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Separation of high-density lipoproteins into apolipoprotein E-poor and apolipoprotein E-rich subfractions by fast protein liquid chromatography using a heparin affinity column

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ABSTRACT

The aim of this paper is to describe a new methodology for the separation of human high-density lipoproteins (HDL) into apolipoprotein (apo) E-poor and apo E-rich subfractions by fast protein liquid chromatography (FPLC) using a heparin affinity column. Recoveries for apolipoproteins AI, AII, CI, CII, CIII, and E were 68.9, 74.7, 71.9, 73.5, 40.0, and 55.8%, respectively. We provide suggestive evidence that apo E-rich HDL is produced from apo E-poor HDL by the displacement of apo AI by apo E. Apo E-poor HDL was the predominant fraction. The molar ratio of apo E to apo AI in apo E-poor HDL was 0.02 and 0.01 for the subjects studied while in apo E-rich HDL it was 1.86 and 1.25. The molar ratios of the C apolipoproteins to apo AI are markedly different between the subfractions.

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INTRODUCTION

Reduced levels of high-density lipoprotein (HDL) cholesterol are associated with an increased risk of coronary artery disease [1,2]. The mechanism by which this occurs is unclear but it has been hypothesized that it may involve the reverse transport of cholesterol from tissues to the liver [3]. Some studies, however, have not found this inverse relationship between HDL cholesterol and risk for coronary artery disease [4,5]. This may be due to the fact that HDL particles are heterogeneous in nature and not all subparticles may be equally protective. HDL particles can be separated into HDL2 and HDL3 subparticles using ultracentrifugation. A further modification has been the separation of subparticles on gradient gels which allows further subdivision of HDL2 and HDL3 based on size [6]. Low HDL levels found in survivors of myocardial infarction have been shown to be due to reduced HDL2b levels [7,8]. This separation, however, is based on density characteristics and has no functional significance. Furthermore, ultracentrifugation is known to result in artifactual changes in apolipoprotein (apo) levels due to shifts between different lipoproteins classes [9,10].

HDL may also be subfractionated on the basis of apolipoprotein content. HDL subparticles containing apo AI (Lp AI) have been shown to have a greater protective effect against atherosclerosis than particles which contain apo AI and apo AII (Lp AI/AII) [11]. These particles have been shown to be functionally and metabolically different [12,13]. Weisgraber and Mahley [14] reported the use of heparin affinity chromatography to separate HDL particles into apo E-rich and apo E-poor subfractions. Marcel *et al.* [15] have also used this method to subfractionate HDL2 and HDL3. Apo E-rich HDL is taken up in the liver by receptors which are specific for this apoprotein [16]. Survivors of myocardial infarction have been shown to have a significantly lower percentage distribution of apo E-rich HDL than healthy controls [7]. Furthermore, although HDL particles have been found to have variable effects on platelet function which may be due to HDL heterogeneity, other investigators [17–21] reported that apo E-rich HDL particles inhibit agonist-induced platelet aggregation in humans. These studies suggest that the subfractions obtained by this separation may have both functional and clinical significance. It should be noted, however, that the initial separation of HDL in these studies was by ultracentrifugation.

We describe the use of fast protein liquid chromatography (FPLC) using a heparin affinity column to separate apo E-rich and apo E-poor HDL from HDL fractions previously isolated from plasma by FPLC using a size exclusion column. Recoveries for lipids and apolipoproteins have never been reported using this technique. We report the recovery rates for cholesterol and apolipoproteins AI, AII, CI, CII, CIII, and E with this methodology. We also report preliminary data on the composition of apo E-poor and apo E-rich HDL obtained by this method.

EXPERIMENTAL

Lipoprotein subfractions were initially separated from 500 μl of human plasma by FPLC using two Superose 6 HR 10/30 columns connected in series (Pharmacia LKB Biotechnology, Uppsala, Sweden). The elution buffer used for this system contained 0.05 *M* NaCl, 0.005 *M* Tris, 0.0005 *M* EDTA, and 0.005% NaN_3. Flow-rate was 0.67 ml/min. The Superose columns have the following dimensions: 24 ml, 300 mm × 10 mm I.D. This system allows the separation of triglyceride-rich lipoproteins, low-density lipoproteins (LDL), and HDL. The collected HDL fraction is then concentrated to a volume of 0.5 ml to enable its injection onto the heparin column. Recoveries for this procedure were first determined.

Concentration of HDL

HDL was obtained from plasma samples of five normal volunteers. After collection of the HDL fraction from the Superose columns it was concentrated using centrifugal concentrator–filtrator tubes (RCF-ConFlit–Hollow Fiber Bundles, Biomolecular Dynamics, Beaverton, OR, USA). The

HDL fraction was centrifuged at 1000 *g* in a Model TJ-6 centrifuge with a swinging bucket rotor for 25 min and brought down to a concentrate volume of 50–100 μl. A final volume of 0.5 ml was then achieved by adding the eluent buffer to each tube (the composition of the eluent buffer was the same as that used to elute the apo E-poor HDL fraction). Recoveries from the concentration step were calculated by comparing the quantity of cholesterol and apolipoproteins in HDL after concentration to the amount in the starting HDL fraction obtained from the Superose column separation.

FPLC using a heparin affinity column

FPLC was performed on a Shimadzu LC-7A Bio-Compatible liquid chromatographic system (Shimadzu Scientific Instruments, Columbia, MD, USA). It consisted of a SIL-6B/9A automatic sample injector, a SIL-6B autoinjector with a high precision 500-μl sample loop, a LC-7A high-performance solvent delivery unit module with an LPM-600 low-pressure mixing proportioning valve, a SCL-6B system controller, a SPD-6AV ultraviolet–visible spectrophotometric detector, a C-R4A Chromatopac data processor, and a SF-2120 fraction collector. A FCV-2AH switching valve was used to direct the injected HDL fraction to a Progel-TSK Heparin-5PW column (Supelco, Bellefonte, PA, USA) which was preceded by a Progel-TSK guard column (Supelco). The apo E-poor fraction was eluted with buffer containing 0.05 *M* NaCl, 0.005 *M* Tris, 0.0005 *M* EDTA, and 0.005% NaN_3 which had previously been filtered and degassed. The apo E-rich fraction was then eluted with the same buffer but with a higher salt concentration (0.8 *M* NaCl). The flow-rate used was 0.5 ml/min. The column eluent was monitored at 600 or 280 nm, respectively, depending on whether the HDL was prestained or not with Sudan Black. The apo E-poor and apo E-rich fractions were collected following a time window mode program in test tubes kept cold on ice and containing 20 μl/ml fraction of a solution of 0.05% aprotinin (Sigma) and 1% bovine serum albumin (Sigma) in eluent buffer. Each run lasted 70 min.

To determine recoveries from the heparin affinity FPLC, twenty plasma samples were obtained in the fasting and postprandial state from two diabetic subjects participating in a study of postprandial lipoprotein metabolism. HDL was obtained by FPLC using two Superose columns in series and concentrated as described above. Recoveries were calculated as the sum of the amount of apolipoprotein or lipid in the apo E-poor and apo E-rich fractions divided by the amount in the starting total HDL fraction obtained from the Superose column separation. It is important to note that our apolipoprotein assays do not detect albumin, hence the contaminating albumin does not interfere with quantification.

Experiment on the incubation of apo E-poor HDL with free apo E

Apo E-poor HDL was obtained from two subjects and concentrated by the methods outlined above. Part of the fraction was incubated with 0.1 mg of free apo E at 37°C for 2 h. A control was obtained by performing a similar incubation but without addition of free apo E. The free apo E was obtained as previously described [22]. Each sample was then injected onto the heparin column, and the unbound (apo E-poor) and bound (apo E-rich) fractions were collected.

Quantitative analysis

Cholesterol was measured using some modifications of the microenzymatic method described by Belcher *et al.* [23]. Aliquots of the apo E-poor and apo E-rich HDL were concentrated by the mechanism described above prior to cholesterol determination. Rapid radioimmunoassays using solid-phase staphylococcus protein were used for assay of apolipoproteins AI [24], AII [25], CI, CII, CIII [26], and E [22]. The apo E assay was performed using 16 m*M* sodium decyl sulphate. Review of our procedures revealed that in analyzing the fractions obtained by FPLC, the final concentration of the decyl sulphate detergent in the mixture had been significantly reduced. This was corrected by raising the final concentration of sodium decyl sulphate to that used in plasma samples. These methods include the use of puri-

fied standards as well as a control plasma and quality control procedures documented previously [22,27] to correct for day-to-day variability.

RESULTS

Fig. 1 illustrates the chromatogram obtained when HDL is separated by FPLC using a heparin-Sepharose affinity column when the column eluent was monitored at 280 and 600 nm. The column eluent was collected at 2-min intervals and cholesterol was assayed in each fraction. Cholesterol was only found in the time periods corresponding to these peaks. The first peak represents the unbound HDL subfraction which is poor in apo E. The concentrations of apolipoproteins AI, AII, CI, CII, CIII, and E in this fraction were 56.71 ± 13.50, 15.82 ± 5.44, 2.31 ± 0.76, 0.52 ± 0.19, 1.50 ± 0.27, and 0.11 ± 0.13 mg/dl, respectively. The second peak is eluted as described in the methodology section with 0.8 *M* NaCl and corresponds to apo E-rich HDL (bound fraction). The concentrations of apolipoproteins AI, AII, CI, CII, CIII, and E in this fraction were 0.27 ± 0.23, 0.06 ± 0.05, 0.12 ± 0.21, 0, 0.08 ± 0.07, and 0.34 ± 0.16 mg/dl, respectively.

The recoveries after concentration were 80–99% for lipids and 100–130% for apolipoproteins. The recoveries of apolipoproteins AI, AII, CI, and CII in apo E-poor and apo E-rich HDL subfractions separated by FPLC on a heparin affinity column ranged from 68.9 to 74.7%. In contrast the recovery for apo E was 55.8%. The mean recovery for cholesterol was 61.4%. The lower recovery for apo CIII (40%) was partly because apo CIII was found in the eluent between the peaks. Other apolipoproteins including apolipoproteins AI, AII, CI, CII, and E were not found in the eluent between the peaks. Recoveries for apo CIII were calculated for two subjects to include the apo CIII found between the peaks. In these cases it improved from 38.1 to 45.8% and from 46.8 to 74%, respectively. The relative apolipoprotein composition of the apo E-poor and apo E-rich fractions is shown in Table I. Apo AI is the major apoprotein found in apo E-poor HDL. This subfraction also contains significant quantities of apo AII. Lesser quantities of CI, CII, CIII, and E are also found in the apo E-poor fraction. Apo E is the main apolipoprotein found in apo E-rich HDL. Apo AI is also found in significant quantities. Apo CI and apo CIII are present in this fraction but apo CII was absent from most apo E-rich fractions assayed.

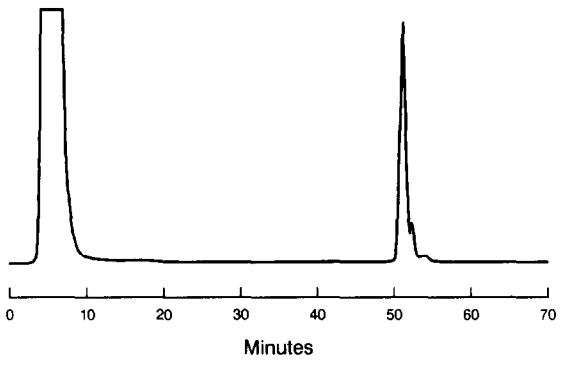

Fig. 1. Chromatogram obtained when HDL is applied to the heparin column. The eluent was monitored at 280 nm. The first peak represents apo E-poor HDL and the second peak represents apo E-rich HDL.

TABLE I

INDIVIDUAL APOLIPOPROTEINS EXPRESSED AS A PERCENTAGE OF TOTAL APOLIPOPROTEIN MASS FOUND IN APO E-POOR AND APO E-RICH HDL ($n = 10$)

Apolipoprotein	Percentage of total apolipoprotein mass (mean ± S.D.)	
	Subject 1	Subject 2
Apo E-poor HDL		
AI	72.0 ± 3.4	73.9 ± 5.0
AII	20.6 ± 3.3	19.8 ± 5.3
CI	3.0 ± 0.6	3.0 ± 0.4
CII	0.6 ± 0.2	0.7 ± 0.2
CIII	2.5 ± 0.5	1.8 ± 0.2
E	1.5 ± 0.7	0.8 ± 0.2
Apo E-rich HDL		
AI	19.5 ± 11.8	28.3 ± 9.5
AII	4.0 ± 2.8	6.0 ± 1.9
CI	8.7 ± 3.3	7.3 ± 1.9
CII	0 ± 0	0 ± 0
CIII	8.3 ± 9.3	5.9 ± 2.3
E	59.5 ± 17.3	52.6 ± 10.5

TABLE II

MOLAR RATIOS OF INDIVIDUAL APOLIPOPROTEINS TO APO AI IN APO E-POOR AND APO E-RICH HDL

Subject	AII/AI	CI/AI	CII/AI	CIII/AI	E/AI
Apo E-poor HDL					
1	0.47	0.18	0.03	0.10	0.02
2	0.44	0.17	0.03	0.07	0.01
Apo E-rich HDL					
1	0.32	1.82	0	1.45	1.86
2	0.30	1.01	0	0.60	1.25

The molar ratios of individual apolipoproteins to apo AI in each patient is shown in Table II. It is apparent that the particles are different compositionally. The apo E-poor particle contains 2 mol of apo AI to each mol of apo AII. Apo CI is the most prominent of the apolipoproteins C with a molar ratio of 0.18. The particle is obviously deficient in apo E relative to apo AI. In comparison the E-rich particle has 3 mol of apo AI for each mol of apo AII. Apo CII was undetectable in most fractions assayed while significant quantities of both apo CI and apo CIII are present. The particles are obviously rich in apo E relative to apo AI.

Apo E-poor HDL is the predominant particle. This particle contains 99.5% of the HDL apo AI, 99.7% of the apo AII, 96.8% of the apo CI, 100% of the apo CII, and 95.4% of the apo CIII. It contains 82.2% of the cholesterol found in HDL. Quantitatively 63.5% of the apo E found in HDL is in the apo E-poor subfraction.

The results of the experiment on the incubation of apo E-poor HDL with free apo E are outlined in Table III. The results of this experiment are expressed in terms of molar ratios of individual apolipoproteins to apo AII. The AI/AII ratio decreased while the E/AII ratio increased in the bound fraction obtained after apo E-poor HDL incubated with apo E was run on the heparin column, suggesting displacement of apo AI by apo E. The ratio of the apolipoproteins C to AII also changes after this experiment.

TABLE III

MOLAR RATIOS OF INDIVIDUAL APOPROTEINS TO APO AII IN BOUND AND UNBOUND FRACTIONS OBTAINED WHEN APO E-POOR HDL SUBPARTICLES ARE INCUBATED WITH AND WITHOUT FREE APO E AND THEN RUN ON THE HEPARIN COLUMN

Fraction	AI/AII	CI/AII	CII/AII	CIII/AII	E/AII
Subject 1					
Unbound					
Incubated with E	1.10	0.20	0.06	0.19	0.01
Incubated without E	1.10	0.39	0.09	0.28	0
Bound					
Incubated with E	0.75	1.38	0.11	0.58	15
Incubated without E	2.10	0	0	0.58	0.34
Subject 2					
Unbound					
Incubated with E	1.50	0.34	0.03	0.23	0.04
Incubated without E	0.89	0.25	0.03	0.15	0
Bound					
Incubated with E	0.37	0.53	0.03	0.22	4.7
Incubated without E	2.43	1.88	0.38	24.00	1.63

DISCUSSION

This paper is the first description of the use of FPLC on a heparin affinity column to subfractionate HDL into apo E-rich and apo E-poor subfractions. HDL is obtained by FPLC and therefore this technique allows the further separation of HDL into subparticles without the use of ultracentrifugation. Apo E-poor and apo E-rich HDL can be separated in 3 h using this methodology. The role of apo E in cholesterol transport and in particular the separate roles performed by apo E-poor and apo E-rich HDL subparticles in reverse cholesterol transport have been reviewed [28]. In this review the role of apo E-poor HDL as a cholesterol acceptor from macrophages and of apo E-rich HDL in binding to the LDL receptor is discussed. Although in humans reverse cholesterol transport can occur via cholesterol ester transfer protein (CETP)-mediated transfer of cholesterol ester from HDL to triglyceride-rich lipoproteins and LDL it is felt that apo E-rich HDL particles probably also play a role [28].

We report for the first time recoveries for apolipoproteins, measured by sensitive radioimmunoassays, after the use of FPLC on a heparin-Sepharose affinity column to subfractionate HDL particles. Recoveries in previous reports [14,15] used total lipoprotein protein recovered as measured by the Lowry method [29]. Recovery rates for individual apolipoproteins have not been reported. Recoveries for individual apoproteins obtained in our experiments are outlined in the results section. We are unable to explain the lower recovery of apo E although it may be due to incomplete elution from the column. Another possibility is that the radioimmunoassay for apo E is insensitive at the low concentrations of apo E found, particularly in the apo E-poor fraction. The lower recovery of apo CIII is due to the presence of this apolipoprotein between the peaks. Other apolipoproteins are absent between the peaks.

Separation of HDL into apo E-poor and apo E-rich fractions was originally described in rats. Hay *et al.* [30] found that apo E-poor HDL was the predominant fraction. In their study apo E was found in both subparticles but an enrichment of apo E relative to apo AI was found in the bound fraction. This study also showed that significant dissociation of apolipoproteins occurred with ultracentrifugation and that this dissociation was especially marked for the apo E-poor HDL fraction. Three other studies have examined subfractionation of HDL using heparin-Sepharose chromatography in rats [31–33]. In all of these studies the apo E-poor HDL fraction was the predominant one. None of these studies, however, used radioimmunoassay to quantitate individual apolipoproteins. Furthermore, the description of the distribution of apolipoproteins CI, CII, CIII, and E was scanty.

Rats differ from humans in having substantial quantities of apo E in HDL [34]. Studies of HDL subfractionation using the rat as a model may therefore not be applicable to man. Weisgraber and Mahley [14] studied HDL subfractionation using heparin affinity chromatography in humans. They used sodium dodecyl sulphate gel electrophoresis to measure apolipoproteins and found that apo E-poor HDL was the predominant subfraction as had been previously reported in the rat model (>85% of the lipoprotein protein). They reported that the apo E-poor fraction contained apolipoproteins AI, AII, and C's while the apo E-rich fraction contained apolipoproteins AI, AII, E and also an apo E–AII complex. Using antisera, Marcel *et al.* [15] reported that the major apolipoprotein of apo E-poor HDL was apo AI and the major apolipoprotein of apo E-rich HDL was apo E. They did find traces of apo E in the apo E-poor fraction. Both fractions contained apo AII, CII, and CIII. They found that between 2 and 12% of the HDL cholesterol was found in the apo E-rich fraction suggesting that apo E-poor HDL is the predominant type of HDL found in the circulation.

As in all previous studies we found that apo E-poor HDL was the predominant type. Although this preliminary report was primarily designed to examine methodology and recovery rates some interesting results regarding composition of these subparticles have emerged. The proportion of various apolipoproteins found in apo E-poor

HDL is seen in Table I. The data can also be expressed in terms of molar ratios of individual apoproteins to apo AI (Table II). The examination of the composition of these particles shows that they are distinct and the presence of lipid and apolipoproteins in both fractions suggests that these fractions represent lipoproteins and not free apolipoproteins.

As in previous studies the apo E-poor HDL fraction is predominant. Approximately 82.2% of the cholesterol found in HDL was in the apo E-poor fraction. This is consistent with the findings of Marcel *et al.* [15]. The ratio of cholesterol to total apolipoprotein mass in the apo E-poor fraction was 0.21 and 0.23 and in the apo E-rich subfraction was 3.2 and 7.5 for the two patients studied. This suggests that relative to the apoprotein mass present the apo E-rich subfraction is cholesterol-enriched.

To examine the hypothesis that apo E-rich HDL can be produced from apo E-poor HDL when apo E displaces apo AI, we incubated apo E-poor HDL with and without free apo E, which was then applied to the heparin column. When apo E-poor HDL is run through the heparin column without prior incubation with free apo E, bound (apo E-rich) and unbound (apo E-poor) fractions are eluted. The bound fraction is similar in composition to apo E-rich HDL. This suggests that the initial separation is not complete and that a second pass of apo E-poor HDL through the heparin column results in further purification of this fraction. The composition of the bound (apo E-rich) fraction is, however, different when apo E-poor HDL is eluted through the heparin column after incubation with free apo E (Table III). Specifically, there is a decrease in the molar ratio of AI/AII and an increase in the molar ratio of E/AII. This suggests that apo E may displace apo AI from apo E-poor HDL subparticles resulting in the production of an apo E-rich HDL subparticle. Changes are also seen in the molar ratios of the C apolipoproteins to apo AII. The composition of the bound fraction is unlikely to be due to the co-elution of free apo E as the changes in apo AI and the C apolipoproteins would not be seen if this were the case.

In summary, we report a rapid method of subfractionating HDL by FPLC using a heparin-Sepharose affinity column. The recovery rates for the individual apolipoproteins are reported and preliminary data on the composition of apo E-poor and apo E-rich HDL subparticles are furnished. Preliminary evidence is presented to suggest that apo E-rich HDL may be produced from apo E-poor HDL by the substitution of apo AI by E in association with changes in the C apolipoproteins.

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