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SEPARATION OF APOLIPOPROTEINS A-I AND A-II BY ION-PAIRED REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The separation of apolipoproteins A-I and A-II, isolated from human high density lipoproteins (HDL), was achieved on either a μBondapak–alkylphenyl or Zorbax-C_8 column. The mobile phase consisted of 1 % triethylammonium phosphate, pH 3.2 with a shallow linear gradient of acetonitrile. These conditions were used to separate mixtures of apolipoproteins A-I and A-II and a mixture of the apoprotein constituents of HDL.

INTRODUCTION

The high-efficiency separation of proteins by reversed-phase high-performance liquid chromatography (HPLC) was made possible by the use of mobile phases which contained ion-pairing reagents[1–8]. Now that the initial development phase has been completed, it is possible to apply this chromatographic technique to the rapid and highly efficient separation of complex mixtures of proteins present in biological samples. Recently, we reported [5] a rapid and highly efficient HPLC separation of the C-apoproteins of human very-low-density lipoproteins (VLDL). This procedure used a μBondapak–alkylphenyl column, with an aqueous mobile phase of 1 % triethylammonium phosphate and with acetonitrile gradients. This publication will describe the application of a similar chromatographic system to the separation of apolipoproteins A-I and A-II, as well as the analysis of a partially purified extract of human high-density lipoproteins (HDL).

EXPERIMENTAL

Apparatus

The analyses were performed on a Waters high-pressure liquid chromato-

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graph equipped with a M-660 solvent programmer (Waters Assoc., Milford, MA, U.S.A.). A Schoeffel 770 UV spectrophotometer was used to monitor the effluent at 220 nm and was linked to an Omniscribe two-channel chart recorder (Houston Instruments, Austin, TX, U.S.A.). Sample injections were made with a Microliter 802 syringe (Hamilton, Reno, NV, U.S.A.).

The columns used were a Waters Assoc. μBondapak–alkylphenyl column (4 × 250 mm), or a Waters Assoc. Radial-Pak A (C_{18}) cartridge (8 × 100 mm) or a DuPont (Wilmington, DE, U.S.A.) Zorbax-C_8 column (4.6 × 250 mm). The C_{18} catridge was used in a radial compression module (Waters Assoc.).

Chemicals

Water was purified by passage through a deionizer, a carbon filter, and then careful distillation in a quartz still (Barnsted, Boston, MA, U.S.A.). The mobile phase was prepared by addition of 10 ml of orthophosphoric acid (AR grade; J. T. Baker, Phillipsburg, NJ, U.S.A.) to 1 l of the purified water. The pH was adjusted to 3.2 with triethylamine (Aldrich, Milwaukee, WS, U.S.A.) to form an approximately 0.17 *M* solution of the ion-pairing reagent.The reagent was then filtered in a stainless-steel funnel (Hydrosol) fitted with a 47-mm Fluoropore (FH) 0.5-μm filter (Millipore, Bedford, MA, U.S.A.), degassed with helium and used as solvent A. Acetonitrile was obtained from Burdick & Jackson Labs. (UV grade; Muskegon, MI, U.S.A.), degassed with helium and used as solvent B. The solvents and chemicals used in this study are identical to those used in a previous paper[5].

Methods

The apolipoproteins A-I and A-II were isolated from HDL which was obtained from normolipemic plasma by ultracentrifugal flotation at density 1.21 g/ml (ref. 9). The apolipoproteins were then isolated by delipidation with ether–ethanol (3:1) and gel filtration chromatography using well established procedures[10]. The purified proteins were shown to be homogeneous by amino acid analytical data and by isoelectric focusing studies. The partially purified HDL apolipoprotein fraction was obtained from a broad pool of the fractions from the gel filtration step. The protein samples were dissolved in 1 % triethylammonium phosphate, 6 *M* guanidine–HCl, pH 7.5, at a concentration of 0.125 mg/ml. The samples were stored at −15°C and immediately before chromatography a 50 to 200 μl sample was treated with 10 μl of orthophosphoric acid to decrease the pH and ensure a large excess of the ion-pairing reagent. All pH values refer to the aqueous phase before addition of the organic component in gradient analysis.

All chromatography was carried out at room temperature (*ca.* 22°C). Other chromatographic conditions were the same as has been described previously[5].

After chromatography, the apolipoprotein samples were collected manually and cooled to 4°C. The pH was adjusted to 6 with triethylamine (5 to 20 μl) and the samples were then lyophilized. This procedure was carried out as quickly as possible to minimize any decomposition of the protein samples when exposed to acidic water–acetonitrile mixtures. The lyophilized samples were then sealed under vacuum with 0.5 ml of 6 *N* HCl, heated at 110°C for 24 h and the amino acid composition was determined on a Beckman 117 amino acid analyzer equipped with an Autolab integrator.

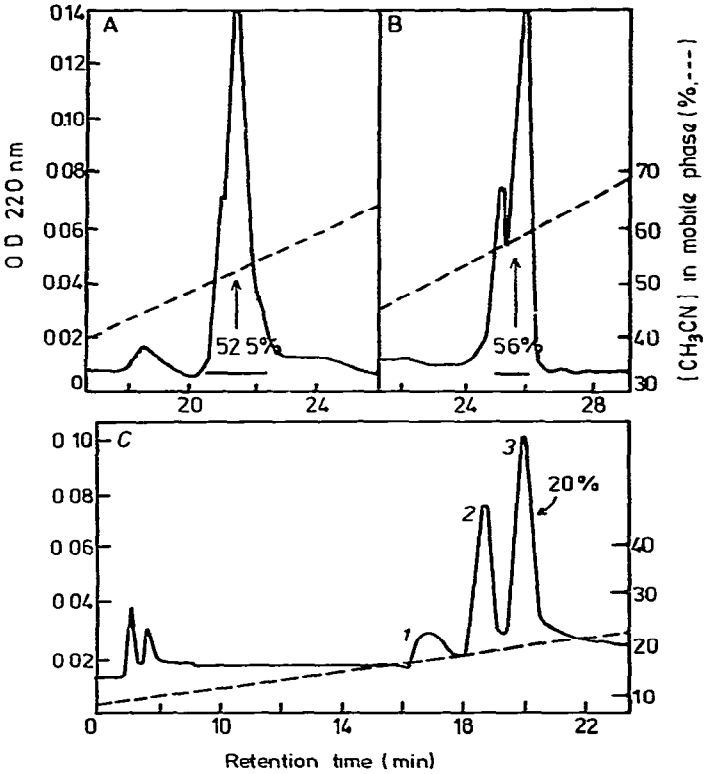


Fig. 1. The elution profile for a sample of apolipoprotein A-I on a μBondapak–alkylphenyl column (A), a Zorbax-C_8 column (B), or a Radial-Pak-C_{18} cartridge (C). In each case the mobile phase was 1 %triethylammonium phosphate, pH 3.2, and a linear gradient of acetonitrile was started at the time of injection. In parts A and B a 30-min gradient of 0 to 80% acetonitrile, and in part C a 30-min graient of 0 to 30% acetonitrile was used. The flow-rate was 1.5 ml/min in each case. In parts A and C, 100 μg, and in part B, 50 μg of protein were injected. The arrow shows the concentration of organic modifier required to elute apolipoprotein A-I.

RESULTS

Fig. 1 shows the elution profile obtained for a sample of apolipoprotein A-I on a μBondapak–alkylphenyl column (part A), a Zorbax-C_8 column (part B), or a Radial-Pak C_{18} cartridge (part C). In each case a shallow, linear gradient of acetonitrile was used at a relatively low flow-rate. These conditions were shown to allow excellent recoveries of the injected protein sample, as well as allowing minimal contamination between different runs. For example, if the analysis shown in Fig. 1A was followed by an injection of 0.2 ml of 1 % triethylammonium phosphate, 6 *M* guanidine–HCl, pH 3.2 and the gradient analysis repeated, less than 0.6% contamination from the previous sample was observed. To reduce contamination to this low level, it was necessary to wash carefully the injector before introduction of the next sample. Also monitoring the reverse gradient did not show any significant amounts of apolipoprotein A-I eluting when the acetonitrile concentration returned to the level suit-

able for elution of the protein. The effect of higher flow-rates on the separation was examined on the Radial-Pak C_{18} column and the results are shown in Fig. 2. In part A a flow-rate of 2 ml/min can be seen to give a similar elution profile as that observed in Fig. 1C where a flow-rate of 1.5 ml/min was used. A high flow-rate, however, can be detrimental to the separation efficiency as can be seen in Fig. 2B where a flow-rate of 5 ml/min was used. The optical density peaks observed in the three chromatograms shown in Fig. 1 were collected and processed as described in the *Methods* section. Representative amino acid compositional data for these peaks are shown in Table I, and it can be seen that the values obtained agree well with theoretical values.

In a similar manner apolipoprotein A-II was successfully chromatographed on a μBondapak–alkylphenyl column (Fig. 3, part A) and a Zorbax-C_8 column (part B). The Zorbax-C_8 column was slightly more retentive for both protein samples than the μBondapak–alkylphenyl column, and in both cases apolipoprotein A-II required a higher level of acetonitrile for elution than did apolipoprotein A-I.

Figs. 4 and 5 show the separation of a mixture of apolipoproteins A-I and A-II on both a μBondapak–alkylphenyl and a Zorbax-C_8 column. As would be expected from the elution profiles obtained for the pure protein standards, the Zorbax-C_8

TABLE I

REPRESENTATIVE AMINO ACID COMPOSITIONAL DATA* OBTAINED IN THE SEPARATION OF APOLIPOPROTEINS A-I AND A-II

ND = Not determined.

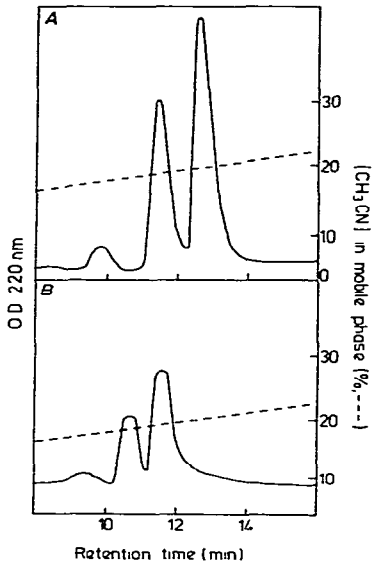
*Amino** acid*	*Expected***		*Fig. 1A*	*Fig. 1C*		*Fig. 3A*	*Fig. 5*		*Fig. 6, Peak 4*
	A-I	*A-II*		*Peak 2*	*Peak 3*		*Peak 2*	*Peak 4*	
Asp	21	6	24.8	25.0	26.1	8.4	20.5	7.3	20.3
Thr***	10	12	10.0	10.9	9.1	13.5	8.1	13.2	15.3
Ser***	15	12	16.3	17.0	19.6	13.7	15.2	13.1	15.1
Pro	10	8	10.4	7.8	ND	8.9	11.4	6.2	8.0
Glu	46	32	47.1	44.6	48.2	37.6	46.3	36.2	46.1
Gly	10	6	12.1	13.4	24.0	7.5	22.1	9.4	11.1
Ala	19	10	16.9	16.2	19.5	9.4	19.6	11.9	16.0
Cys	0	2	0	0	0	ND	ND	ND	0
Val	13	12	11.3	11.7	11.5	11.6	13.4	12.0	14.3
Met	3	2	2.65	2.2	3.1	2.1	2.5	1.5	2.6
Ile	0	2	0	0	0	1.6	0	1.2	0.3
Leu	37	16	34.6	32.5	31.8	14.0	38.1	16.2	36.5
Tyr	7	8	8.1	7.1	6.5	8.2	7.4	8.5	8.7
Phe	6	8	6.3	6.4	6.2	7.9	6.1	8.4	8.3
His	5	0	3.8	4.1	3.0	0	5.2	0	ND
Lys	21	18	22.1	19.4	21.1	17.0	22.5	18.8	23.6
Arg	16	0	14.8	14.3	18.1	0	16.7	0	10.5
Recovery (%)§			71			65	88	85	

* Expressed as number of residues per molecule.

** Theoretical values obtained from ref. 11.

*** Uncorrected.

§ Calculated from amino acid analysis of samples before and after HPLC.



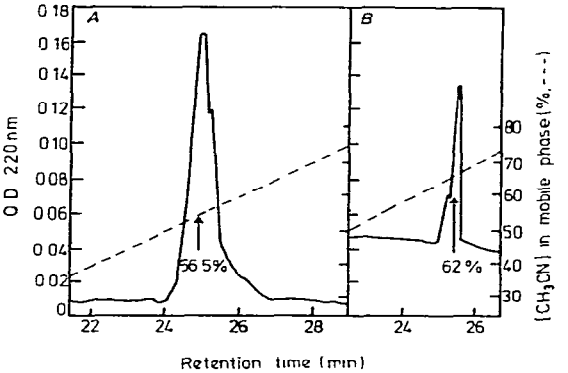


Fig. 2. The elution profile obtained for apolipoprotein A-I on a Radial-Pak-C_{18} cartridge at different flow-rates. All chromatographic conditions were the same as described in Fig. 1C except that a flow-rate of 2 ml/min (part A) and 5 ml/min (part B) was used.

Fig. 3. The elution profile for a 100-μg sample of apolipoprotein A-II on a μBondapak–alkylphenyl column (A) and a Zorbax-C_8 column (B). All chromatographic conditions were identical to those described in Fig. 1.

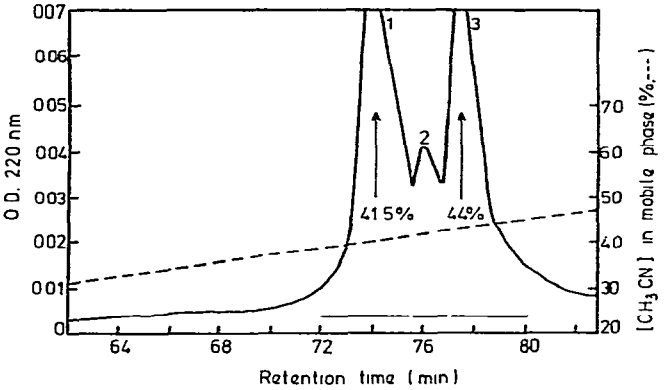


Fig. 4. The elution profile for a mixture of apolipoproteins A-I and A-II on a μBondapak–alkylphenyl column. A 50-μg sample of both proteins was injected and then a 2-h gradient of 0 to 80% acetonitrile was started. All other chromatographic conditions were as described in Fig. 1. The peaks were collected as described in the *Methods* section (see bars in Figure) and subjected to acid hydrolysis and amino acid analysis. Peak 1 was attributed to apolipoprotein A-I, 2 was unidentified and peak 3 was apolipoprotein A-II.

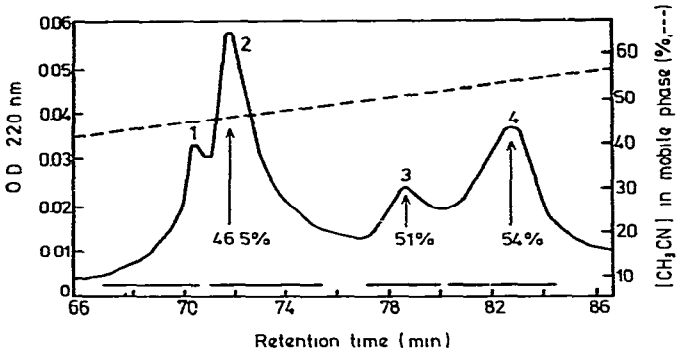


Fig. 5. The elution profile for a mixture of apolipoprotein A-I and A-II on a Zorbax-C_8 column. All chromatographic conditions were identical to those described in Fig. 4. Peaks 2 and 4 were identified by amino acid analysis as apolipoprotein A-I and A-II, respectively.

column clearly gave a better resolution of the mixture. The eluted apolipoproteins (peaks 1 and 3 in Fig. 4, and 2 and 4 in Fig. 5) were identified by amino acid analysis (see Table I). The minor peaks did not contain sufficient material for unequivocal identification, although peaks 1 and 3 in Fig. 5 gave similar analyses to the theoretical values for apolipoprotein A-I and A-II, respectively. Since the 30-min gradient of the individual protein samples gave similar retention times, a longer 2-h gradient was used in analysis of the mixture to improve resolution. Although the same size sample was loaded (100 μg) in each case, the resolution of the mixture with the phenyl-column was in fact the same for both gradients (difference, Δ of 3.5% acetonitrile). With the Zorbax-C_8 column, an improvement in resolution was observed with the shallower gradient (Δ of 8.5 and 5.5% acetonitrile). At this stage the reasons for these differences are not clear. The shallow gradients were successful, however, in allowing elution of both apolipoprotein A-I (41.5 *vs.* 52.5% and 46.5 *vs.* 56%) and A-II (44 *vs.* 56.5% and 54 *vs.* 62%) at lower concentrations of acetonitrile for both columns. The elutions of the protein samples with lower concentrations of organic modifier resulted in higher recoveries of eluted material (see Table I). The recovery of apolipoprotein A-I increased from 71% (30-min gradient) to 88% (2-h gradient) and A-II from 65 to 85%. The importance of the use of shallow gradients of organic modifers for protein separations was also demonstrated in other studies[5,6,12].

Fig. 6 shows the elution profile obtained for the analysis of a partly purified sample of HDL apoproteins. Peaks 1 to 3 were attributed to C-apolipoproteins based on previous studies on the separation of these apoproteins[5], while peaks 4 and 5 were attributed to apolipoprotein A-I and A-II, respectively. Due to the lower levels of the other proteins in HDL, only apolipoprotein A-I could be identified unambiguously by amino acid analysis, although the analysis of peak 5 was consistent with the assignment of apolipoprotein A-II. The C-apolipoproteins were identified by chromatography of authentic standards. A number of minor peaks were observed at other regions in the gradient analysis, which is consistent with the observations by others[11] of additional proteins such as apolipoproteins D and E which are present at low levels in HDL.

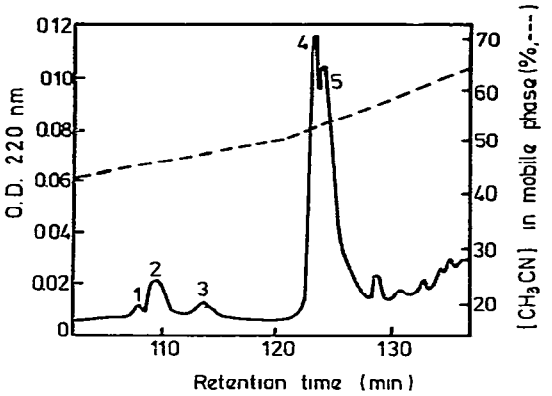


Fig. 6. The analysis on a μBondapak–alkylphenyl column of a partially purified sample of the apoproteins present in HDL. The sample volume was 200 μl. All other chromatographic conditions were identical to those used in Fig. 5 except that a 2-h gradient 0 to 50% was used, and was followed by a 30-min gradient of 50 to 80% acetonitrile.

DISCUSSION

HDL have the greatest density and hence protein content (up to 55% of the mass of the particle) of the four main lipoprotein particles[11]. The major protein constituents of HDL are apolipoproteins A-I and A-II which account for up to 70 and 25% of the protein content of the particle. In addition this lipoprotein also contains smaller amounts of C-, D- and E-apolipoproteins[13]. In the past, the apoprotein constituents of human lipoproteins have been analysed best by electrophoretic techniques, such as polyacrylamide gel electrophoresis or isoelectric focussing[14]. The analysis of HDL levels in plasma samples has assumed a greater significance since it was proposed that high levels of this lipoprotein could protect an individual from premature atherosclerosis[15].

Recently we published a new approach to the separation of the C-apolipoprotein constituents by reversed-phase HPLC[5]. In this separation a μBondapak–alkylphenyl column was used. A column of greater hydrophobicity, *e.g.* μBondapak C_{18}, gave poor resolution of the mixture. The apolipoproteins contain significant non-polar regions and thus interact too strongly with the C_{18}-packing for effective separation. In addition, the apolipoproteins are sensitive to silanol group interactions, and bind irreversibly to the reversed-phase support in the absence of a significant concentration of an amine phosphate in the mobile phase. A 1% solution of triethylammonium phosphate gave excellent recoveries of the C-apolipoproteins after separation on the alkylphenyl column[5].

The elution profiles described in the Results section demonstrate that both apolipoprotein A-I and A-II, the major protein constituents of HDL, can be successfully chromatographed with the system described above. The A-apolipoprotein samples interacted too strongly with a Zorbax-ODS column with the result that elution could only be achieved with very high levels of organic modifier (up to 90% acetonitrile). Figs. 1 and 3 show that column packings of intermediate polarity, namely Zorbax-C_8, μBondapak–alkylphenyl and Radial-Pak-C_{18} could be used to

chromatograph apolipoproteins A-I and A-II. The mobile phase of 1% triethylammonium phosphate, was used successfully in this study. Fig. 5 shows that the Zorbax-C_8 column gave a better separation of the mixture of the two apolipoproteins than did the μBondapak–alkylphenyl column (Fig. 4). The Zorbax-C_8 column has a much higher loading of the reversed-phase [15 *vs.* 10%(w/w) of C] than the Waters alkylphenyl column. In addition the C_8-packing consists of 5-μm particles, while the alkylphenyl packing consists of 10-μm particles. Since the peak shapes observed in the two elution profiles are similar, the improved separations on the C_8 column can be related to differences in selectivity rather than particle size differences between the two packings.

The Radial-Pak-C_8 cartridge contains a significant concentration of free silanol groups and was shown to allow the separation of protein samples via a mixed mode mechanism[4]. Fig. 1C shows that this column is capable of separating polymorphs of apolipoprotein A-I[16], whereas the C_8 and alkylphenyl columns show very little separation of these materials (see Fig. 1A and B). These studies show that the nature of the reversed-phase column is important to the success of a particular apolipoprotein separation. With the C-apolipoprotein mixture the alkylphenyl-column was best[5], while the C_8 column was superior for the separation of apolipoproteins A-I and A-II and the Radial-Pak-C_{18} column for the polymorphs of apolipoprotein A-I.

Recently Lewis *et al.*[6] demonstrated that the pore size of most commercial reversed-phase packing materials (generally about 80 Å) was inadequate for effective protein separations. These authors found that peak shapes were significantly affected by moderate flow-rates, and that the columns could be readily overloaded by the injection of larger sample loadings (0.2 mg and greater). Fig. 2 shows that the peak shapes obtained for apolipoprotein A-II were severely broadened at a flow-rate of 5 ml/min. The separation of the mixture of apolipoproteins A-I and A-II (Figs. 4 and 5) was not as good as one would expect from the chromatography of the individual proteins (see Figs. 1 and 3), particularly as a more shallow gradient of acetonitrile was used in separation of the mixture. Since both these proteins are of relatively high molecular weight [apoLP(A-I), 28,000 and apoLP(A-II), 17,400], access to the interior regions of the silica particles could be severely restricted. In this situation, even moderate sample loads may lead to lower separation efficiencies.

Since the separation of C-apolipoproteins could be achieved at a lower concentration of acetonitrile than apolipoproteins A-I and A-II[5], it was decided to investigate the separation of a mixture of these proteins. Fig 6 shows the successful separation of a partially purified extract of HDL which contains A- and C-apolipoproteins.

In conclusion, reversed-phase HPLC has been shown to allow the rapid and selective isolation of the major constituents of human HDL. The application of this technique to the analysis of these apolipoproteins in patients with various types of hyperlipidemia is currently under study.

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