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RESOLUTION AND QUANTITATION OF APOLIPOPROTEINS A-I AND A-II FROM HUMAN HIGH-DENSITY LIPOPROTEIN BY SIZE EXCLUSION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Apolipoproteins A-I and A-II, extracted from human high-density lipoprotein (HDL), were resolved and quantified by size exclusion high-performance liquid chromatography on TSK 125 and TSK 250 analytical columns connected in series without the use of chemical denaturants or detergents in the eluent buffer. The columns were pre-equilibrated with a solution containing 0.1 *M* sodium phosphate, pH 7.2, 0.2 *M* sodium chloride at a flow-rate of 1 ml/min. Delipidated HDL (1 mg protein per ml) was resolved into two populations of apolipoprotein (apo) A-I: one representing the apo A-I monomer and the other, a self-associated form with a molecular weight of approximately 120,000 daltons. The column eluates were screened for immunoreactivity to apo A peptides, and the identity of each peak was confirmed by sodium dodecyl sulfate—polyacrylamide gel electrophoresis followed by immunoblot analysis. Apo A-I peptides isolated by high-performance liquid chromatography disrupted unilamellar phospholipid vesicles to form smaller phospholipid particles that eluted on gel filtration columns within the size range of HDL. Thus, a rapid method for the isolation and quantitation of non-denatured apolipoproteins from HDL has been developed using size exclusion high-performance liquid chromatography.

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INTRODUCTION

The levels of plasma high-density lipoprotein (HDL) have been correlated inversely with mortality from cardiovascular disease [1, 2], and increased amounts of cholesterol in HDL appeared to protect against development of atherosclerosis [3]. Recent studies indicated that the levels of apolipoprotein (apo)A-I, the major protein component of HDL, may be a better metabolic marker for coronary artery disease than cholesterol levels [4], and for that reason efforts were directed toward the development of a rapid, reliable assay for apolipoprotein A levels.

Size exclusion high-performance liquid chromatography (HPLC) provides a high-recovery system for the detection, isolation and quantitation of proteins and other macromolecules. The size exclusion columns are capable of separating apolipoprotein molecules utilizing denaturants such as urea or guanidinium chloride [5, 6]. However, we have demonstrated that delipidated human HDL (apo-HDL) can be resolved by size exclusion HPLC without the use of these chemicals, thus providing a rapid method for quantification and recovery of native apolipoproteins from human HDL.

EXPERIMENTAL

Materials

Size exclusion protein standards, Biogel A-15 M, and protein assay kit II were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). Human apolipoproteins A-I, A-II and C-III, and antisera to apolipoprotein A were obtained from Calbiochem-Behring (San Diego, CA, U.S.A.). Sodium phosphate, sodium chloride, and HPLC-grade water were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). Electrophoretic reagents and protein standards were supplied by Bethesda Research Labs. (Bethesda, MD, U.S.A.). Phosphatidylcholine (egg), sphingomyelin, cholesterol, cholesteryl oleate, oleic acid, triolein, and bovine serum albumin were obtained from Sigma (St. Louis, MO, U.S.A.). [^{3}H]Methyl choline dipalmitoyl phosphatidylcholine and [^{125}I]protein A were obtained from New England Nuclear (Boston, MA, U.S.A.).

Preparation of apo-HDL

Plasma was obtained from normolipidemic, fasting donors, and HDL was isolated by the sequential floatation method of Lindgren et al. [7]. In order to isolate very low density lipoprotein (VLDL) and low density lipoprotein (LDL), the plasma was adjusted to a density (d) of 1.063 g/ml by addition of a solution containing 0.96 *M* sodium chloride, 7.572 *M* sodium bromide, 1 m*M* EDTA (d = 1.48 g/ml) and centrifuged at 190,000 *g* for 24 h at 12°C using a Sorvall (DuPont, Wilmington, DE, U.S.A.) 65.13 rotor in a Sorvall OTB-75B ultracentrifuge. The VLDL and LDL were removed by aspiration, and the infranatant was adjusted to d = 1.21 g/ml using the same salt solution (d = 1.48 g/ml) and centrifuged for 36 h under the same conditions. The HDL fraction was recovered and dialyzed against nitrogen-saturated HPLC-grade water adjusted to 1.0 *M* sodium chloride. HDL was delipidated by diethyl ether and ethanol extractions according to the method of Shore and Shore [8]. Protein

content was determined with the Bio-Rad protein assay kit using bovine serum albumin as a standard. The protein concentration of the final apo-HDL preparations ranged from 1.0 to 1.5 mg/ml and recoveries of protein averaged 85%. Each HDL and apo-HDL preparation was extracted by the method of Folch et al. [9], and thin-layer chromatography of these extracts was performed on silica gel G 20 × 20 cm plates (Fisher Scientific). The absence of phospholipid in the apo-HDL preparations was confirmed by separation using a solvent mixture of chloroform—methanol—water (65:25:4, v/v/v) [10] as the developing solvent, and the absence of neutral lipid was determined using a solvent system of hexane—diethyl ether—acetic acid (70:30:1, v/v/v) [11]. Phospholipid and neutral lipid in HDL and standards were quantified by a modification [12] of the densitometric procedure of Katz et al. [13]. Each apo-HDL preparation was stored in liquid nitrogen until chromatographed.

Chromatographic system

The HPLC system consisted of one 6000A pump, a 720 system controller, a U6K injector all from Waters Assoc. (Milford, MA, U.S.A.). Column eluate was monitored for ultraviolet absorbance using a Waters 440 detector (280 nm), and relative areas of each peak were determined using a Waters 730 data module. Two size exclusion columns, a TSK 125 connected in series to a TSK 250 (Bio-Rad Labs.) were equilibrated with the eluent buffer consisting of 0.1 *M* sodium phosphate, pH 7.2, 0.2 *M* sodium chloride at a flow-rate of 1 ml/min. The columns were calibrated with molecular weight standards (Bio-Rad Labs.), and no significant variation was observed between column and protein standard lots.

Hybridot screening of column eluates

A 150-μg sample of human apo-HDL was resolved by size exclusion HPLC and collected into 0.3-ml fractions. A 100-μl aliquot of each fraction corresponding to the appropriate peaks was applied to nitrocellulose using a hybridot apparatus (Bethesda Research Labs.). The nitrocellulose sheet was suctioned to dryness and processed with a 1:50 dilution of antisera against apo A peptides (Calbiochem-Behring) according to the method of Burnette [14]. Also, bovine serum albumin, standard apo A-I and A-II injections were chromatographed and collected as described for hybridot analysis.

Polyacrylamide gel electrophoresis

Fractions collected from HPLC resolution of human apo-HDL were dialyzed against 0.1 *M* sodium phosphate, pH 7.4, containing 1% sodium dodecyl sulfate (SDS) in a microdialysis unit from Bethesda Research Labs. and subjected to SDS—polyacrylamide gel electrophoresis (PAGE) on 15% gels using the method of Laemmli [15]. Paired PAGE gels were run using the Bio-Rad Labs. Protean double-slab electrophoresis cell with identical sample volumes applied to corresponding lanes of each gel. Proteins on one gel were visualized by silver staining [16], and the proteins on the other gel were transferred to nitrocellulose and analyzed by the immunoblot procedure of Burnette [14] using a 1:50 dilution of antisera against apo A peptides.

Incubation of vesicles with apo-HDL fractions

Unilamellar phospholipid vesicles were prepared and radiolabeled with tritiated lecithin as previously described [17]. Appropriate fractions from size exclusion HPLC of apo-HDL (167 μg protein per ml) were incubated with egg yolk phosphatidylcholine vesicles (445 μg lecithin per ml) in a total volume of 0.6 ml (2.6 × 30 cm) and chromatographed as described previously [17]. The Biogel A-15M column was calibrated using human HDL, bovine serum albumin and unilamellar vesicles. When incubation mixtures were applied to the Biogel A-15M columns, eluted fractions were analyzed for radioactivity using Aquasol (New England Nuclear) as the scintillation cocktail, counting efficiencies for tritium were approximately 50% and data were expressed as dpm tritium per ml eluted fraction.

RESULTS

Calibration of TSK 125 and TSK 250 size exclusion HPLC columns

When a mixture of molecular weight standards was separated by size exclusion HPLC with serial TSK 125 and TSK 250 columns, a linear relationship was obtained between log molecular weight and retention time (Fig. 1). Thyroglobulin (MW = 670,000), eluting at the void volume of these columns with a retention time of 10.50 min, was excluded from this plot. Therefore, these columns provided an estimate of protein molecular weight from the retention

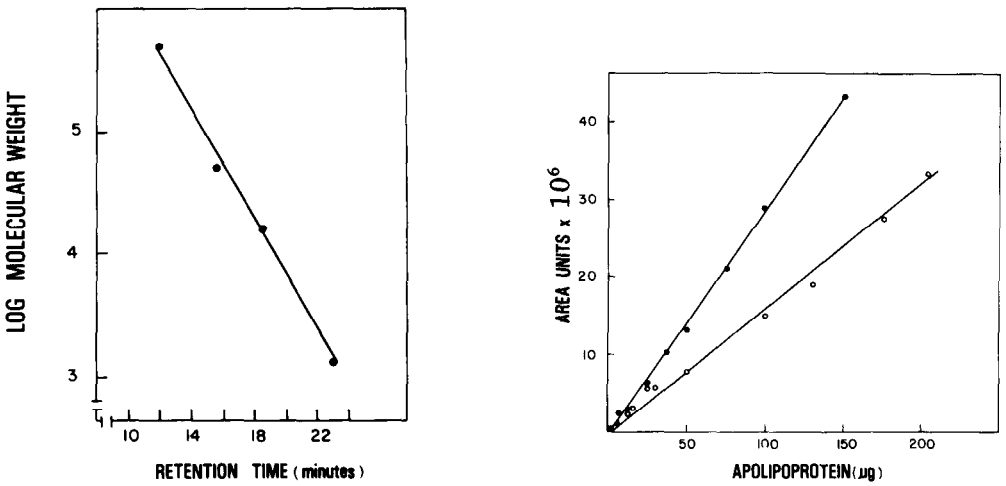

Fig. 1. Plot of log molecular weight versus retention time. A mixture of thyroglobulin, γ-globulin, ovalbumin, myoglobin, and cyanocobalamin was resolved on TSK 125 and 250 size exclusion HPLC columns as described. Thyroglobulin (MW = 670,000) eluted at the void volume of the columns with a retention time of 10.50 min (data not shown). The retention time of each protein was plotted against log molecular weight.

Fig. 2. Plot of apo A-I and apo A-II standard amounts versus area units. Varying amounts of apo A-I (•) and apo A-II (○) were resolved by size exclusion HPLC as previously described. The area of each peak was determined and each peak area was plotted against the amount of protein (μg). Each data point represents the average of at least three separate injections of each apoprotein in a concentration of 0.5 μg/μl eluent buffer. The coefficient of linearity for apo A-I was 0.999, and for apo A-II 0.994.

time values. Varying amounts of apo A-I and apo A-II (Calbiochem-Behring) were chromatographed, and a linear correlation between amount (μg) protein and the area of the resultant peak was shown (Fig. 2). Peak retention times and peak areas of standard apo A-I and apo A-II chromatograms were measured to ascertain levels of apo A-I and apo A-II present. A standard curve was regenerated prior to each analysis and less than 5% variation was observed.

Chromatography of the apo A-I standard (50 μg) in a concentration of 18 μM resulted in an elution profile shown in Fig. 3. Fractions were collected and analyzed for protein content, and approximately 46.4 μg were detected, representing a recovery of 93%. Similar results were obtained for the apo A-II standard and human apo-HDL isolates (data not shown). Therefore, this methodology provided a high-recovery system for the resolution and quantitation of apo A-I and apo-A-II on the basis of a molecular weight separation by HPLC.

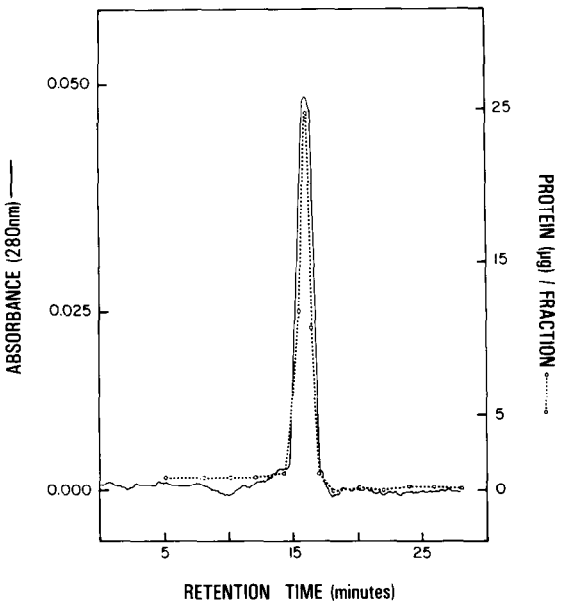

Fig. 3. Elution profile of apo A-I on TSK 250 and TSK 125 columns. Apo A-I, 50 μg in a concentration of 0.5 μg/μl, was resolved by HPLC as described, and column eluates were monitored for protein content at 280 nm (—). Fractions of 0.3 ml volume were collected and analyzed for protein content using the Bio-Rad microassay (o- - -o).

Chromatography of apo-HDL on TSK-125 and TSK-250 HPLC columns

Size exclusion HPLC of human apo-HDL resulted in an elution profile as depicted in Fig. 4B. Human apo-HDL was resolved into three major peaks with retention times of 13.50 min, 15.62 min, and 17.09 min. When a mixture of commercially available apo A-I, A-II, and C-III peptides was resolved by size exclusion HPLC apo A-I eluted at 15.50 min, apo A-II at 17.16 min, and apo C-III at 18.91 min (Fig. 4A). The major difference between these elution profiles was the appearance in the human apo-HDL profile of a major peak with retention time 13.50 min. This peak at 13.50 min represented a protein or

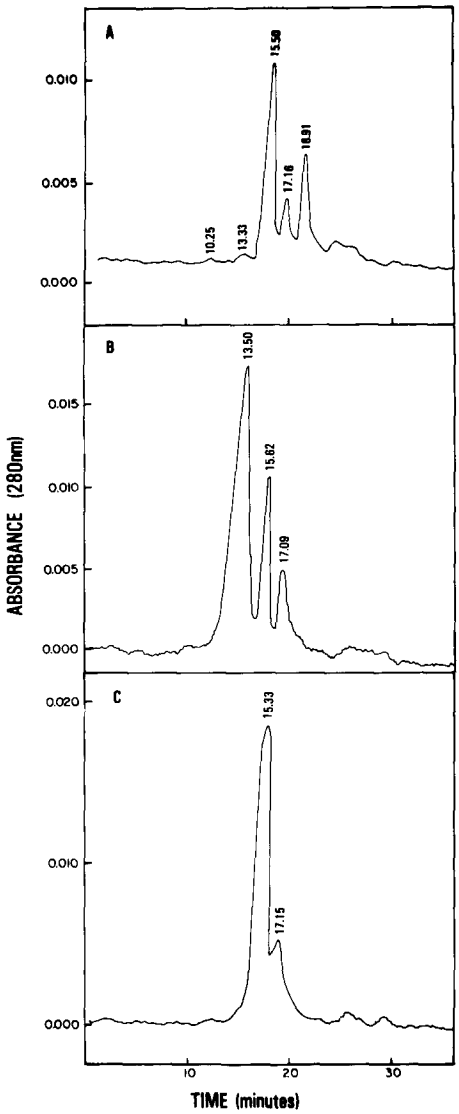

Fig. 4. Size exclusion HPLC of apolipoproteins. (A) A mixture of apo A-I (15 μg), apo A-II (3 μg), and apo C-III (3 μg), each obtained from Calbiochem-Behring, was resolved by size exclusion HPLC under previously described conditions. Retention times for each apolipoprotein were determined by separate injection of each protein (data not shown). Apo A-I, apo A-II, and apo C-III eluted with retention times of 15.50 min, 17.16 min, and 18.91 min, respectively. (B) An aliquot of 25 μl human apo-HDL (1 μg/μl protein) was chromatographed under the same conditions. Three peaks were resolved: peak I, retention time 13.50 min; peak 2, retention time 15.62 min, corresponding to 4.5 μg apo A-I; and peak 3, retention time 17.09, representing 3.8 μg apo A-II. (C) Prior to resolution by HPLC, 30 μl of the apo-HDL preparation was adjusted to 0.1% SDS at 25°C. The aliquot was chromatographed immediately after mixing on size exclusion HPLC columns, and two protein peaks were observed. The peak eluting at 15.33 min corresponded to 24.65 μg apo A-I and the second peak, with retention time 17.15 min to 3.5 μg apo A-II. All protein amounts were determined by comparing integrated areas of each peak to the areas obtained from standard apolipoprotein injections of known concentrations.

protein complex eluting from the TSK 250 and 125 columns with an apparent molecular weight, MW = 120,000. Adjustment of the eluent buffer from 0.2 *M* to 2.0 *M* sodium chloride had no disruptive effect on the peak at 13.50 min. When the respective peaks were collected and injected again, each peak chromatographed with the same retention time, although the peaks were broader and some material (< 20%) eluted at the void volume (data not shown). When apo-HDL was adjusted to 0.1% SDS (w/v) the peak at 13.50 min was abolished and an increase in the 15.33 min peak was observed (Fig. 4C). Also in Fig. 4C, the resolution of apo A-II was obscured somewhat by the descending shoulder of the monomeric A-I peak.

Hybridot analysis of eluates from size exclusion HPLC of apo-HDL

In order to determine the composition of the peaks obtained from size exclusion HPLC of the human apo-HDL isolate, column eluate fractions were analyzed immunologically for the presence of apo-A peptides. Fractions were collected and subjected to hybridot analysis against antisera to apo A peptides (Fig. 5). All three protein peaks resolved by HPLC of apo-HDL displayed immunoreactivity to antisera against apo A peptides. Separate elutions of standard apo A-I, apo A-II, and bovine serum albumin were screened with apo A antisera under described conditions. Apo A-I and apo A-II standards isolated directly from HPLC displayed reactivity (Fig. 5), but bovine serum albumin did not (data not shown).

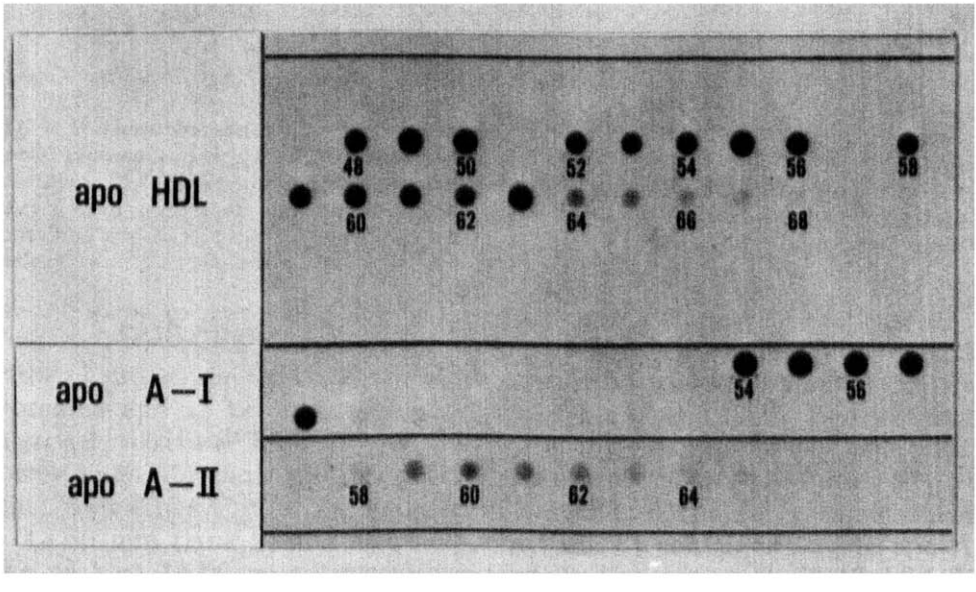

Fig. 5. Autoradiographic screening of HPLC column eluates for reactivity to antisera against apo A peptides. Apo-HDL, apo A-I and apo A-II were chromatographed separately and collected as described. For the human apo-HDL isolate: fractions 48—52 represent peak 1 (t_R 13.50 min); fractions 53—58, peak 2 (t_R 15.62 min); and fractions 59—68, peak 3 (t_R 17.09 min). The apo A-I standard displayed immunoreactivity in fractions 54—58 and the apo A-II standard reacted with antisera in fractions 58—64.

SDS—PAGE

In order to establish the identities of the peaks obtained from HPLC resolution of apo-HDL. fractions corresponding to the appropriate peaks were analyzed by SDS—PAGE (Fig. 6). The total apo-HDL isolate was subjected to SDS—PAGE in lane 5 and appeared as two major bands corresponding in mobility to apo A-I and apo A-II. When fractions collected from HPLC resolution of human apo-HDL were analyzed by SDS—PAGE, both the first peak, t_R = 13.50 min (lane 2), and the second peak, t_R = 15.62 min (lane 3) contained predominately a protein with approximate MW of 24,000. This protein comigrated with apo A-I standard, applied either directly (lane 6) or after HPLC (lane 9). The third peak t_R = 17.09 min, was resolved into two components with MW = 24,000 and 17,500 (lane 4). Although contaminated with apo A-I, this fraction was enriched in apo A-II as shown by co-migration with the apo A-II standard (lane 7).

In order to confirm the identities of the protein bands resolved by SDS—PAGE analysis, parallel slab gel was subjected to immunoblot analysis (Fig. 7). Each apolipoprotein demonstrated immunoreactivity to anti-apo A antisera. Total apo-HDL contained three cross-reacting proteins (lane 5). The

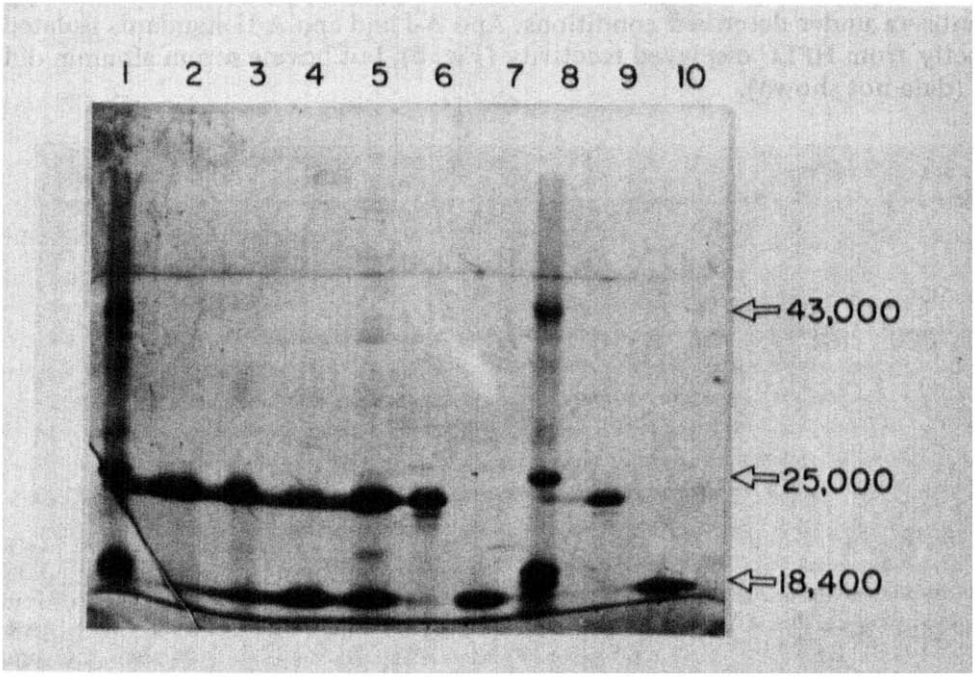

Fig. 6. SDS—PAGE of apolipoprotein standards and apo-HDL before and after resolution by HPLC. Ten injections each of 35 μg apo-HDL were chromatographed, collected, and prepared for electrophoresis as previously described. Lanes 1 and 8 contained as protein molecular weight standards ovalbumin, α-chymotrypsinogen, β-lactoglobulin, lysozyme/cytochrome c. Lanes 2, 3, and 4 contained human apo-HDL isolates from HPLC representing the peaks eluting with retention times of 13.50, 15.62, and 17.09 min, respectively. Lane 5 contained apo-HDL prior to resolution by HPLC. Lanes 6 and 7 contained commercial apo A-I and apo A-II standards prior to injection, respectively, and lanes 9 and 10 contained apo A-I and apo A-II after resolution by HPLC, respectively. Proteins were visualized by silver staining.

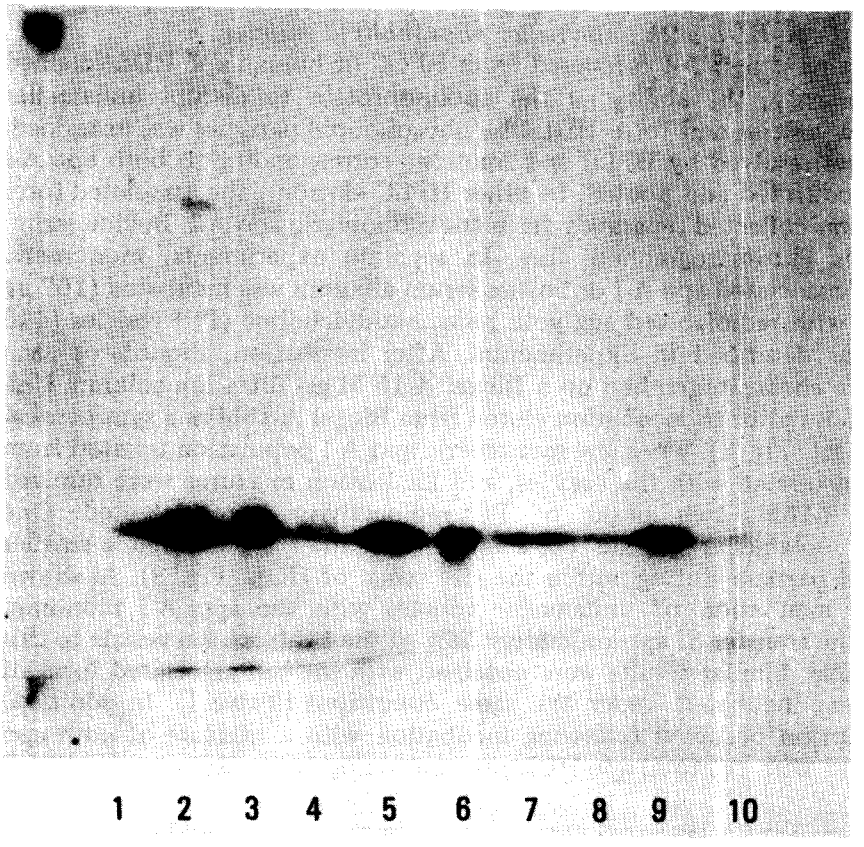

Fig. 7. Immunoblot detection of apolipoproteins resolved by SDS—PAGE. Apolipoproteins were prepared and subjected to SDS—PAGE and transferred to nitrocellulose as previously described. Lanes 10 and 9 represented aliquots of apo A-II and apo A-I isolated from HPLC, lanes 7 and 6 represented apo A-II and apo A-I prior to resolution by HPLC. Lane 5 contained apo-HDL prior to resolution by HPLC. Lanes 2, 3, and 4 correspond to apo-HDL peaks eluting with retention times of 13.50, 15.62, and 17.09 min, respectively.

predominant protein band comigrated with apo A-I standards (lane 6 or 9). Two faster migrating bands in the total apo HDL lane were detected faintly with the antiserum. One of these bands comigrated with the dye front and may represent apo C peptides. The other band, slightly above the dye front, migrated with the apo A-II standards (lane 7 or 10). Lanes 2, 3, and 4 corresponded to peak 1 (13.50 min), peak 2 (15.62 min) and peak 3 (17.09 min), respectively, after fractionation of human apo HDL by HPLC. The peaks at 13.50 min (lane 2) and at 15.62 min (lane 3) contained largely apo A-I. The peak at 17.09 min (lane 4) was identified as apo A-II. The commercial antisera (Calbiochem-Behring) appeared to react predominantly with apo A-I and much less with apo A-II. The relative lack of radioactivity bound at the apo A-I position in the peak 3 fraction (lane 4) indicated much better resolution of apo A-II from apo A-I than indicated by silver-stained gel (Fig. 6, lane 4). Since silver staining occurs predominantly on the gel surface, the apo A-I protein content detected by silver staining may be over-estimated from possible leakage of sample down the gel surface.

Incubation of apo-HDL with unilamellar phospholipid vesicles

To determine if apo A-I recovered from HPLC of human apo-HDL retained biological activity, the ability of the apolipoprotein to disrupt unilamellar phospholipid vesicles and form HDL-like phospholipid particles was examined. Apo-HDL was resolved by HPLC and fractions corresponding to both apo A-I peaks were collected and pooled. In other HPLC elutions, the associated form of apo A-I was collected separately from the monomeric apo A-I. Bovine serum albumin was chromatographed also. In separate experiments, monomeric apo A-I, self-associated apo A-I or bovine serum albumin was incubated (167 μg protein/ml) with radiolabeled egg yolk phosphatidylcholine (PC) vesicles (445 μg/ml PC) as described in Experimental. After incubation, aliquots of each mixture were chromatographed on a Biogel A-15 M gel filtration column. Unilamellar vesicles prior to incubation eluted from Biogel A-15M as a symmetrical peak at 104 ml (Fig. 8). When the monomeric apo A-I population isolated from HPLC was incubated with the vesicles, and incubation mixtures were resolved on Biogel A-15M, two peaks of PC radioactivity were detected. One represented unilamellar vesicles (104 ml) and another represented a smaller phospholipid particle eluting within the size range of HDL (Fig. 8). As shown in Table I, incubation of unilamellar vesicles with the apo A-I monomer resulted in the transfer of approximately 58% of the PC from the vesicle to this smaller particle. Similar results were obtained with the self-associated form of apo A-I when incubated under the same conditions (Table I). In addition, vesicle disruption occurred following incubation with a mixture of both apo A-I peaks and with the total apo-HDL preparation prior to resolution by HPLC

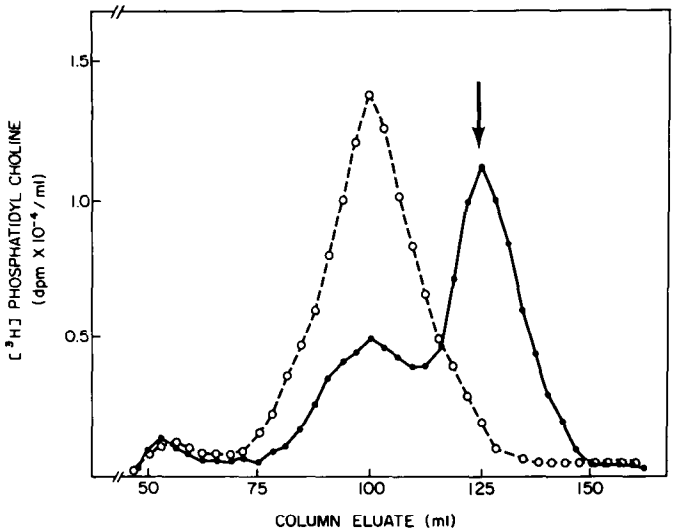

Fig. 8. Effect of apo-A-I isolated by HPLC on unilamellar vesicles. [³H]labeled egg PC vesicles (265 μg) were incubated in a total volume of 600 μl for 18 h at 37°C in the absence (○- - -○) and presence (●—●) of the apo A-I monomer (100 μg) isolated by HPLC. Aliquots of 550 μl were resolved on a Biogel A-15M gel filtration column (2.6 × 30 cm) and eluted fractions were analyzed for radioactivity. Recovery of radioactivity averaged 90%. The void volume of the column occurred at 56 ml, and the internal volume was found at 164 ml. The arrow indicated the elution volume of HDL.

TABLE I

EFFECT OF DIFFERENT PROTEIN FRACTIONS UPON THE DISRUPTION OF PHOSPHOLIPID VESICLES

All incubations included tritium-labeled vesicles (445 μg/ml egg yolk PC) and were conducted for 18 h at 37°C. Aliquots were applied to a Biogel A-15M column for estimation of lecithin disruption. The values shown are the average of at least three separate experiments.

Substance added to vesicles	Percentage labeled phospolipid disrupted
No addition	1
Bovine serum albumin* (167 μg/ml)	1
Apo-HDL** (167 μg/ml)	65
Apo A-I monomer* (167 μg/ml)	58
Apo A-I aggregate* (167 μg/ml)	46
Apo A-I monomer* (84 μg/ml) + apo A-I aggregate* (84 μg/ml)	49
Apo A-I*** (167 μg/ml)	60

*After resolution by HPLC.
**Not resolved by HPLC.
***Calbiochem-Behring, prior to HPLC.

(Table I). No vesicle disruption was detected when bovine serum albumin was incubated with the unilamellar vesicles. Thus, both monomeric and associated apo A-I isolated from size exclusion HPLC can disrupt unilamellar vesicles to form smaller phospholipid particles eluting in the size range of human HDL.

DISCUSSION

A non-denaturing size exclusion HPLC method has been developed for the isolation and quantitation of apo A-I and apo A-II from human HDL. By utilizing the TSK 250 and TSK 125 analytical columns in series, it was possible to obtain reliable estimates of apolipoprotein molecular weight. Also, a linear response was obtained for the amount of apo A-I and apo A-II resolved, thus providing a high-recovery means for determining apolipoprotein concentrations. Human apo-HDL was resolved into three separate peaks in the absence of denaturants. These peaks were identified as a self-associated form of apo A-I, monomeric apo A-I, and apo A-II. Furthermore, apo C peptides were resolved from apo A-I and apo A-II peaks, when a mixture of these proteins was applied to the column. Apo C peptides were not readily detected in apo HDL fractionations because of their relatively low concentrations in apo HDL preparations.

Our data suggested that native apo A-I separated from apo-HDL existed in both polymeric and monomeric forms. The polymeric form observed in the first HPLC peak (t_R = 13.50 min) appears to be a tetrameric complex of apo A-I. Vitello and Scanu [18] have observed the association of human apo A-I into dimers, tetramers, and octomers by resolution on Sephadex G-75 using a non-denaturing buffer. Within the concentration range of $5 \cdot 10^{-7}$ *M* and $5 \cdot 10^{-4}$ *M*, bovine apo A-I molecules displayed both tetrameric and

monomeric forms [19]. In our initial solutions of human apo-HDL, apo A-I was present at approximately $2 \cdot 10^{-5}$ *M* and would be within this concentration range. It is of interest to note that apo A-II also has been reported to self-associate in aqueous solution in a concentration dependent manner [20]. In our HPLC system, we observed no apo A-II association, perhaps because the apo A-II levels were too low. The apo A-I standard from Calbiochem-Behring did not form tetramers as readily as the apo A-I molecules isolated from human donors in this laboratory. It is possible that the commercial apo A-I may have been altered by the electrophoretic conditions used for purification such that it no longer readily self-associated within the concentration ranges described by Jonas [19]. Other investigators have resolved apolipoproteins from apo-HDL utilizing conventional gel filtration columns equilibrated with 6—8 *M* urea [19, 21, 22]. Also, apolipoproteins from HDL have been isolated by preparative size-exclusion HPLC using buffered 6 *M* urea or 6 *M* guanidinium chloride in the column eluates [5, 6]. On Sephadex G-150 bovine apo-HDL was resolved into three major peaks [19]. The peak with the highest molecular weight had an estimated molecular weight of 80,000 daltons; the second was 27,000 daltons, and the third approximately 17,000 daltons. Jonas [19] proposed that the first peak represented a tetrameric form of apo A-I and our results appear to confirm this observation with human apo A-I. We have not utilized urea, guanidinium chloride, or other agents known to solubilize apolipoproteins. In our hands, apo-HDL has remained in solution when dialyzed against either 1.0 *M* sodium chloride for ether extractions or 0.2 *M* sodium chloride for ethanol—diethyl ether extractions. The preparation was quickly frozen in liquid nitrogen at a concentration of 1—1.5 mg/ml protein and defrosted within 5 min of its removal from liquid nitrogen. This storage did not alter elution profiles or protein content, and apo-HDL solutions have been stored for as long as six months. Another factor that may have contributed to the successful fractionation without denaturants may have been the rapid resolution of apo-HDL components accomplished by size exclusion HPLC. Within 20 min these components were resolved with minimal dilution of the sample. Since gel filtration columns can take as long as 8 h to elute, it is possible that more column—apolipoprotein interactions may develop necessitating the use of chemical denaturants.

The monomeric and polymeric forms of apo A-I after resolution by HPLC retained the ability to disrupt unilamellar phospholipid vesicles. When spherical HDL was mixed with phospholipid dispersions or vesicles, apo A-I disassociated from HDL to disrupt vesicle bilayers and form discs [23]. It has been proposed that disc-like precursor HDL molecules, composed largely of phospholipid and apo A-I, give rise to mature HDL molecules through the action of lecithin cholesterol acyl transferase [24, 25]. Thus the insertion of apolipoprotein molecules into lipid bilayers is a critical event for the assembly of plasma lipoproteins, and regardless of the origin of HDL molecules, apo A-I is involved in interactions with phospholipids at various stages in HDL metabolism [26]. In aqueous solution apo A-I molecules may self-associate in order to stabilize the amphiphilic apoprotein molecule in the absence of phospholipid. Apo A-I molecules are most stable when associated with a phopsholipid bilayer to form a discoidal particle, followed by association with spherical HDL, and are least

stable in aqueous solution without lipid [23]. In our experiments, both monomeric and polymeric A-I molecules when incubated with phospholipid associated with phospholipid bilayers leading to disruption of vesicles to form smaller HDL discs. Jonas [19] proposed that the bovine tetrameric A-I aggregate may bind preferentially to protein rather than phospholipid based on the more non-polar components of bovine HDL as compared to human HDL composition. Since bovine apo A-I interacts with a larger amount of apolar lipid, it may develop stronger interprotein bonding than human apo A-I [19]. Therefore, our data suggest that both apo A-I fractions isolated here retained biological activity.

Using this method, native apolipoproteins can be isolated directly from HPLC columns and utilized for metabolic studies concerning apolipoprotein interaction with phospholipids and other models of in vivo phenomenon. Furthermore, this system provided a reliable means to determine apo A-I and apo A-II levels in human HDL and can be utilized in clinical studies to determine metabolic markers for coronary artery disease in humans.

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