A rapid radioassay procedure for plasma lecithin-cholesterol acyltransferase

Uri Piran and Robert J. Morin

Harbor-UCLA Medical Center, Department of Pathology, Torrance, CA 90509

Summary A rapid and accurate single step procedure is described for the assay of lecithin-cholesterol acyltransferase activity. After incubation, using radiolabeled cholesterol as the substrate, an ethanolic solution of digitonin is added directly to the incubation mixture to extract the lipids. Excess cholesterol is then added, and the labeled cholesterol-digitonide along with denatured proteins are sedimented by low speed centrifugation, leaving the labeled esterified cholesterol in solution. An aliquot of the supernatant is counted in an aqueous scintillation mixture. The method correlates well with the established thin-layer chromatographic procedure using either lecithin-cholesterol vesicles or heat-inactivated plasma as the substrate for lecithin-cholesterol acyltransferase.—**Piran, U., and R. J. Morin.** A rapid radioassay procedure for plasma lecithin-cholesterol acyltransferase. *J. Lipid Res.* 1979. **20:** 1040–1043.

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Several methods have been developed for the determination of lecithin-cholesterol acyltransferase (LCAT.E.C.2.3.1.43). These include radioassays using labeled cholesterol (1–9) and chemical (10), enzymatic (11), or gas–liquid chromatographic (12) analysis of the change in free cholesterol. The radioassay has been the method of choice in most studies, because the linear portion of the reaction is short (up to 5% of change in cholesterol) (13), requiring a highly sensitive method that measures rate of appearance of product rather than disappearance of substrate.

Measurement of the radioactivity of the cholesteryl esters formed during the incubation has usually been done by extracting the lipids with organic solvents, evaporating under nitrogen, separating free from esterified cholesterol by thin-layer chromatography, and counting portions of the chromatogram by liquid scintillation spectrometry (1–9). To replace the laborious and time-consuming thin-layer chromatography with a single step procedure, we have adapted the cholesterol-digitonide precipitation reaction for the radioassay of LCAT.

MATERIALS AND METHODS

Egg yolk phosphatidylcholine, egg yolk lysophosphatidylcholine, cholesterol, cholesteryl oleate, triolein, fatty acid free bovine serum albumin (BSA), betamercaptoethanol and digitonin were purchased from the Sigma Chemical Company, St. Louis, MO. [7(*n*)-^{3}H]Cholesterol was obtained from Amersham Corp., Arlington Heights, IL, and [4-^{14}C]cholesteryl

Abbreviations: LCAT, lecithin-cholesterol acyltransferase; TLC, thin-layer chromatography; ApoA-I, apolipoprotein A-I; EDTA, ethylenediamine tetraacetate; HDL, high density lipoproteins, d = 1.063–1.21 g/ml.

oleate from New England Nuclear, Boston, MA. The radioactive materials were purified by thin-layer chromatography on Silica Gel G using hexane–diethyl ether–acetic acid 90:10:1 (v/v) as the developing solvent, and were stored in ethanol under nitrogen at −20°C. Organic solvents were obtained from Fisher Scientific Company, Pittsburgh, PA, and silicic acid-impregnated glass fiber sheets, type ITLC-SA from the Gelman Instrument Co., Ann Arbor, MI. All other chemicals were reagent grade.

Potassium bromide was dissolved in fresh citrated human plasma to raise the density to 1.21 g/ml, and the lipoproteins were floated by ultracentrifugation (13). The d > 1.21 g/ml fraction was dialyzed against 50 mM phosphate buffer, pH 7.4, containing 1 mM ethylenediamine tetraacetate (EDTA) and used as the enzyme preparation. The lipoproteins were further fractionated by centrifugations to isolate the high-density lipoproteins (HDL) (13), and apoA-I was purified from HDL by delipidation, gel filtration, and DEAE column chromatography (14).

Single bilayer vesicles containing egg yolk phosphatidylcholine (PC) and labeled cholesterol, molar ratio 4:1, were prepared by the method of Batzri and Korn (15) in phosphate buffer, pH 7.4, and were used as the substrate for LCAT. For some recovery studies, lipid dispersions containing labeled cholesteryl oleate and additional lipids, as indicated, were prepared by sonication (4). Incubations were carried out in 50 mM sodium phosphate buffer, pH 7.4, containing 1 mM EDTA, 1% BSA, 150 mM NaCl, and 10 mM beta-mercaptoethanol in a final volume of 250 μl. One hundred nmoles phosphatidylcholine and 25 nmoles cholesterol (0.2 μCi) in vesicles, plus 20 μg apoA-I were used per assay. The substrate for the Glomset and Wright (8) assay was prepared by heating fresh human plasma at 56°C for 45 min, and labeling with a bovine serum albumin-stabilized cholesterol emulsion (9). In this assay 100 μl of substrate containing 118 nmoles free cholesterol (0.2 μCi) were used per 250 μl incubation mixture without purified apoA-I.

The mixtures were incubated in duplicate at 37°C under N_2 in covered tubes and the reaction was stopped by addition of 2 ml of 1% digitonin in 95% ethanol. The contents of the tubes were mixed on a vortex stirrer for 15 sec to denature the proteins and extract the lipids, 0.5 mg cholesterol was added in 100 μl ethanol, and the mixtures were vortexed again. The proteins and cholesterol digitonide were sedimented by centrifugation at 1500 *g* for 10 min, and a 0.5 ml aliquot was taken from the supernatant using a semiautomatic pipetter (Selectapette, Clay Adams, Parsippany, NJ). This aliquot was added directly to 3 ml of aqueous scintillation cocktail ACS (Amersham Corporation).

In control incubation mixtures, buffer or heated enzyme preparations were substituted for the enzyme to obtain the background supernatant radioactivity. This radioactivity was subtracted from that of the complete incubation mixture to correct the enzyme activity. Additional controls were treated with 2 ml of 95% ethanol not containing digitonin, in order to determine the total cholesterol radioactivity in the incubation mixture. Aliquots from all types of incubations caused identical degrees of quenching as determined by internal standardization, thus obviating the need for routine quench corrections when sampling the same volumes. Quenching was 30% in the 3 ml of ACS used throughout this study; it could be reduced to 11% if the aliquot was counted in 10 ml of scintillation solution.

For comparison to the TLC method, lipids were extracted from the incubation mixtures according to Folch, Lees, and Sloane Stanley (16), dried under nitrogen and fractionated on polysilicic acid-impregnated glass fiber sheets using hexane–diethyl ether–acetic acid 90:10:1 (v/v) as the developing solvent. Portions of the sheets were cut and placed in 3 ml ACS for counting. LCAT was also assayed by the rate of loss of free cholesterol as determined by gas–liquid chromatography. Incubation media contained vesicle substrates and cofactors at the same concentrations, but were made at 1 ml final volume, or 100 nmoles cholesterol per assay. The reaction was stopped by addition of 1 ml ethanol containing 40 μg stigmosterol as an internal standard, and the total lipids were extracted (16), dried, and redissolved in 20 μl CS_2. Aliquots were injected into a Barber-Colman Model 5000 gas chromatograph with flame ionization detector, using 3% OV 17 on Gaschrom Q 120/140 mesh (Applied Science Labs) at 260°C and a nitrogen carrier gas flowrate of 45 ml/min.

Correlation coefficients were calculated by regression analysis using an Olivetti Programma 101. Cholesterol was determined enzymatically using reagents purchased from Boehringer Manheim, (Germany); phospholipid phosphorous was measured by the method of Bartlett (17), and protein by the method of Lowry et al. (18).

RESULTS

In preliminary experiments, not shown here, we found that treatment of incubation mixture with 1% digitonin dissolved in ethanol–water 1:1 (v/v), as used for the determination of free cholesterol in serum

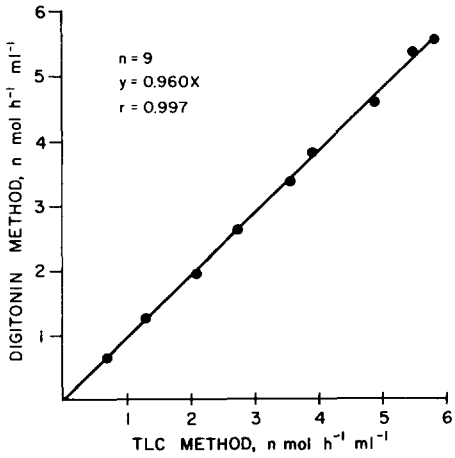

Fig. 1. Comparison of the present digitonin method with the TLC method for determination of LCAT activity using the vesicle substrate. Results are expressed as nmoles cholesteryl esters formed per ml in 1 hour incubation.

(19), was not suitable for the assay of LCAT, due to difficulty in denaturing the proteins and the occurence of extensive co-precipitation of cholesteryl esters. The ethanol concentration of the digitonin reagent was therefore increased to 95%. When 2 ml of this solution was mixed for 15 sec with the 0.25 ml control incubation mixtures, most of the protein and 80% of the radioactive free cholesterol were precipitated upon centrifugation, leaving 20% of the label in the supernatant. Addition of 0.5 mg unlabeled cholesterol resulted in further digitonide precipitation, and the free cholesterol radioactivity in the supernatant was reduced to 2.35% ± 0.03 (mean and standard deviation of 5 determinations). Sonicated dispersions of phosphatidylcholine containing various concentrations of cholesterol, cholesteryl oleate, triolein, egg yolk phosphatidylcholine, egg yolk lysophosphatidylcholine, [7(*n*)-^{3}H]cholesterol and [4-^{14}C]cholesteryl oleate were prepared to determine the effect of these lipids on the recoveries of the labeled free and esterified cholesterol in the supernatant. It was found that the recovery of the [7(*n*)-^{3}H]cholesterol from incubation mixtures containing up to 80 nmoles/ml of each lipid, in addition to the standard substrate, remained 2.35%. The recovery of [4-^{14}C]-cholesteryl oleate was 96.6% ± 1.3 (five determinations using same dispersion) and remained unaffected by the additional lipids. Moreover, when up to 600 μl human plasma (containing 210 mg% cholesterol) was added per ml incubation mixture, the recovery of ^{14}C remained at the same level, and that of ^{3}H was again unchanged. The order of addition of the precipitating reagents was found to be important; when the carrier, unlabeled cholesterol, was added before digitonin, the recovery of labeled cholesteryl oleate under all conditions decreased and varied between 85–90%, while the background ^{3}H radioactivity increased to approximately 2.88%. The carrier cholesterol was therefore added after the digitonin in all subsequent experiments.

The new method was compared with the established TLC procedure used in the assay of LCAT. Substrate vesicles were incubated with 2–10 μl enzyme preparation (80–400 μg protein) for 1 hour and cholesterol esterification was determined by the two methods. Regression analysis showed excellent agreement (r = 0.997) between these methods (**Fig. 1**). When apoA-I was omitted from the incubation mixture LCAT activities measured by both methods were completely abolished due to the low concentration of activator protein present in the enzyme preparation. The precision of the two methods was compared by five determinations of the activity of 5 μl of enzyme preparation. Means and standard deviations of 2.94 ± 0.06 and 3.06 ± 0.07 nmoles cholesteryl ester per ml per hour were obtained with the digitonin and TLC methods, respectively. The digitonin method was further validated by the direct measurement of the consumption of medium free cholesterol by LCAT using gas–liquid chromatography. This was conducted in an experiment similar to that described in Fig. 1, except that, due to the lower sensitivity of the latter method, enzyme concentrations and incubation times were doubled and medium volume was increased fourfold. LCAT activities determined by gas–liquid chromatography correlated well with those obtained by the new method (r = 0.998, y = 1.06x + 0.5, n = 9; y represents the digitonin method; x represents the gas–liquid chromatographic method).

The digitonin method was also applied to the Glomset and Wright assay (8), incubating 5–40 μl (0.02–1.6 mg protein) enzyme preparation and 118 nmoles labeled free cholesterol per assay for 5 hours. With this version the new method again showed an excellent correlation with the TLC method (r = 0.998) (**Fig. 2**). The activity of 5 μl enzyme preparation in this assay (five determinations) was 3.91 ± 0.07 and 4.04 ± 0.08 nmoles cholesterol esters per ml per 5 hour incubation, using the digitonin and TLC procedures, respectively.

DISCUSSION

The present report describes a rapid and simple single step procedure for quantitation of the labeled cholesteryl esters formed during the exogenous substrate radioassay of LCAT. This procedure is in routine use in our laboratory in place of the TLC method, resulting in considerable saving in time and

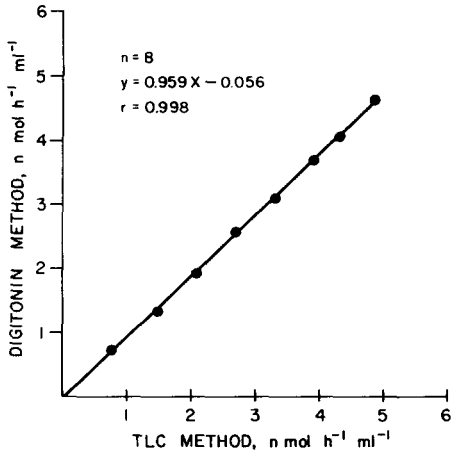

Fig. 2. Comparison of the present digitonin method with the TLC method for determination of LCAT activity using heat inactivated plasma as substrate. Results are expressed as nmoles cholesteryl esters formed per ml in 1 hour.

labor without sacrificing accuracy or precision. Because the recovery of cholesteryl esters is 96.6%, the results are consistently 3–4% lower than those obtained by TLC. The specificity of the present method is lower, because cholesteryl esters are not identified by their chromatographic mobility but only by the modification of the 3 beta-hydroxyl group of cholesterol. Except for LCAT, however, no other factor in plasma is presently known to modify this hydroxyl group. In addition, omission of apoA-I from the assay mixture with the artificial vesicle substrate can serve as a control against other factors, including other possible cholesterol esterifying enzymes.

Lack of interference by lipids or proteins suggests that the method may be easily adapted to other versions of LCAT assays (7, 9). In addition, it may be utilized for radioassays of other enzymes, such as acyl-CoA-cholesterol acyltransferase (E.C. 2.3.1.26), cholesterol ester hydrolase (E.C.3.1.1.13), and cholesterol oxidase (E.C.1.1.3.6).

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