akanuma et al. (22) between males and females. This may be related to the different method they used for LCAT determination. However, other investigators (19) observed a difference between males and females only in the fractional cholesterol esterification rate, even though these authors used endogenous cholesterol for LCAT determination.

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