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Separation of human apolipoproteins A-IV, A-I and E by reversed-phase high-performance liquid chromatography on a TSK Phenyl-5PW column

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ABSTRACT

A method has been developed for the rapid separation of the medium-molecular-weight apolipoproteins A-IV, A-I and E by high-performance liquid chromatography. Separations were achieved using a commercially available column of very low hydrophobicity (TSK Phenyl-5PW) in the reversed-phase mode rather than the conventional mode of hydrophobic interaction. Delipidated apolipoproteins were dissolved in 20 m*M* orthophosphoric acid (pH 2.3), applied to the column which was pre-equilibrated with the same buffer, and eluted with an increasing gradient of acetonitrile. Purified apolipoproteins were identified by a combination of sodium dodecyl sulphate–polyacrylamide gel electrophoresis, amino acid analysis and N-terminal sequence analysis. In one step the method can be used to separate the major human chylomicron apolipoproteins A-IV, A-I and E, following preliminary removal of apolipoprotein A-II and the C apolipoproteins by size-exclusion chromatography.

INTRODUCTION

Purification of the various plasma apolipoproteins (apo) has enabled investigation of their structure and function and consequently the assignment of physiological roles to many of them[1]. Of special interest to this laboratory has been the separation of the medium-molecular-weight apolipoproteins, apo A-IV, apo A-I and apo E. These proteins have traditionally been purified by a series of steps including size exclusion, anion exchange or chromatofocussing[2–6], usually in the presence of denaturants such as urea or guanidinium hydrochloride. However, none of these techniques alone produces satisfactory purification of apolipoproteins and most lipoprotein labora-

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tories use preparative gel electrophoresis as a final step to obtain homogeneous preparations of apolipoproteins, particularly when these proteins are needed for antibody production or functional investigations. More recently, two groups[7,8] have described the application of reversed-phase high-performance liquid chromatography (RP-HPLC) to the separation of apolipoproteins. These groups have used standard reversed-phase columns of high hydrophobicity (C_{18}) in order to separate apo A-IV from apo A-I (ref. 8) or apo A-I from apo E (ref. 7). In our hands, the use of such columns does not allow the efficient separation of apo A-IV, apo A-I and apo E in a single chromatographic step. We have, however, developed a method which achieves such a separation by the unconventional use of a column (TSK Phenyl-5PW) of much lower hydrophobicity than the standard C_8 or C_{18} reversed-phase columns. This method is of particular use in the separation of the components of human lymph chylomicrons.

EXPERIMENTAL

Reagents

High-purity reagents (HPLC grade) were used throughout. Water and acetonitrile were purchased from Mallinckrodt, while orthophosphoric acid and trifluoroacetic acid (TFA) were obtained from Merck and Pierce Chemicals, respectively.

HPLC columns

TSK Phenyl-5PW columns (75 mm × 7.5 mm I.D.) were purchased from Toyo Soda (Japan). The packing material consists of a 10-μm porous (1000 Å pore size) hydroxylated polyether support with a low density of covalently bound surface phenyl groups. μBondapak C_{18} (P/N 27324, 300 mm × 3.9 mm I.D.) and Activon RP300 C_8 (30 mm × 4.6 mm I.D.) columns were purchased from Beckman (Australia) and Applied Biosystems (Australia), respectively. The packing material for these two columns consists of porous spherical silica particles (10 μm diameter for μBondapak, 7 μm diameter for RP300) to which are covalently bonded C_{18} or C_8 alkyl chains.

HPLC methodology

Reversed-phase separations were performed on a Hewlett-Packard HP1090 HPLC system equipped with a Rheodyne sample injector (2-ml loop) and a diode array detector. Solvent A was 20 m*M* H_3PO_4 in water (pH 2.3) and solvent B was 20 m*M* H_3PO_4 in acetonitrile–water (60:40). Purified apolipoproteins were dissolved or diluted in solvent A and applied to the column at flow-rates of 1.35 ml/min (TSK Phenyl-5PW) or 0.5 ml/min (μBondapak, RP300). Column temperature was maintained at 45°C. Elution of proteins was effected by a linear (50 min) gradient from solvent A to solvent B. Protein elution was monitored simultaneously at 215 nm, 254 and 280 nm. Fractions were collected on a Cygnet fraction collector (Iscom).

Purification of apolipoproteins

Apolipoprotein A-I was purified from delipidated human high-density lipoprotein by ion-exchange chromatography in 6 *M* urea on DEAE-Sephacel[6]. Apolipoproteins A-IV and E were isolated from delipidated human lymph chylomicrons by preparative sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and electroelution[9].

Analytical methods

Samples for SDS-PAGE were run on 8–20% polyacrylamide gels and stained with Coomassie blue, using a Phastgel system (Pharmacia).

For amino acid analysis, samples were transferred to WISP vials (Waters Assoc.) containing 4 nmol of amino-guanidino propionic acid hydrochloride (AGPA) as an internal standard. Gas-phase hydrolysis was performed for 24 h at 110°C by the method described by Meltzer *et al.*[10] except that thioglycolic acid was omitted. Separation and quantitation of amino acids were performed on a Beckman System 6300 high-performance analyzer.

Amino acid sequences were determined using an Applied Biosystems Model 470A protein sequencer, equipped with an on-line Model 120A PTH analyzer.

RESULTS AND DISCUSSION

TSK Phenyl-5PW columns are designed for use in hydrophobic interaction chromatography (HIC) rather than for RP-HPLC. While HIC and RP-HPLC columns both contain stationary phases which consist of hydrophobic groups covalently bonded to hydrophilic supports, the HIC columns contain hydrophobic groups which are packed at a lower density[11]. It might thus be expected that the use of a HIC column for the reversed-phase separation of very hydrophobic proteins, such as apolipoproteins, might lead to improved separation of these proteins. To test this hypothesis, purified apolipoproteins A-IV, A-I and E were applied to a TSK Phenyl-5PW column and eluted with an increasing gradient of acetonitrile, as described in Methods. The resulting chromatograms are shown in Fig. 1A–C. Apolipoproteins A-IV and A-I eluted as single peaks with retention times of 10.1 and 17.2 min, respectively. Apolipoprotein E eluted as two peaks, with retention times of 21.7 and 23.9 min. The separation of a mixture of apolipoproteins A-IV, A-I and E is shown in Fig. 1D. The identity and purity of individual peaks from Fig. 1D were confirmed by amino acid analysis (Table I), SDS-PAGE (Fig. 2) and N-terminal sequencing of the first 10 amino acids. In a single step, purified apolipoproteins A-IV, A-I and E were obtained with yields in excess of 95% (as determined by amino acid analysis).

In contrast, we have found that poor separation of apolipoproteins A-IV, A-I and E resulted when these proteins were run on standard RP-HPLC columns under the same conditions as used for the separation on TSK Phenyl-5PW. The results of an attempted separation of these proteins on a standard C_8 RP-HPLC column (RP300, Activon, 30 × 4.6 mm) are shown in Fig. 3. Apolipoproteins A-IV, A-I and E eluted very late in the gradient with retention times of 37.9, 39.0 and 39.1 min respectively (Fig. 3, A–C). A mixture of these three proteins chromatographed as a single peak (Fig. 3D). Very similar results were obtained by using a μBondapak C_{18} column (P/N 27324, 300 × 3.9 mm), with apolipoproteins A-IV and A-I eluting with retention times of 43.9 and 45.2 min, respectively. Apo E eluted as two peaks, with retention times of 45.2 and 47.2 min (chromatograms not shown).

Two other groups have reported on the purification of apolipoproteins by RP-HPLC. Hughes *et al.*[7] described a RP-HPLC system with the capacity to separate apo A-I from apo E, although apo E eluted in several broad peaks and the chromatography of apo A-IV was not examined in that study. Weinberg *et al.*[8] used

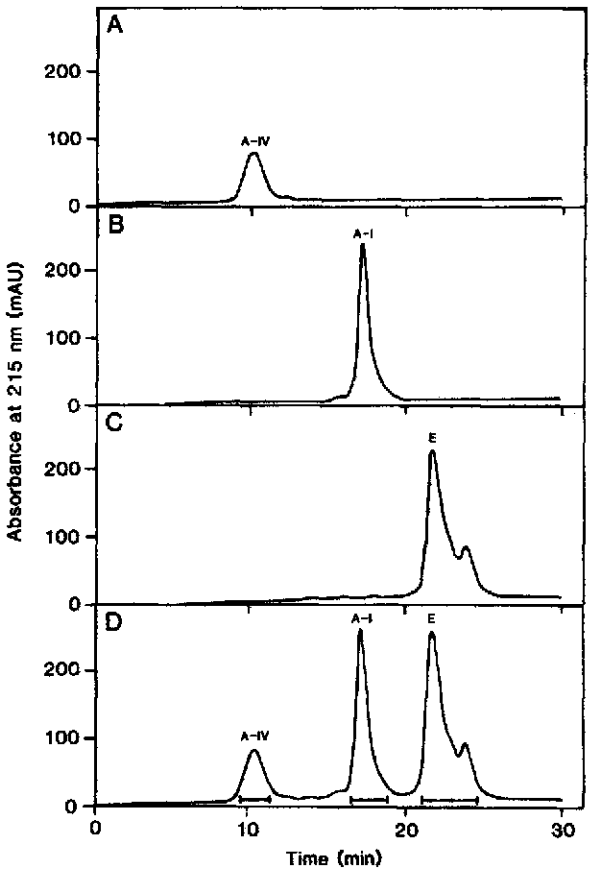

Fig. 1. RP-HPLC chromatograms of apolipoprotein separations on a TSK Phenyl-5PW column. Gradient conditions were as described in the text. Apolipoproteins A-IV (A, 30 μg), A-I (B, 50 μg) and E (C, 50 μg) were applied to the column separately (A–C) and as a mixture (D). Fractions were collected as indicated (|——|).

RP-HPLC to successfully separate apo A-IV from apo A-I, but in our hands that system does not separate apo A-I from apo E (date not shown). In both of the above procedures high concentrations of acetonitrile were used in the mobile phase, with very shallow gradients on standard C_{18} columns. Apart from the improved separation, RP-HPLC of apolipoproteins A-IV, A-I and E on the TSK Phenyl-5PW column has other advantages over the above methods. The lower concentration of acetonitrile needed for elution and the shorter residence times on the column may favour recovery of biological activity[11], while the steeper gradient elution might be expected to yield more reproducible separations of proteins[11].

The use of the hydrophilic ion-pairing reagent phosphoric acid as a mobile phase modifier has often been found to decrease column retention times for peptides and proteins in RP-HPLC and hence lead to sharper peaks[12]. In agreement with this, we have found that replacing phosphoric acid with TFA in our reversed-phase system leads to markedly increased retention times and lower resolution for apolipoprotein

TABLE I

AMINO ACID COMPOSITION[a] OF PURIFIED APOLIPOPROTEINS

Amino acid	*Expected*[b]			*Obtained (Fig. 1D)*		
	A-IV	*A-I*	*E*	*A-IV*	*A-I*	*E*
Asx[c]	36	21	12	36.8	21.6	12.9
Threonine	13	10	11	11.6	8.1	9.0
Serine	18	15	14	12.9	9.7	8.9
Glx[c]	92	46	71	104.4	52.5	80.0
Proline	12	10	8	11.0	9.8	8.5
Glycine	15	10	17	17.6	11.2	18.2
Alanine	30	19	35	32.5	20.1	35.9
Cysteine	0	0	1	ND[d]	ND	ND
Valine	19	13	22	19.3	13.7	22.7
Methionine	5	3	7	2.1	1.7	5.0
Isoleucine	5	0	2	4.8	0	2.1
Leucine	53	37	37	55.4	39.6	39.6
Tyrosine	6	7	4	5.1	5.5	3.3
Phenylalanine	11	6	3	11.1	6.3	3.3
Histidine	8	5	2	8.5	5.2	2.1
Lysine	28	21	12	28.4	21.4	12.5
Tryptophan	1	4	7	ND[d]	ND	ND
Arginine	24	16	34	23.0	16.7	34.9

[a] Expressed as number of residues per molecule.

[b] Theoretical values obtained from Swissprot data base (University of Geneva, Geneva, Switzerland).

[c] Asx = Asparagine plus aspartate; Glx = glutamine plus glutamate.

[d] ND = Not determined.

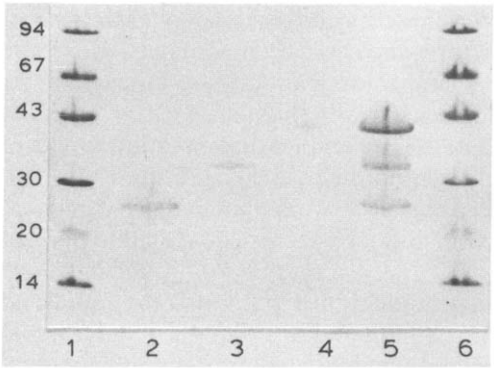

Fig. 2. Analysis of fractions from the TSK Phenyl-5PW column by SDS-PAGE and Coomassie staining. Sample loading buffer includes β-mercaptoethanol. Lanes 1 and 6 contain molecular weight standards (mol.wt. $\times 10^{-3}$). Lane 5 contains a mixture of apo A-IV, apo A-I and apo E. Lanes 2 (apo A-I) and 3 (apo E) and 4 (apo A-IV) correspond to the fractions collected as shown in Fig. 1D.

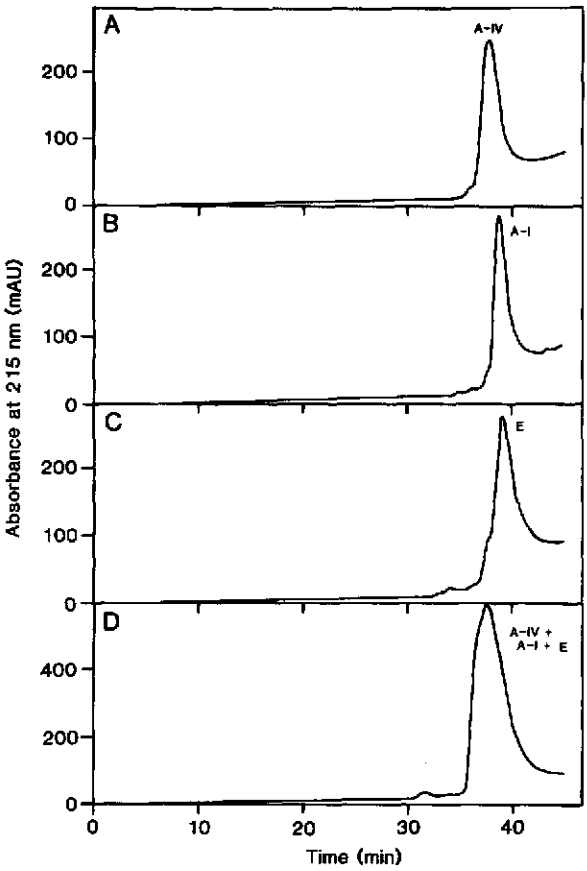

Fig. 3. RP-HPLC chromatograms of apolipoprotein separations on an Activon RP300 (C_8) column. Gradient conditions were as described in the text. Apolipoprotein samples were as described for Fig. 1.

separations (data not shown), and we therefore recommend the use of phosphoric acid as a mobile phase modifier. Another factor which has been found to affect the retention times of proteins in RP-HPLC is temperature: an increase in temperature usually leads to decreased retention[11]. In agreement with this also, we have observed that lowering the column temperature from 45°C to ambient temperature results in a significant increase in retention time for apolipoproteins A-IV, A-I and E (to 18.4, 23.3 and 26.4 min, respectively) on the TSK Phenyl-5PW column under the gradient conditions used, with some loss of resolution although the peaks are still separated baseline to baseline.

In conclusion, we have described a method which facilitates the rapid and efficient separation of apolipoproteins A-IV, A-I and E. Furthermore, we have found that the method is of particular use in the separation of the apolipoprotein components of human lymph chylomicrons, provided that the insoluble B apolipoprotein, apo A-II and C apolipoproteins are first removed by size-exclusion chromatography since some of the C apolipoproteins co-elute with apo A-IV and apo A-II coelutes with apo A-I (data not shown). An example of such a separation is shown in Fig. 4, which illustrates

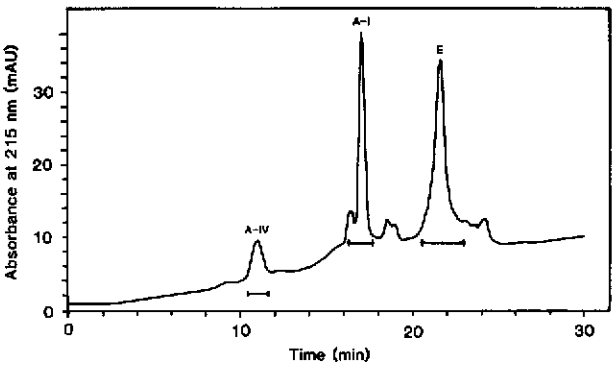

Fig. 4. Analytical RP-HPLC separation of the apolipoprotein components of human lymph chylomicrons on a TSK Phenyl-5PW column. Gradient conditions were as described in the text. The sample applied to the column was a fraction from the size-exclusion chromatography of delipidated human lymph chylomicrons on a Sephacryl S-300 column. Fractions were collected as indicated (|——|).

the analytical separation of apolipoproteins A-IV, A-I and E which were contained in a fraction obtained by size-exclusion chromatography of delipidated human lymph chylomicrons through Sephacryl S-300 equilibrated with 4 *M* guanidinium hydrochloride in 50 m*M* Tris–HCl pH 8.0. This type of gel filtration is the most widely reported technique used to isolate apolipoproteins from plasma or lymph triglyceride-rich lipoproteins[2,13], but never provides a satisfactory separation of the medium-molecular-weight apolipoproteins which are often the subject of investigation. However, as shown in Fig. 4, the method described in this paper achieves their isolation; the identity and purity of individual peaks from Fig. 4 were confirmed by SDS-PAGE, amino acid analysis and N-terminal sequencing.

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