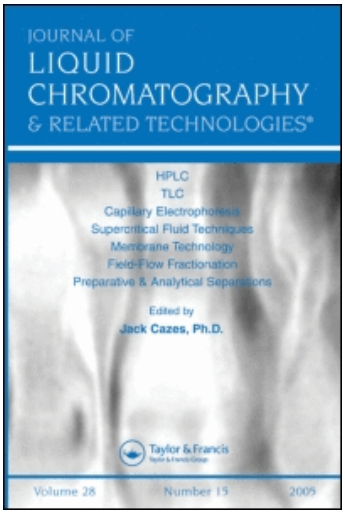

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Direct Determination of Apolipoproteins in Plasma by High Performance Liquid Chromatography

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DIRECT DETERMINATION OF APOLIPOPROTEINS IN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY*

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ABSTRACT

A simple and sensitive high performance liquid chromatographic (HPLC) method is described for the direct quantitation of apolipoproteins (apo) A-I, A-II and B in plasma. The apolipoproteins are well resolved, which allows for quantitation of apo A-I, A-II and B and calculation of the ratio of A-I/A-II. Only 10 μl is required for a single determination of these three apolipoproteins. Compared to other methods requiring separation of HDL by ultracentrifugation and delipidation of apoproteins with organic solvents, this method avoids any loss of apolipoproteins. The HDL in supernatant of plasma precipitated with phosphotungstic acid/magnesium chloride reagent contained essentially all of the plasma apo A-I and apo A-II. However, this supernatant also contained about 37% of plasma apo B, suggesting incomplete precipitation of apoproteins in very low and low density lipoproteins. Thus, this HPLC method can rapidly determine three apolipoproteins in a small volume of plasma without any loss and also can detect incomplete separation of apolipoproteins of very low and low density lipoproteins from HDL fraction.

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INTRODUCTION

High density lipoproteins (HDL) play a central role in cholesterol transport and metabolism via their interaction with lecithin-cholesterol acyl transferase (1). Low concentrations of HDL in the plasma are associated with increased risk of atherosclerotic coronary artery disease (2). The protein fraction of HDL is composed of a number of chemically and immunochemically distinct polypeptides referred to as apolipoproteins (apo), of which apo A-I and apo A-II are the predominant species (80-90% of the total protein) (3). Recently, studies with transgenic mice have elucidated the importance of apoproteins A-I and A-II in the development of atherosclerosis. Warden et al. (2) showed that fatty streak atherosclerotic lesions were formed in transgenic mice which exhibited elevated HDL levels and overexpressed apo A-II. Transgenic mice overexpressing human apo A-I also showed elevated plasma HDL but the development of aortic fatty streaks was reduced, even when the mice were maintained on atherogenic diets high in fat and cholesterol (4-6). This suggests that the ratio of apo A-I to apo A-II in HDL is an important criterion indicative of pathogenesis of atherosclerosis.

A number of different methods have been developed to measure apo A-I and A-II levels in HDL. These include immunoassays (1,7,8), capillary electrophoresis (9) and high performance liquid chromatography (HPLC). The latter includes both reversed-phase (10,11) and gel exclusion chromatography (12-14). All of the above methods, with the exception of an immunoturbidimetric assay (7), require purification of the HDL fraction from plasma by ultracentrifugation. Further, most of these methods (1, 7, 8, 10, 11, 13) require delipidation of the apolipoproteins with some organic solvent(s). Both of these steps introduce the possibility of sample loss.

Kinoshita et al. (13) solubilized the HDL fraction with sodium dodecyl sulfate (SDS) and then separated apolipoproteins by using a gel exclusion HPLC column. We have standardized conditions to apply this technique directly to plasma samples and to the HDL fractions

in the supernatant after precipitating the very low density lipoproteins (VLDL) and low density lipoproteins (LDL) fractions with a standard precipitating reagent. Our method eliminates the need to separate HDL by ultracentrifugation and delipidation with organic solvents. Thus, there is no loss of apolipoproteins due to preparatory steps. There is an added advantage that very small samples (10 μl plasma or supernatant) are required for analysis of the major apolipoproteins (A-I, A-II and B) and the determination of the ratio of apo A-I to apo A-II.

MATERIALS AND METHODS

Reagents

Apolipoprotein A-I, A-II, B calibration serum (from human serum) was obtained from Boehringer Mannheim Biochemica (Lot No. 162794-66, exp. 31.Jul.94, Laval, Quebec, Canada). This serum contained (mg/dl) 163 apo A-I, 49 apo A-II, and 96 apo B. Phosphotungstic acid/magnesium chloride reagent (Pta/$MgCl_2$) (procedure no. 352-4, lot no. 52H-6132) for precipitating LDL and VLDL fractions from plasma and total cholesterol reagent (procedure no. 352, lot no. 033H6129) were obtained from Sigma Chemical Company, St. Louis, MO, U.S.A. All other chemicals were of reagent grade.

The calibration serum was reconstituted with 1 ml distilled and deionized water. Aliquots (10 μl) were removed and diluted with 190 μl of 0.1 M phosphate buffer, pH 7.0, containing 0.2% SDS. The stock solution was frozen at -20°C. The diluted calibration serum was stored at 4°C and analyzed within two days.

Apparatus and Chromatographic Conditions

The HPLC system consisted of a model P200 Spectra Series binary pump (Spectra-Physics Analytical Canada Ltd., Toronto, Ontario, Canada) equipped with a Rheodyne model 712NS syringe-loading sample injector (Rheodyne, Cotati, CA, U.S.A.), and a Datajet single-

channel integrator (Spectra-Physics). The apolipoproteins were detected with a variable wavelength ultraviolet detector (Model UV2000, Spectra-Physics) set at 280 nm. Data was also captured to a Trillium 386SX IBM compatible computer using "Winner-on-Windows" software package (Spectra-Physics Canada Ltd.).

The mobile phase consisted of 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1% SDS; the flow rate was 0.33 ml/min. The solvent was filtered through a 0.45 um filter (Millipore Canada, Mississauga, Ontario, Canada) under vacuum, then allowed to sit overnight to remove the foaming from the SDS.

Analysis was performed on a TSK-Gel G3000SW gel filtration HPLC column (7.5 mm i.d. X 60 cm, particle size 10 um, TosoHaas, Montgomeryville, PA, U.S.A.), equipped with a TSK-Gel SW guard column (7.5 mm i.d. X 7.5 cm, particle size 10 um, TosoHaas).

Assay procedures

Blood samples were taken with heparinized syringes from the vena cava of adult male guinea pigs. The blood samples were centrifuged at 3000 rpm for 15 minutes at 4°C to obtain plasma.

Samples of plasma (30 μl) were placed in 1.5 ml polypropylene centrifuge tubes (Eppendorf, Hamburg, Germany), the required volume of phosphate buffer containing SDS was added to each tube, and the tubes were capped and heated at 60°C for five minutes to delipidate the apolipoproteins. Aliquots (10 μl) of each sample were injected onto the HPLC column.

Quantitation of apolipoproteins in the HDL fraction of plasma samples was performed as follows. Plasma samples (125 μl) were incubated with 25 μl of Sigma Pta/$MgCl_2$ reagent for five minutes at room temperature to precipitate LDLs and VLDLs, and the tubes centrifuged for ten minutes at 3000 rpm. The supernatants (30 μl) were transferred to 1.5 ml Eppendorf tubes and the required volume of phosphate buffer containing 0.2% SDS was added. Samples were heated as for plasma and 10 μl aliquots were analyzed by HPLC. The amounts of apoproteins in biological samples were quantitated by comparison to peak areas obtained by injecting known amounts of standard apolipoproteins.

Analytical variables

The relationship between area count response and injection size was determined using the diluted calibration standard. Aliquots of 2, 5, 10 and 15 μl, corresponding to 0.62, 1.54, 3.08 and 4.62 μg of total apoproteins A-I, A-II and B, respectively, were injected onto the HPLC column, and the correlation coefficient was determined for each apoprotein.

Reproducibility of injection was determined by triplicate analyses of 10 μl aliquots of plasma samples by HPLC.

RESULTS AND DISCUSSION

Chromatography

Elution with sodium phosphate buffer containing 0.1% SDS resulted in well-resolved peaks for the three main apoproteins. The HPLC chromatogram for the apolipoprotein calibration standard is shown in Figure 1 (a). The retention times for apoproteins B, A-I and A-II were 33.0, 39.9 and 54.2 minutes, respectively. The identities of the three major apolipoproteins were confirmed by injecting purified standards from human sera (Sigma Chemical Co., cat. no. A9284 (A-I), A8909 (A-II), A 9910 (B)) onto the HPLC column. A shoulder eluting just after apo B may represent some of the minor apolipoproteins (apo E, A-IV, B48).

Kinoshita et al. (14) found that the relation between the peak area of each apolipoprotein fraction separated by HPLC and the volume of sample applied to the column was linear for protein contents of 9 to 72 μg. This was confirmed in the present study with both the calibration standard and the plasma samples for smaller protein concentrations of 0.3 to 2.3 μg (r^2=0.999).

Reproducibility of injection was determined by injecting 10 μl of the same sample three times, and calculating the percent standard deviation in the area counts for the resulting peaks. The variation in peak areas was found to be 0.7%, 1.0% and 1.1%, for apolipoproteins A-I, A-II and B, respectively.

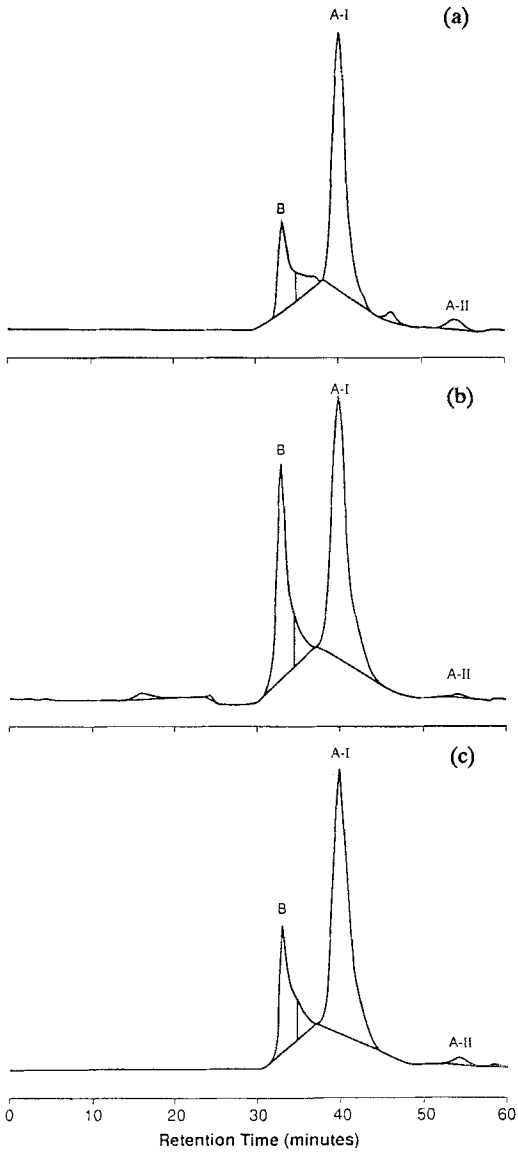

FIGURE 1.

HPLC separation of apolipoprotein from (a) calibration standard, (b) guinea pig plasma and (c) its HDL fraction. Chromatographic separation conditions are given in the text.

TABLE 1

Effect of Sodium Dodecyl Sulphate Concentration on Solubilization of Apolipoproteins

Guinea pig #	Total cholesterol (mg/dl)	SDS[1] in buffer (%)	Dilution[2] (x)	Apoproteins A-I (mg/dl)	A-II (mg/dl)	B (mg/dl)	Ratio of A-I/A-II
1	52.2	0.1	5	48.8	5.6	77.0	8.7
		0.2	10	51.1	6.7	71.6	7.6
		0.2	15	68.1	9.9	91.9	6.9
		0.5	5	39.0	5.2	56.9	7.5
2	67.8	0.2	15	52.3	6.0	61.7	8.7
		0.2	20	68.0	9.4	86.4	7.2
3	289.0	0.2	15	74.5	105.3	23.9	4.4
		0.2	20	85.2	126.3	29.2	4.3
		0.5	15	76.6	101.5	23.7	4.3

[1] Sodium dodecyl sulphate.
[2] Times (x) plasma was diluted with phosphate buffer containing SDS.

Analysis of biological samples

Guinea pig plasma samples and HDL samples (prepared by precipitation of LDLs and VLDLs) directly assayed by our method produced clear separation of apolipoproteins A-I, A-II and B as shown in Figure 1, (b) and (c), respectively. The amounts of apolipoproteins A-I, A-II, and B in plasma of guinea pigs containing 52.2 to 289 mg/dl cholesterol are shown in Table I. It is apparent that the use of 0.2% SDS solutions maximized the amounts of each of the above three apolipoproteins. A 20-fold dilution of plasma with 0.2% SDS compared to 15-fold dilution also markedly increased the amounts of apoproteins determined. The use of 0.5% SDS solution had a tendency to decrease the levels of various apolipoproteins, particularly for samples low in total cholesterol.

The levels of apolipoproteins A-I and A-II determined in guinea pig plasma were very similar to the values obtained by analyzing the HDL fraction prepared by Pta/$MgCl_2$ precipitation of

TABLE 2

Amounts of Apolipoproteins Present in Guinea Pig Plasma and HDL Fraction

	Apolipoproteins (mg/dl)			
Guinea pig #	A-I	A-II	B	A-I/A-II
Plasma				
4	47.6	4.0	40.3	11.9
5	43.7	6.3	47.9	6.9
6	44.3	6.6	47.0	6.7
HDL				
4	47.2	4.6	15.4	10.3
5	44.6	6.0	16.7	7.4
6	41.3	5.6	18.0	7.4

VLDL and LDL fractions (Table 2). However, there was about 37% of the plasma apo B present in the HDL fraction, although HDL particles are not known to contain any apo B (15). Thus, the HDL fraction prepared by precipitation of VLDL and LDL fractions is not pure in respect of the apolipoproteins.

Summarily, we have developed an HPLC method for clear separation and quantitation of apolipoproteins A-I, A-II and B directly in plasma without any fractionation by ultracentrifugation or delipidation by organic solvents. This procedure requires a very small sample of plasma and does not incur any losses in the preparatory steps. It can be used for the determination of the apo A-I to apo A-II ratio and for detecting any apo B contamination of HDL fractions.

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