BBA 57786

AN OPTIMIZED METHOD FOR MEASURING LECITHIN : CHOLESTEROL ACYLTRANSFERASE ACTIVITY, INDEPENDENT OF THE CONCENTRATION AND QUALITY OF THE PHYSIOLOGICAL SUBSTRATE

MARGRET BARTHOLOMÉ, D. NIEDMANN, H. WIELAND and D. SEIDEL

Medical University Hospital, Department of Clinical Chemistry, University of Göttingen, Robert-Koch-Strasse 40, 3400 Göttingen (F.R.G.)

Summary

The aim of the study was to achieve the measurement of lecithin : cholesterol acyltransferase (EC 2.3.1.43) activity independent of and uninfluenced by equilibration of lipids between different lipoproteins, their molar ratios or by possible differences in their substrate quality. A mixture of sodium phosphotungstic acid/$MgCl_2$ was added to serum samples to achieve total precipitation of all plasma lipoproteins. The filtrate containing the total plasma lecithin : cholesterol acyltransferase activity, apolipoprotein A-I and major plasma proteins was used for the assay. Liposomes comprised of phosphatidylcholine, cholesterol and dicetylphosphate served as substrate. The decrease in free cholesterol was determined enzymatically after incubation for 60 min at 37°C.

The assay followed zero-order kinetics and was linear for more than 60 min. The following K_m values for various substrates were obtained: liposomes, 0.43 mM; HDL, 0.63 mM; LDL, 0.0; VLDL, 0.0; abnormal lipoprotein, found in cholestasis, (LP-X), 0.0. Comparison with generally used lecithin : cholesterol acyltransferase assay methods revealed similar activities for healthy controls, but in different forms of dyslipoproteinemia higher values were obtained with our method and, in special cases, the activity could be enhanced by addition of apolipoprotein A-I.

Introduction

The enzyme lecithin : cholesterol acyltransferase is synthesized in the liver [1] and circulates in plasma in its active form. Under normal conditions it acts on high density lipoproteins and catalyzes the transfer of the fatty acid moiety from the C-2 position of phosphatidylcholine to unesterified cholesterol [2]. By this reaction the enzyme promotes the formation of cholesterol esters and lysophosphatidylcholine [3]. The action of lecithin : cholesterol acyltransferase on HDL facilitates the transfer of newly formed cholesterol esters from HDL to LDL and VLDL. Thus, this enzyme plays an important role in the metabolism of the different lipoprotein classes and may be of relevance for the removal of cholesterol from peripheral tissue [3]. The reaction is activated by apolipoprotein A-I [4] and possibly also by apolipoprotein D [5], and it has been suggested that apolipoprotein A-II may act as an inhibitor [4].

Several groups of methods are available for measuring the activity of lecithin : cholesterol acyltransferase. One group is based on the determination of cholesterol ester formation from radioactively labeled cholesterol in whole plasma [6—8]. The accuracy of this assay depends on the availability of an efficient and physiological substrate. Moreover, the incorporation of radioactive cholesterol into plasma lipoproteins by in vitro exchange is not standardized and the uncertainties in the relative distribution of labeled cholesterol after incubation with serum containing all the various lipoprotein fractions may be a significant source of error [9]. In another group of methods the rate of disappearance of unesterified cholesterol is measured during incubation of freshly obtained plasma in the presence or absence of dipalmitoyl phosphatidylcholine as activator [10,11]. Enzyme activities obtained with these methods depend not only on (a) the concentration of the enzyme but also on (b) the quality and concentration of the patients' plasma lipoproteins.

We herein describe a simple procedure to assay the activity of lecithin : cholesterol acyltransferase after removal of the plasma lipoproteins using liposomes optimized as substrate.

Material and Methods

Preparation of samples. Blood samples were obtained from patients of the Department of Medicine, University Hospital of Göttingen, and from healthy volunteers after an overnight fast. Blood was drawn and allowed to clot for 10 min at room temperature. For the separation of serum, centrifugation was performed at $6000 \times g$ for 5 min and after separation the samples were kept at 4°C until used (not later than 24 h after drawing). Complete delipoproteinization was achieved by precipitation of whole serum or plasma with phosphotungstic acid and $MgCl_2$ in a final concentration of 0.182 M $MgCl_2$ and 1.8% sodium phosphotungstic acid [12,13]. The completeness of the precipitation was checked by lipoprotein electrophoresis on agarose gel. After centrifugation for 5 min at $20\,000 \times g$ $MgCl_2$ was complexed from the supernatant using sodium citrate in a final concentration of 0.182 M, i.e. equimolar to $MgCl_2$. Such a preparation contains the total plasma lecithin : cholesterol acyltransferase activity (see Results) and usually sufficient apolipoprotein A-I, i.e. more than

5 mg/100 ml, as determined by rate nephelometry [14].

Assay conditions for lecithin : cholesterol acyltransferase reaction. 0.3 ml of the supernatant obtained after phosphotungstic acid precipitation was mixed with the liposome preparation to give a final cholesterol concentration of 1.29 mM. This mixture was incubated for 1 h at 37°C. Cholesterol was determined immediately after addition of the liposome preparation to the supernatant and after 1 h of incubation. Lecithin : cholesterol acyltransferase activity is expressed as the rate of decrease of unesterified cholesterol over a period of 1 h.

Preparation of liposomes. Liposomes were prepared in principle according to the method of Gregoriadis and Ryman [15]. Phosphatidylcholine, cholesterol and dicetylphosphate were dissolved in 5 ml chloroform in a molar ratio of 7 : 2 : 1, which equals 29.4, 4.4 and 3.1 mg, respectively. Phosphatidylcholine, pure grade, was provided by Merck, Darmstadt. The solvent is removed under reduced pressure in round-bottom glass flasks and the dry lipids were then suspended in 0.9% NaCl under constant stirring for 15 min with several glass rods at room temperature. The suspension is allowed to stand for 1 h and sonicated for four periods of 1 min with a titanium microtip and 60 W, during which the solution was kept in a water/ice bath. The liposomes were purified by chromatography on Sepharose 6B and recovered in the void volume. Such a liposome preparation is stable for up to 3 weeks kept at 4°C.

Cholesterol determination. Free and total cholesterol were determined enzymatically using the oxidation reaction of iodine for detection of H_2O_2 (Merck, Darmstadt, F.R.G.).

Preparation of lipoprotein fractions. Lipoprotein fractions were isolated from fresh plasma by sequential ultracentrifugation at 10°C and 45 000 rev./min using a titanium rotor, type 65, and the Heraeus Christ Omega II ultracentrifuge (Heraeus Christ, Osterode, F.R.G.). Each ultracentrifugation step was carried out for 18 h. The isolated fractions (VLDL density, $d < 1.006$ g/ml, LDL density range, d 1.019—1.063 g/ml, and HDL density range, d 1.073—1.21 g/ml) were recentrifuged twice and purity was checked by lipoprotein electrophoresis and double immunodiffusion using the appropiate and specific antisera [16,17]. LP-X was isolated according to our previously described method using a combination of ultracentrifugation and Cohn fractionation [18].

For special experiments a fraction containing all lipoproteins was isolated by ultracentrifugation at a solvent density of d 1.21 g/ml.

Apolipoprotein A-I measurement. Determination of apolipoprotein A-I was achieved by a newly developed rate nephelometric technique [14] using purified sheep antibodies against apolipoprotein A-I isolated from human HDL by isoelectric focusing.

Results

Assay conditions for lecithin : cholesterol acyltransferase reaction

The rate of cholesterol esterification in the incubation mixture containing approx. 1.3 mM cholesterol (liposome cholesterol) is proportional to the incubation time (Fig. 1) and to the amount of lecithin : cholesterol acyltransferase present in the assay (Fig. 2). The rate of cholesterol esterification is linear for
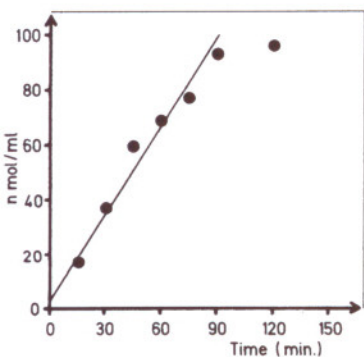
Fig. 1. An aliquot of lecithin : cholesterol acyltransferase solution was incubated with liposomes (1.3 mM cholesterol) for different intervals of time at 37°C.
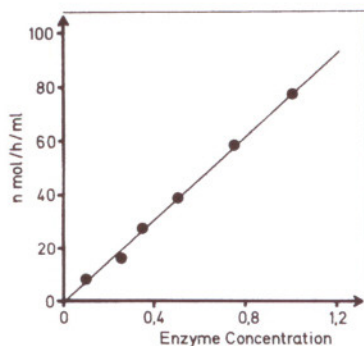
Fig. 2. Effect of varying dilutions of lecithin : cholesterol acyltransferase on the rate of cholesterol esterification. The concentration of free cholesterol in the assay was 1.3 mM and the incubation time was 60 min at 37°C.

approx. 90 min (Fig. 1). The enzyme activity is temperature-dependent with a maximum activity between 37 and 42°C (Fig. 3).

Precision of the method

A coefficient of variance of 6.6% was determined in one series ($n = 20$) and of 7.9% for the day-to-day variation ($n = 20$). Samples obtained from the same donor on different days ($n = 20$) showed a coefficient of variance of 10.8%.

Substrate specificity of lecithin : cholesterol of different lipoproteins from human plasma

Kinetic studies were performed with isolated lipoprotein fractions (HDL, VLDL and LP-X). The double-reciprocal plot (Lineweaver-Burk plot) of the
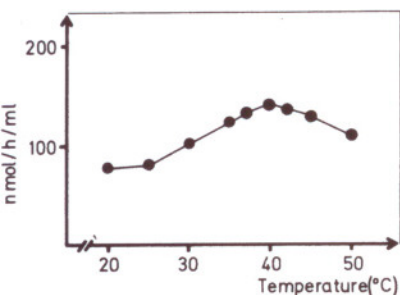

Fig. 3. Effect of temperature on the esterification rate by lecithin : cholesterol acyltransferase. The time of incubation was 60 min with liposomes as substrate.
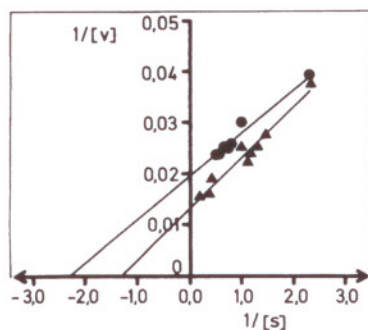
Fig. 4. Double-reciprocal plot of lecithin : cholesterol acyltransferase activity as a function of increasing concentration of HDL or liposomes as substrate. Incubation was carried out at 37°C for 60 min. ▲, HDL; ●, liposomes.

velocity vs. concentration of HDL or liposomes as substrates was linear with an apparent K_m value of 0.43 mmol for liposomes ($\bar{x}$ of six different preparations), and 0.63 mmol ($\bar{x}$ of seven different preparations) for isolated HDL (Fig. 4). The K_m value for the liposomes is almost identical to that calculated for HDL. VLDL, LDL and LP-X do not act as substrates. For these lipoprotein fractions no esterification of their free cholesterol was measured under the chosen assay conditions. In agreement with this, the Lineweaver-Burk plot of a mixture of HDL and VLDL (cholesterol HDL: cholesterol VLDL, 1 : 1) revealed the same K_m value as for HDL alone (Fig. 5). As VLDL does not interact with the enzyme the K_m value of HDL in a mixture is not affected by the presence of VLDL. Thus our results clearly indicate that any method for assaying lecithin : cholesterol acyltransferase activity using lipoproteins from the patients' plasma as substrate depends on normal concentrations and possibly on the composition of these particles primarily of the HDL density class. This requirement, however, does not hold for many clinical instances, particularly not for liver disease in which striking abnormalities of the lipoprotein profile and lecithin : cholesterol acyltransferase activity have been reported [19—22]. The usefulness of a lipoprotein-independent lecithin : cholesterol acyltransferase test system optimized for the substrate is strongly supported by comparison of the values for lecithin ; cholesterol acyltransferase activity measured with different assay techniques in various pathological cases (Table I).

Case 1: Acute hepatitis. With the method using HDL from patients as substrate (method of Nagasaki and Akanuma [10]) we found low activity (12.2 nmol/h per ml). After delipoproteinization and with liposomes as substrate (our technique) the activity was higher (30.6 nmol/h per ml) and after addition of isolated apolipoprotein A-I (25 µg per test) it increased to subnormal levels of 58.9 nmol/h per ml. Further addition of apolipoprotein A-I had no influence on this activity.

Case 2: Biliary obstruction. No activity was determined with the method of Nagasaki and Akanuma [10], but residual activity was found using our technique (38.3 nmol/h per ml) with or without addition of apolipoprotein A-I.

TABLE I

COMPARISON OF LECITHIN : CHOLESTEROL ACYLTRANSFERASE ACTIVITY IN PATHOLOGICAL SERA MEASURED WITH DIFFERENT METHODS

n.d., not determined.

Case	Method		
	Nagasaki and Akanuma [10]	Ours	Our method + addition of apolipoprotein A-I
1. Acute hepatitis	12.2	30.6	58.9
2. Biliary obstruction (LP-X, +++)	0	38.3	38.7
3. Chronic pancreatitis (LP-X, +)			
Before operation	174.4	176.7	n.d.
1 day after	36.5	84.7	n.d.
8 days after	78.9	86.3	n.d.

Case 3: Chronic pancreatitis (Whipple operation). Before the operation measurement with both methods showed a very high esterification rate (greater than 170 nmol/h per ml). 1 day after the operation, when both HDL and apolipoprotein A-I concentrations were drastically lowered by 45% as measured by quantitative lipoprotein electrophoresis and rate nephelometry, respectively, we found low enzyme activity using the method of Nagasaki and Akanuma [10], which measured esterification of the patients' own lipoproteins (36.5 nmol/h per ml) but normal values with our method (84.7 nmol/h per ml). 8 days after the operation the lecithin : cholesterol acyltransferase activity determined with both methods was comparable and within the normal range, being 78.9 nmol/ml per h and 86.0 nmol/ml per h, respectively.

Interpretations

Case 1: The differences in the measured activity are most likely due to an apparent lack of HDL which influenced the Nagasaki and Akanuma system. The lower enzyme activity measured with our technique is due, in part, to a reduced concentration of apolipoprotein A-I which may be compensated through the addition of isolated apolipoprotein and, in part, to an absolute decrease of enzyme concentration.

Case 2: The high concentration of LP-X, possibly in combination with a low HDL concentration, may have influenced the Nagasaki and Akanuma system at the low level of lecithin : cholesterol acyltransferase concentration. For the interpretation of the measured activity in such patients it is important to realize that LP-X does not serve as substrate for the lecithin : cholesterol acyltransferase reaction, as is clearly demonstrated in this communication.

Case 3: The differences between the two methods 1 day after operation can easily be explained by the low HDL concentration, which does not have any influence on our test system.
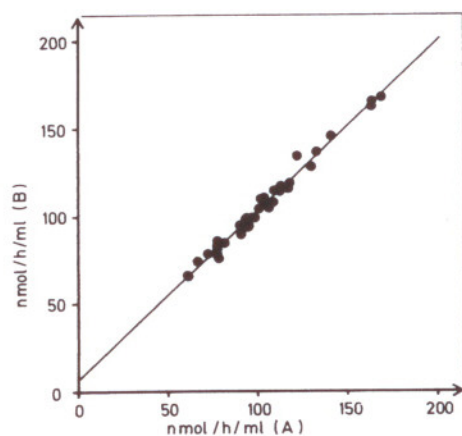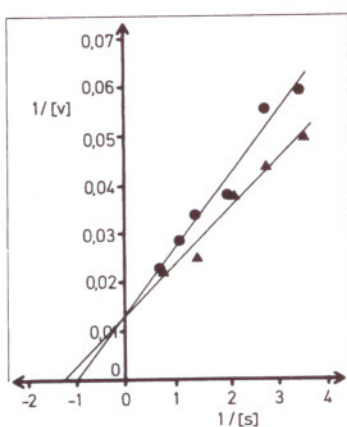

Fig. 5. Double-reciprocal plot of lecithin : cholesterol acyltransferase activity as a function of varying concentrations of a mixture of HDL plus VLDL or HDL as substrate. The incubations were carried out at $37°C$ for 60 min. ●, HDL plus VLDL; ▲, HDL.

Fig. 6. Comparison of lecithin : cholesterol activity measured with the method of Nagasaki and Akanuma [10] (A) and our method (B). The incubation were carried out at $37°C$ for 60 min. $n = 38$, $r = 0.99$, $j = 0.97x + 6.5$.

Comparison of lecithin : cholesterol acyltransferase activity in normal subjects measured with our method and with the generally used method [10]. Comparison of the two methods showed a high correlation ($r = 0.99$) in a group of 38 healthy volunteers (Fig. 6). Considering the K_m values of liposomes and HDL as substrate points out that no enzyme activity is lost during the precipitation of the lipoproteins.

Discussion

The method presented in this communication has the advantage of measuring lecithin : cholesterol acyltransferase activity independent of its physiological substrate, i.e. the patients' own lipoproteins. These particles, in particular the HDL fraction as the preferential substrate, may be abnormal in composition and concentration and thus alter the esterification kinetics, even in the case of normal enzyme concentration. this is an important factor to realize and to consider for a valid interpretation of lecithin : cholesterol acyltransferase activity in pathological states. A good correlation exists between methods reflecting 'in vivo esterification' [10] and our lecithin : cholesterol acyltransferase method with 'optimized substrate conditions' in healthy subjects. This agreement between these methods, however, is not apparent in various forms of liver disease and biliary obstruction, in which an abnormal HDL, the occurrence of LP-X and high or low lecithin : cholesterol acyltransferase concentration may occur. This situation and the opportunity of comparing 'in vivo esterification' [10] with 'optimized enzymatic measurement' of the activity of lecithin : cholesterol acyltransferase may offer new scope for a better interpretation of the activity of this enzyme in health and disease.

The method does not need radioactively labeled compounds, it is easy to perform and is precise, as determined for both single series and from day-to-day variation. Thus the technique described in this communication may be recommended for clinical and biochemical purposes.

Acknowledgement

References

1 Simon, J.B. and Boyer, J.L. (1971) Biochim. Biophys. Acta 218, 549—551
2 Glomset, J.A. (1968) J. Lipid Res. 9, 155—167
3 Glomset, J.A. and Norum, K.R. (1973) Adv. Lipid Res. 11, 1—65
4 Fielding, C.J., Shore, U.G. and Fielding, P.E. (1972) Biochem. Biophys. Res. Commun. 46, 1493—1498
5 Kostner, G. (1974) Scand. J. Clin. Lab. Invest. 33, Suppl. 137, 19—21
6 Glomset, J.A. and Wright, J.L. (1964) Biochim. Biophys. Acta 89, 266—272
7 Stokke, K.T. and Norum, K.R. (1971) Scand. J. Clin. Lab. Invest. 27, 21—27
8 Akanuma, Y. and Glomset, J.A. (1968) J. Lipid Res. 7, 638—648
9 Rose, H.G. (1968) Biochim. Biophys. Acta 152, 728—741
10 Nagasaki, T. and Akanuma, Y. (1977) Clin. Chim. Acta 75, 371—375
11 Patssch, W., Sailer, S. and Braunsteiner, H. (1976) J. Lipid Res. 6, 506—511
12 Kostner, G. and Alaupovic, P. (1972) Biochemistry 11, 3419—3428

13 Burstein, M., Scholaick, H.R. and Norfin, R. (1970) J. Lipid Res. 11, 583—595

14 Weinstock, N., Bartholomé, M. and Seidel, D. (1981) Biochim. Biophys. Acta 663, 279—288

15 Gregoriadis, G. and Ryman, B.E. (1972) Eur. J. Biochem. 24, 485—491

16 Havel, R.J., Eder, H.A. and Bragdon, J.H. (1955) J. Clin. Invest. 34, 1345—1353

17 Seidel, D., Greten, H., Geisen, H.P., Wengeler, H. and Wieland, H. (1972) Eur. J. Clin. Invest. 2, 359—367

18 Seidel, D., Alaupovic, P., Furman, R.H. and McConathy, W. J. (1970) J. Clin. Invest. 49, 2396—2407

19 Seymour, M., Sabesin, M.D., Harry, L., Hawkins, M.D., Lois Kuikken, B.S. and Ragland, J.B. (1977) Gastroenterology 72, 510—518

20 Wengeler, H., Greten, H. and Seidel, D. (1970) Eur. J. Clin. Invest. 2, 372—378

21 Calandra, S. Martin, M.J. and McIntyre, N. (1971) Eur. J. Clin. Invest. 1, 352—360

22 Ritland, S., Blomhoff, J. and Gjone, E. (1973) Clin. Chim. Acta 49, 251—259