Analytic and preparative separation of apolipoproteins A-I, A-II, and A-IV by reverse phase high pressure liquid chromatography

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Summary We have developed a rapid reverse phase high pressure liquid chromatographic technique capable of separating apolipoproteins A-I, A-II, and A-IV and their constituent isoforms. The separations were performed on a 30 cm × 3.9 mm C18 reverse-phase silica-based column (Waters Associates) using a 47–55% gradient of acetonitrile in 0.1% trifluoroacetic acid. Injection of a mixture of equal weights of apolipoproteins A-I, A-II, and A-IV yielded a single peak for apoA-IV, two peaks of apoA-I isoforms, and three peaks of apoA-II isoforms, with negligible overlap of each peak. When applied to the isolation of apoA-IV from a crude protein mixture obtained by incubation of phospholipid-triglyceride emulsion particles with lipoprotein-depleted plasma, the technique yielded a single peak of highly pure apoA-IV cleanly separated from isoforms of apoA-I and higher molecular weight proteins. A positive linear correlation was observed between apoprotein hydrophobicity and column retention time, thus indicating that the system provided a true reverse phase separation. We conclude that reverse phase high pressure liquid chromatography can separate human apolipoprotein A-IV from isoforms of apoA-I and apoA-II on the basis of differences in mean molecular hydrophobicity. The technique has direct application to the isolation of human apolipoprotein A-IV.—**Weinberg, R., C. Patton, and B. DaGue.** Analytic and preparative separation of apolipoproteins A-I, A-II, and A-IV by reverse phase high pressure liquid chromatography. *J. Lipid Res.* 1988. **29:** 819–824.

Supplementary key words apoA-I isoforms • apoA-II isoforms • molecular hydrophobicity

Traditionally, isolation and purification of human apolipoproteins have been carried out using low pressure gel filtration or anion exchange chromatography in denaturant-containing buffers (1). Although these techniques provide excellent yields, they have the disadvantages of exacting column preparation and maintenance, long separation times, and the need for desalting following chromatography. Recently, high pressure liquid chromatography (HPLC) has been successfully adapted to the purification of apolipoproteins, providing the advantages of rapidity, high purity, and run-to-run reproducibility (2–13). Most such HPLC methods have utilized analogues of the traditional preparative approaches, that is gel filtration (6–11) or anion exchange (4, 13). However, as apolipoproteins differ in their hydrophobic properties, reverse phase separations based upon differences in mean molecular hydrophobicity are also feasible (3, 5, 12). To expedite the isolation and purification of human apolipoprotein A-IV, we have developed a rapid reverse phase technique that is capable of separating apolipoproteins A-I, A-II, and A-IV and their constituent isoforms. An additional advantage of this method is that denaturants are not necessary to effect the separation, and hence no desalting step is necessary.

METHODS

Reagents

The mobile phase solutions were prepared with HPLC grade reagents. High purity water was prepared with a Milli Q system (Millipore Corporation). Solvent A consisted of 0.1% trifluoracetic acid (Pierce Chemicals) in water. Solvent B consisted of 0.1% trifluoroacetic acid in acetonitrile (Mallinckrodt Chemicals). The mobile phases were degassed for 5 min prior to use.

Columns

All columns were obtained from Waters Associates (Milford, MA). The column used for analytical separations was a 30 cm × 3.9 mm C18 reverse phase μBondapak silica-based column (PN 27324). Preparative separations were carried out with a 30 cm × 7.7 mm C-18 semipreparative column (PN 84176). A Guard-PAK C-18 guard cartridge was used with both columns.

Instrumentation

Separations were performed on a Waters system consisting of a U6-K injector with a 2-ml sample loop, a 680 automated gradient controller which operated a model 510 and a model M-45 solvent pump, a 450 variable wavelength detector, and a Waters 750 data module recorder/integrator. Fractions were collected on a dedicated LKB 2212 Helirac Fraction Collector (LKB). Analytic separations were performed at an eluant flow rate of 1 ml/min. The solvent program consisted of a 5-min isocratic elution with 47% solvent B followed by a 30-min linear gradient to 55% solvent B. Isocratic elution at 55% solvent B was then continued for an additional 15 min. Samples were applied to the column with a Hamilton syringe. Protein elution was monitored at 280 nm, or at 210 nm which afforded an approximately 40-fold increase in sensitivity. Protein peaks were lyophilized following collection. Peak identities were confirmed by SDS gel electrophoresis and isoelectric focusing.

Abbreviations: HPLC, high pressure liquid chromatography; VLDL, very low density lipoproteins.

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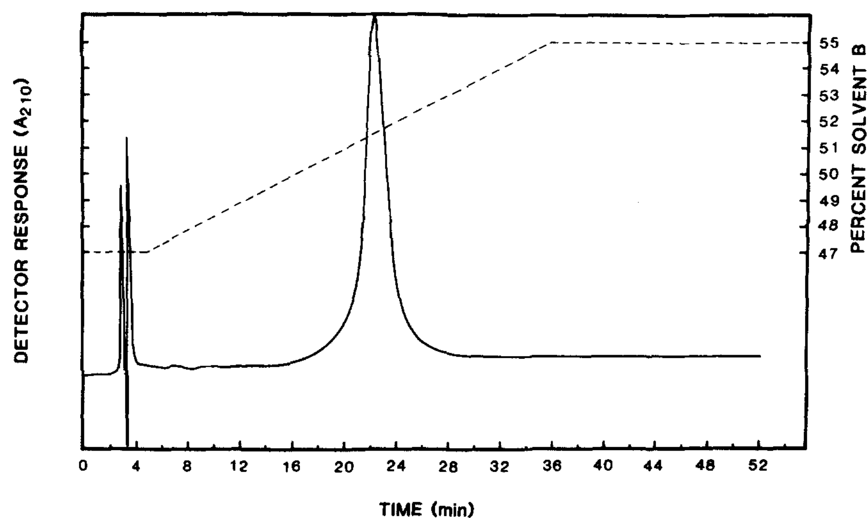

Fig. 1. Reverse phase HPLC chromatogram of 50 μg of pure human apolipoprotein A-IV. Column and gradient conditions as described in text. The peak corresponds to lane 1 in Fig. 3.

Preparation of human apolipoproteins

Apolipoprotein A-IV was purified by adsorption to particles of a phospholipid-triglyceride emulsion, followed by delipidation and anion exchange chromatography in 6 M urea (14). Apolipoproteins A-I and A-II were isolated from delipidated high density lipoproteins (1.125–1.210 g/ml) by a combination of gel filtration chromatography on Sephacryl S-200 (Pharmacia) and anion exchange chromatography in 6 M urea on DEAE cellulose (DE-52, Whatman) (1). Apolipoproteins C-II, C-III_1, and C-III_2 were purified from delipidated VLDL by anion exchange chromatography in 6 M urea on DEAE cellulose (DE-52 Whatman) (1). Apolipoprotein fractions were collected with continuous monitoring at 280 nm; desalted and concentrated by diafiltration against 1 mM ammonium bicarbonate, pH 8.6, using a YM10 membrane (Amicon Corporation); and sterilized by passage through a 0.45-μm Millipore filter.

Analytic techniques

The concentrations of aqueous apoprotein solutions were determined using the bicinchoninic acid assay (Pierce Chemicals) with bovine serum albumin as a standard (15). The molecular weights were estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis in 12% acrylamide gel. Isoelectric focusing in 7.5% acrylamide contain-

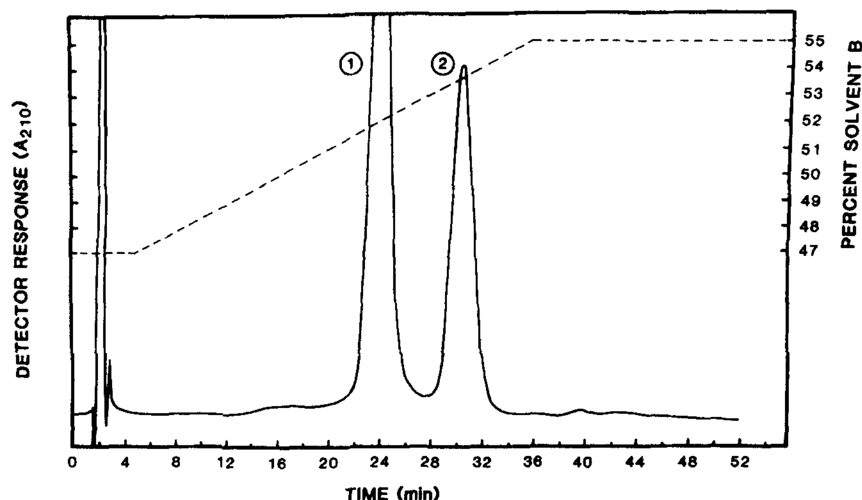

Fig. 2. Reverse phase HPLC chromatogram of 50 μg of pure human apolipoprotein A-I. Peaks 1 and 2 are isoforms of apoA-I, and correspond to lanes 2 and 3 in Fig. 3.

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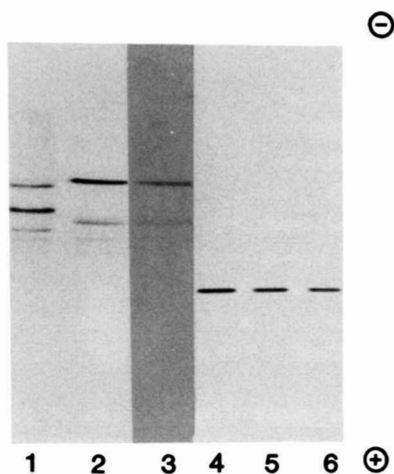

Fig. 3. Isoelectric focusing of apolipoprotein peaks obtained by reverse phase HPLC. The gel is 7.5% acrylamide and contains 2% pH 4-6 ampholine and 8 M urea. Lane 1, apoA-IV obtained from separation in Fig. 1; lanes 2 and 3, peaks 1 and 2 from apoA-I separation in Fig. 2; lanes 4, 5, 6, peaks 1, 2, and 3 from apoA-II separation in Fig. 4.

ing 2% pH 4-6 ampholine (LKB) and 8 M urea was performed as described by Gidez, Swaney, and Murnane (16).

RESULTS AND DISCUSSION

Elution profiles of individual apolipoproteins were determined using injections of 50 μg of each purified apolipoprotein. As anticipated, these apolipoproteins eluted from the column in the order of increasing hydrophobicity. Apolipoprotein A-IV eluted in a single peak with a retention time of 21.5 min **(Fig. 1).** Although apoA-IV undergoes high affinity self-association in aqueous solutions, even at very low concentrations (17), no oligomeric peaks were observed, most likely because the self-association of apoA-IV does not occur at the acidic pH of 0.1% trifluoroacetic acid (17). Apolipoprotein A-I eluted in two peaks: the first peak, containing 61% of the total mass, eluted with a retention time of 23.7 min; a second peak, containing 39% of the mass, eluted with a retention time of 29.8 min **(Fig. 2).** These two peaks had identical molecular weights of 28,000, but had different isoelectric focusing profiles **(Fig. 3),** consistent with apoA-I isoforms. Apolipoprotein A-II had the longest retention time and eluted in a series of three peaks: the first peak comprised 56% of the total injected mass and had a retention time of 41.6 min; the second peak consisted of 29% of total injected mass, and had a retention time of 45.6 min; and a final, broader peak consisted of 15% of total injected mass, and had an elution time of 51.9 min **(Fig. 4).** SDS gel electrophoresis showed that each isoform had a molecular weight near 18,000. Each of these isoforms focused in a single band with identical isoelectric points (Fig. 3). Heterogeneity of apoA-II, previously observed by isoelectric focusing and two-dimensional gel electrophoresis, has been ascribed to differences in sialic acid content and extent of amidation (18, 19). In the present case, however, the isoforms appear to have identical charges and similar molecular weights, suggesting that the observed heterogeneity may be based on other molecular modifications that influence hydrophobicity.

Apoproteins of the C family were retained for long times on the column. Purified apoprotein C-III_1 and C-III_2 coeluted with a retention time of 40.4 min, and apolipoprotein C-II eluted with a retention time of 41.1 min, thus over-

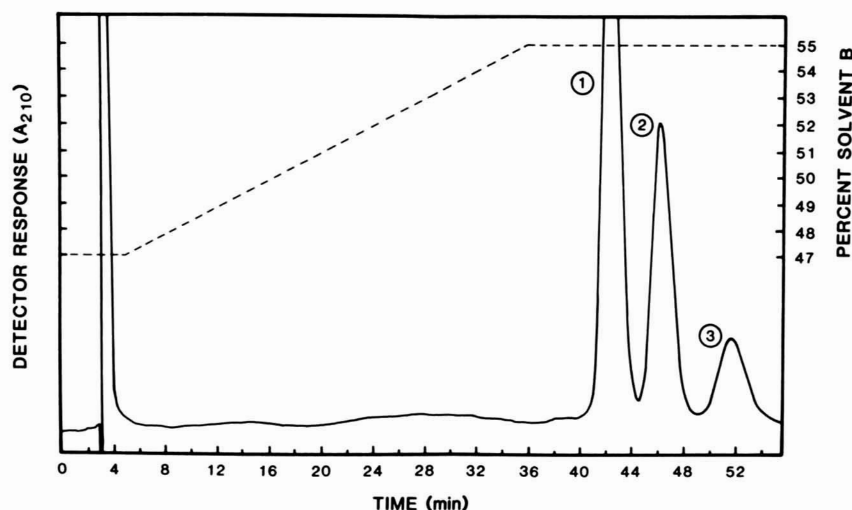

Fig. 4. Reverse phase HPLC chromatogram of pure human apolipoprotein A-II. Peaks 1, 2 and 3 are isoforms of apoA-II and correspond to lanes 4, 5, and 6, respectively, in Fig. 3.

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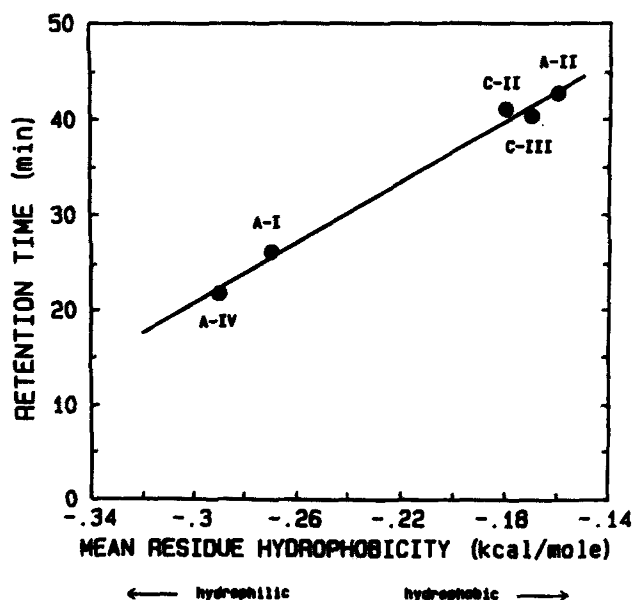

Fig. 5. Column retention time of purified apolipoproteins as a function of mean residue hydrophobicity. The mean residue hydrophobicity of each apolipoprotein was calculated from its amino acid composition (20) using the hydrophobicity values of Eisenberg et al. (21), and plotted against retention time. For apoA-I and A-II the retention times are calculated as a weighted average of the isoform retention times.

lapping the peaks of the apoA-II isoforms. Attempts to improve the separation of these apoproteins from apoA-II by changing the total gradient time, the gradient slope, or by placing an isocratic hold on the percentage of solvent B towards the end of the run were unsuccessful. Previous attempts to separate C apolipoproteins by reverse phase HPLC have been similarly disappointing, in that peak overlap persisted despite the use of exceedingly shallow gradients and long run times (3, 5).

Hancock and Sparrow (3) have pointed out that elution of apolipoproteins from silica-based alkylphenyl reverse phase columns may be extremely sensitive to the composition of the buffer systems, and that under certain circumstances, a normal rather than reverse phase separation may occur. However, a positive linear correlation was observed between mean residue hydrophobicity (calculated from apolipoprotein amino acid compositions (20) using the hydrophobicity scale of Eisenberg et al. (21)) and retention time **(Fig. 5).**

Injection of a mixture of equal weights of apolipoproteins A-I, A-II, and A-IV yielded a separation with negligible overlap of each peak **(Fig. 6),** and little significant change in retention times. Hence, this system was next utilized in the isolation and purification of apoA-IV. Particles of a phospholipid-triglyceride emulsion were incubated with lipoprotein-depleted serum, reisolated, and delipidated (14). The protein precipitate was solubilized in 6 M guanidine hydrochloride, 50 mM Tris, pH 8.6, dialyzed against 1 mM ammonium bicarbonate, and filtered through a 0.45-μm Millipore filter. Application of this crude apoA-IV solution to the column yielded three peaks **(Fig. 7):** large peak near the origin containing salt and higher molecular weight apolipoproteins (peak 1), and two well-resolved peaks consisting of apoA-IV (peak 2) and apoA-I (peak 3). The purification could be scaled up to accommodate injections of up to 7 mg of crude protein by using the semipreparative column with a modified solvent program consisting of a 10-min isocratic elution with 45% solvent B, followed by a 50-min linear gradient to 52% solvent B. ApoA-IV obtained using HPLC as the final isolation purification step

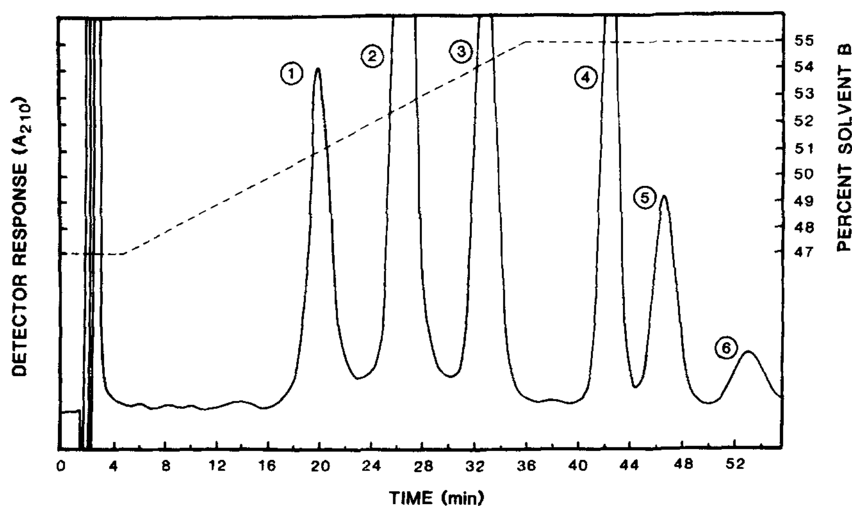

Fig. 6. Reverse phase HPLC separation of a mixture of 50 μg each of human apolipoproteins A-I, A-II, and A-IV. Identities of the peaks are: peak 1, apoA-IV; peaks 2 and 3, isoforms of apoA-I; peaks 4, 5, 6, isoforms of apoA-II.

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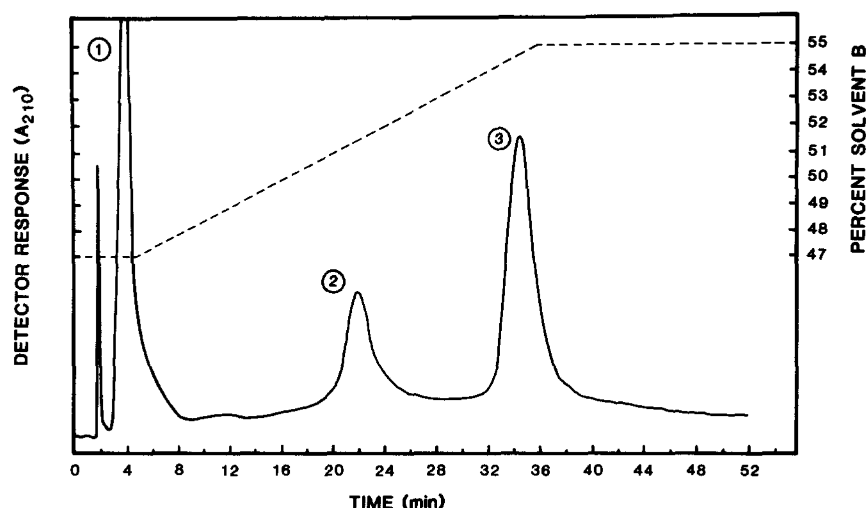

Fig. 7. Reverse phase HPLC purification of human apolipoprotein A-IV from a crude mixture obtained from delipidated phospholipid-triglyceride emulsion particles (14). Identities of the peaks are: peak 1, proline-rich protein and other higher molecular weight proteins; peak 2, apoA-IV; peak 3, apoA-I.

was identical to apoA-IV obtained by anion exchange when analyzed by SDS gel electrophoresis, isoelectric focusing, and immunodiffusion.

In summary, reverse phase HPLC can separate human apolipoprotein A-IV from isoforms of apoA-I and apoA-II on the basis of differences in mean molecular hydrophobicity. Our method utilizes volatile buffers, which obviates the need for a final desalting step. The technique has direct application to the final step in the purification of human apolipoprotein A-IV, and with a preliminary step to eliminate C apoproteins, could be used to directly isolate isoforms of apoA-I and apoA-II from delipidated high density lipoproteins.

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