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ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF HUMAN SERUM APOLIPOPROTEINS

GARY S. OTT and VIRGIE G. SHORE*

Lawrence Livermore National Laboratory, Biomedical Sciences Division, University of California, P.O. Box 5507 L-452, Livermore, CA 94550 (U.S.A.)

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SUMMARY

The rapid separation of seven urea-soluble apolipoprotein species from delipidated human serum very low density lipoproteins (VLDL) and high density lipoproteins (HDL) has been achieved by high-performance liquid chromatography on an anion-exchange column of Syn-Chropak AX 300. Effluent chromatographic peaks were detected by absorbance at 280 nm in a flow-through cell. Peaks corresponding to apolipoproteins AI_1, AI_2, AII, CI, CII, $CIII_1$, and $CIII_2$ were identified by amino acid analysis, gel electrophoresis, and isoelectric focusing. Maximum efficient loading of semipreparative columns (250 × 9.0 mm) was established to be ca. 20 mg HDL apolipoprotein. Minimum detectable protein was shown to be ca. 1 μg on an analytical-scale column (300 × 4.5 mm). Chromatographic resolution is comparable to that of conventional DEAE-cellulose column chromatography. The ratio of $apoAI_1$ to $apoAI_2$ was considerably greater in high-performance liquid chromatography, suggesting that the variants seen in conventional chromatography and isoelectric focusing are in part artifactual.

INTRODUCTION

The apolipoproteins of human serum are important determinants of lipoprotein metabolism by virtue of their lipid-binding capacity and transport roles, interparticle exchangeability, enzyme-cofactor roles, and affinity for specific receptors [1, 2]. The human apolipoprotein population includes AI_{1-3}, AII, CI, CII, $CIII_{0-2}$, E_{1-4}, several minor species that are urea-soluble, and apoB, the major apolipoprotein of low density lipoprotein (LDL) and very low density lipoprotein (VLDL), which usually becomes insoluble upon delipidation. The urea-soluble apolipoproteins are present in very different ratios in high density lipoproteins (HDL), in which apoAI and apoAII constitute ca. 90% of the protein mass, than in VLDL, in which apoC's and apoE constitute 40—60% [3, 4].

The continuing elucidation of lipoprotein metabolism, structure, and func-

tion requires both routine isolation of pure apolipoproteins and analytical determination of the apolipoprotein composition of both HDL and VLDL. The advent of SynChropak AX 300, a protein-compatible anion-exchange support [5, 6] for high-performance liquid chromatography (HPLC), provided the opportunity to apply the well documented resolution of anion-exchange chromatography [7, 8] to the separation of the HDL apolipoproteins. We have identified seven of the chromatographic peaks obtained by Tris · HCl gradient elution of a semipreparative (250 × 9 mm) column of SynChropak AX 300 and demonstrated the loading and sensitivity levels that limit anion-exchange chromatography for preparative and analytical applications.

EXPERIMENTAL

Preparation of HDL and apoHDL

HDL (ρ = 1.065—1.210 g/ml) was isolated from the fresh sera of normal males by the sequential flotation ultracentrifugation procedure of Lindgren et al. [9]. VLDL and LDL were removed by centrifugation of a 1.063 g/ml solution for 18 h at 14°C at 103,000 *g* in a Beckman Type 40.3 rotor (Beckman Instruments, Palo Alto, CA, U.S.A.). Total HDL was isolated by centrifugation of a ρ = 1.21 g/ml solution for 48 h under similar conditions. The lipoprotein composition of these fractions was analyzed by gradient polyacrylamide gel electrophoresis on 2.5—27% gradient polyacrylamide gels (Isolab, Akron, OH, U.S.A.). Albumin and LDL contamination was minimal. Salt was removed by dialysis (2 × 100:1, v/v) against nitrogen-saturated double-distilled water. The HDL was delipidated with ethanol—diethyl ether as previously reported [10]. The protein concentration of the apoHDL was determined by the method of Lowry et al. [11].

Isolation of the urea-soluble VLDL apolipoproteins

VLDL (ρ < 1.007 g/ml) of a 12-h fasted Type IV hyperlipidemic male was isolated from the fresh serum by the ultracentrifugal method of Chung et al. [12] using a Beckman VT 50 rotor. The upper 2 ml contained the VLDL, the purity of which was verified by gradient polyacrylamide gel electrophoresis on 2—16% polyacrylamide gels (Pharmacia, Uppsala, Sweden). Delipidation was performed by the tetramethylurea (TMU) extraction method of Kane et al. [13], which precipitates apoB.

Anion-exchange HPLC

Anion-exchange HPLC of apoHDL and apoVLDL was performed with SynChropak AX 300, an oxirane-crosslinked polyethyleneimine-coated 10-μm macroporous spherical silica support. Lots 363, 374 and 403 from SynChrom (Linden, IN, U.S.A.) were used with some variation in results. Both semipreparative (250 × 9 mm) and analytical-scale (300 × 4.5 mm) columns were fabricated from stainless-steel tubing and Whatman high-pressure fittings (Whatman, Clifton, NJ, U.S.A.) and then packed at 400 bar with a slurry of 0.5 g AX 300 support per ml of methanol with a slurry packer (Model CP111, Jones Chromatography, Columbus, OH, U.S.A.). Columns were fitted with 60 × 2 mm guard columns (Whatman) of SynChropak GPC 100, which were replaced

after operating pressures exceeded 140 bars. After 10 min preequilibration with starting buffer, the AX 300 columns were eluted at 1 ml/min with linear gradients of Tris · HCl in 6 *M* urea at pH 7.9 at room temperature (ca. 22°C) and at 40—133 bar, using a pair of Waters 6000A HPLC pumps (Waters Assoc., Milford, MA, U.S.A.). Gradients were programmed with a Waters Model 660 solvent programmer. All injections were made manually with Hamilton syringes (Hamilton, Whittier, CA, U.S.A.) into a Waters U6K injector. Samples for semipreparative columns were injected in 2 ml of 6 *M* urea, 0.01 *M* Tris · HCL at pH 7.9. Samples for analytical columns were stored and injected in nitrogen-bubbled, double-distilled water. Analytical-scale samples preincubated for ca. 20 min at room temperature in 0.01 *M* Tris · HCl, 6 *M* urea at pH 7.9 behaved similarly to those injected in water. Apolipoproteins were detected by absorbance at 280 nm using a Waters Model 440 detector.

Gel electrophoresis and isoelectric focusing

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels, and polyacrylamide gel isoelectric focusing (PAGIF) in the pH range of 4—8 on 6% gels containing 6 *M* urea, were performed by methods previously described by Shore et al. [14]. These methods were (with modifications) those of Weber and Osborne (SDS-PAGE) [15] and Wrigley (PAGIF) [16]. PAGIF pH gradients were determined by slicing of replicate gels, followed by overnight aqueous extraction of gel slices, and subsequent determination of the pH of the aqueous extract.

Amino acid analysis

Amino acids were determined as described by Shore et al. [14]. Proteins were hydrolyzed with 4 *N* methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole for 40 h at 110°C in sealed ampoules. Composition of hydrolysates was determined by the method of Liu and Chang [17] with a Beckman Model 121 Amino Acid Analyzer.

RESULTS

SynChropak AX 300 HPLC of HDL apolipoproteins

The HDL apolipoproteins are resolved into at least ten species by anion-exchange HPLC on a SynChropak AX 300 column using an optimized adaptation of the conditions successfully employed for DEAE-cellulose chromatography of apolipoproteins (Fig. 1a). The separation achieved in 180 min by anion-exchange HPLC is similar to that obtained by a 30-h elution of DEAE-cellulose columns. The absorbance profile at 280 nm has a major species at 73 min (peak 1) and a major multiplex between 88 and 100 min with two predominant peaks (peaks 2 and 3). DEAE-cellulose-purified apoAII coelutes with peak 1, apoAI_1 with peak 2 and apoAI_2 with peak 3. The ratio apoAI_1/AII is similar to that observed with DEAE-cellulose chromatography (2.4:1 on DEAE-cellulose, 2.6:1 on AX 300) while the ratio AI_1/AI_2 is substantially higher in anion-exchange HPLC chromatograms (3.0:1 on DEAE-cellulose, 6.4:1 on AX 300) [7]. Repeated chromatography of AX 300-purified apoAII and apoAI_1 produced single peaks with the expected mobilities (data not shown).

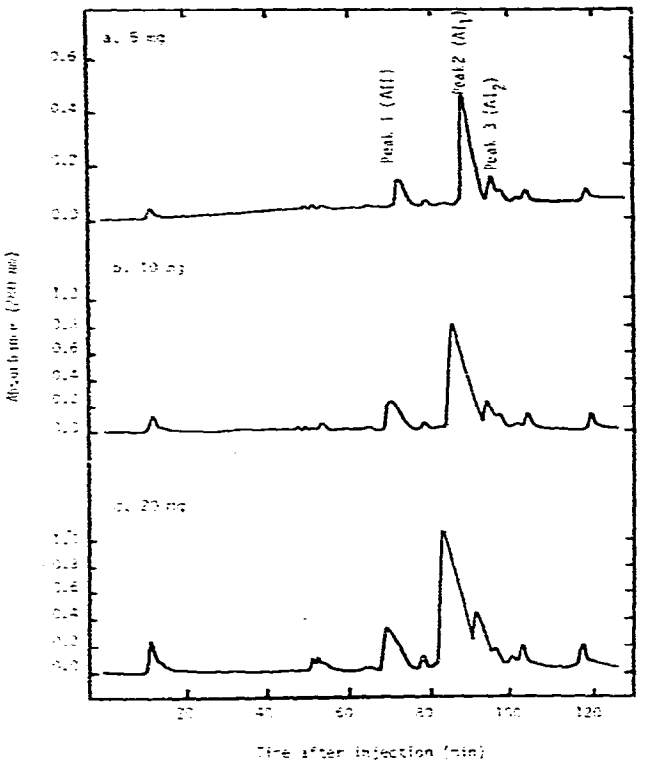

Fig. 1. Semipreparative-scale AX 300 chromatography of HDL apolipoproteins. HDL apolipoproteins were eluted from a 250 × 9 mm column of AX 300 with a linear, 180-min gradient of 0.02—0.20 *M* Tris · HCl in 6 *M* urea at pH 7.9 and a flow-rate of 1 ml/min. After each run the column was returned to initial conditions and reequilibrated for 10 min. (a) 5-mg, (b) 10-mg, and (c) 20-mg samples of HDL apolipoproteins in 6 *M* urea, 0.01 *M* Tris · HCl, pH 7.9, were each loaded in a single 2-ml injection.

The potential preparative utility of anion-exchange HPLC was attractive enough to justify the determination of loading capacity and the purity of effluent peak material. The chromatograms in Fig. 1a, b, and c were obtained with 5-, 10-, and 20-mg inputs, respectively, of apoHDL on a semipreparative column. Increased loading of AX 300 columns results in shorter elution times for the major species (reduced apparent binding constants and decreased resolution). The AI band broadens substantially as the input load is increased from 5 to 20 mg. Resolution in the region between AI_1 and AII is reduced and the separation between AI_1 and AI_2 is diminished. Input of loads > 20 mg does not appear to be useful.

The purity of the three major peaks obtained by chromatography of 10 mg apoHDL was analyzed by SDS-PAGE, PAGIF, and amino acid analysis of isolated proteins. The composition of a protein from peak 1 (Fig. 1a) corresponds to that derived from the published sequence of apoAII [18], with an average deviation of 5.6%. Hydrolysates from peaks 2 and 3 (Fig. 1a) have compositions that agree with that derived from the sequence of apoAI [19], with average deviations of 4.6% for $apoAI_1$ and 5.0% for $apoAI_2$ (Table I). Apparent molecular weights derived from SDS-PAGE (Fig. 2) for peaks 2 and 3 were

TABLE I

AMINO ACID COMPOSITION OF HDL APOLIPOPROTEINS

Single determination of peak material isolated by HPLC on an AX 300 anion-exchange column. Values are expressed as mol per 10^3 mol of amino acids.

Amino acid	AII peak 1	AII expected [18]	AI_1 obtained, peak 2	AI_2 obtained, peak 3	AI expected [19]
Lys	113	117	88	81	86
His	0	0	18	19	18
Arg	0	0	67	56	65
Asp	44	39	91	89	85
Thr	79	78	36	37	40
Ser	79	78	64	60	60
Glu	208	195	193	193	188
Pro	53	52	43	40	43
Gly	45	39	45	45	42
Ala	63	65	76	78	78
Val	72	78	48	54	55
Met	9	13	10	8	12
Ile	12	13	0	0	0
Leu	107	104	152	162	154
Tyr	53	52	28	28	29
Phe	50	52	23	20	24
Trp	0	0	12	16	16

27,000. Values obtained for peak 1 were 16,000 and 8500, respectively, for samples preincubated with and without mercaptoethanol. These apparent molecular weights also agree well with sequence data. PAGIF analysis of the isolated apoAI isoforms gave apparent isoelectric points of 5.6 for peak 2, and 5.5 for the major constituent of peak 3 (Fig. 3). The apparent isoelectric point of apoAII (peak 1) was 5.0 (data not shown). These values are in good agreement with those derived by thin-layer isoelectric focusing for apoAII, $apoAI_1$, and $apoAI_2$ [20]. The identification of the minor C apolipoproteins of HDL is given in the following section on HPLC of apoVLDL and a mixture of apoHDL and apoVLDL.

PAGIF analyses of $apoAI_1$ fractions consistently produced minor amounts of $apoAI_2$ and $apoAI_3$ that were not apparent upon repeated chromatography. The multiple peaks observed for PAGIF analysis of $apoAI_2$ represent in part the tailing of the $apoAI_1$ peak into the $apoAI_2$ region, and possibly in part, interconversion of the variants. Pure $apoAI_2$ has not been obtained by a single anion-exchange HPLC separation.

In addition to semipreparative usage, anion-exchange HPLC has the potential for quantitative analytical application. Chromatographic separations of 1.5, 15, and 75 μg of apoHDL are displayed in Fig. 4a, b, and c, respectively. With an input of 1.5 μg of apoHDL (ca. 0.9 μg $apoAI_1$ and 0.5 μg apoAII), $apoAI_1$ is clearly detectable but apoAII, whose absorption coefficient at 280 nm is about half that of apoAI, is not detectable (Fig. 4a). Input of 15 μg apoHDL allows reasonable detection of $apoAI_1$, $apoAI_2$, apoAII, and several minor components

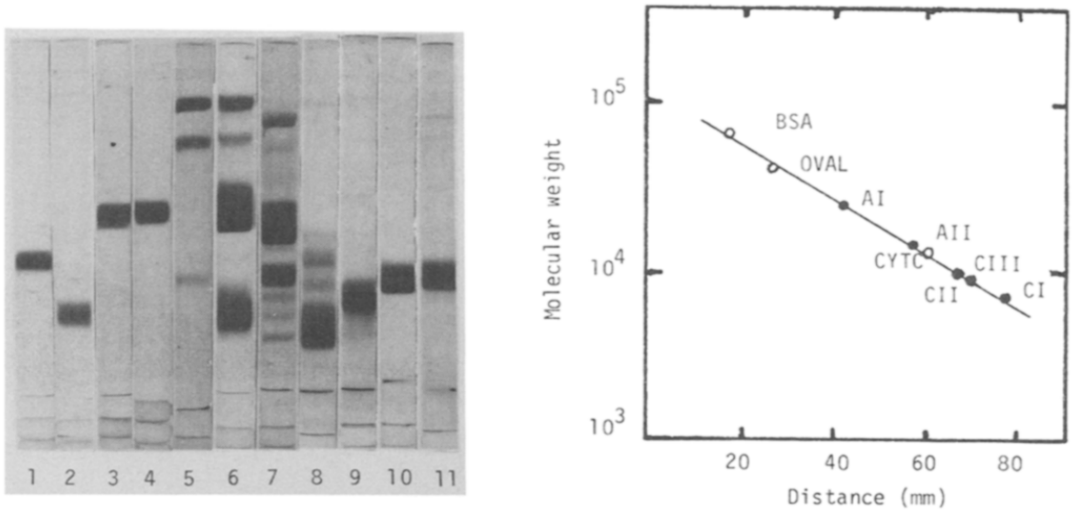

Fig. 2. SDS-PAGE of apolipoproteins. Electrophoresis of apolipoproteins was performed on 10% polyacrylamide gels in 0.2% SDS. Samples (10—100 μg) were run in the following gels: 1, apoAII (peak 1); 2, apoAII (peak 1) + mercaptoethanol; 3, $apoAI_1$ (peak 2); 4, $apoAI_2$ (peak 3); 5, standard mixture of bovine serum albumin (BSA), ovalbumin (OVAL), and cytochrome C (CYTC); 6, HDL apolipoproteins + mercaptoethanol; 7, HDL apolipoproteins; 8, apoCI (peak 1'); 9, apoCII (peak 2'); 10, apoCIII (peak 3'); 11, $apoCIII_2$ (peak 4').

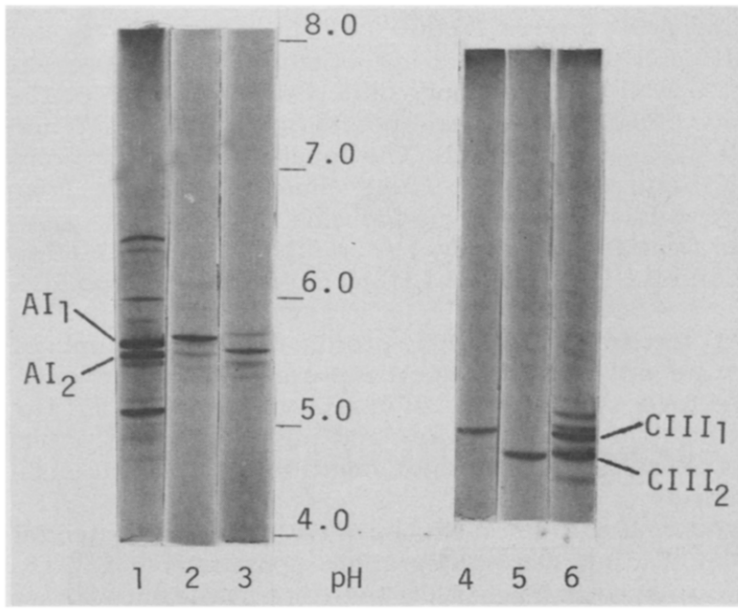

Fig. 3. PAGIF of apolipoproteins. Apolipoproteins were focused in 6% polyacrylamide gels containing 6 *M* urea and 2% pH 4—8 ampholines for 5 h at 400 V. Samples (10—100 μg) were focused in the following gels: 1, HDL apolipoprotein; 2, $apoAI_1$ (peak 2); 3, $apoAI_2$ (peak 3); 4, apoCIII (peak 3'); 5, $apoCIII_2$ (peak 4'); 6, urea-soluble VLDL apolipoproteins.

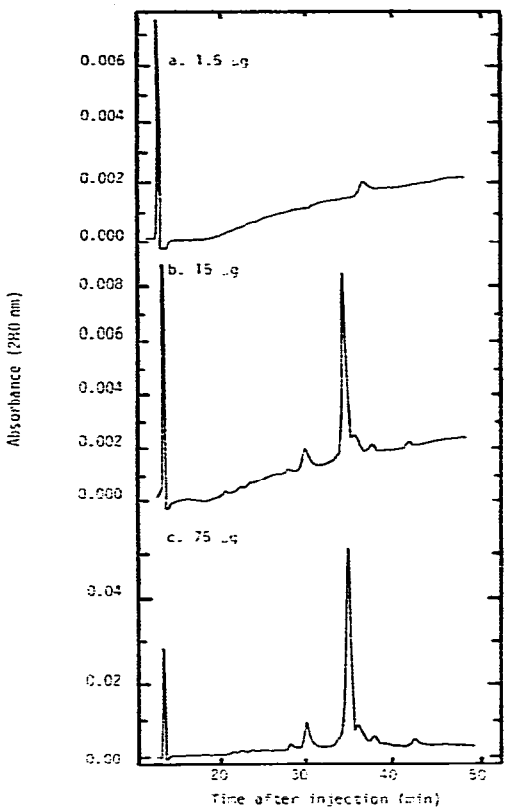

Fig. 4. Analytical-scale AX 300 chromatography of HDL apolipoproteins. Apolipoproteins were eluted from a 300 × 4.5 mm column of AX 300 with a linear 45-min gradient of 0.02—0.15 *M* Tris · HCl in 6 *M* urea at pH 7.9 and a flow-rate of 1 ml/min. (a) 1.5-µg, (b) 15-µg, and (c) 75-µg samples in double-distilled water were each loaded in a single 100-µl injection.

(Fig. 4b). The chromatography remains similar at 75 µg (Fig. 4c) and analyses of multiple species are possible with 15- to 75-µg inputs. It should be noted that the apoAII peak is contaminated with apoCII when 45-min gradients (Fig. 4) are used. This is not the case when 180-min gradients are employed (Fig. 1).

AX 300 chromatography of apoVLDL and a mixture of apoVLDL and apoHDL

The urea-soluble apolipoprotein moiety of VLDL shares several species in common with the HDL apolipoproteins, but the relative abundances are quite different. The chromatogram of Fig. 5 was obtained by elution of 6 mg of urea-soluble VLDL apolipoprotein from the semipreparative AX 300 column under the conditions employed for separation of the HDL apolipoproteins. The identities of four apoC peaks were assigned and purities estimated on the basis of SDS-PAGE, PAGIF, and amino acid analysis of the collected fractions corresponding to the peaks in the chromatogram. Peak 1′ (Fig. 5) contains apoCI in addition to unidentified contaminants. SDS-PAGE analysis reveals a major band with an apparent molecular weight of 6000 (Fig. 2) and several higher

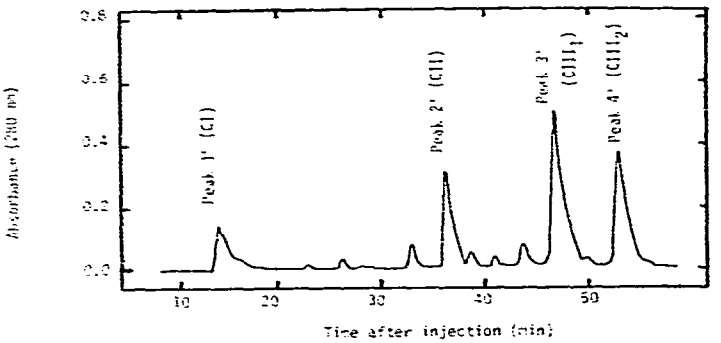

Fig. 5. Semipreparative-scale AX 300 chromatography of urea-soluble VLDL apolipoproteins. Urea-soluble VLDL apolipoprotein (6 mg in 6 *M* urea and 0.001 *M* Tris · HCl, pH 7.9) were eluted from a 250 × 9 mm AX 300 column with a linear 45-min gradient of 0.02–0.15 *M* Tris · HCl in 6 *M* urea at pH 7.9 and a flow-rate of 1 ml/min.

molecular weight species. The amino acid composition reflects these impurities, differing from sequence-derived composition [21] by ca. 23% per residue (Table II). Peak 2' (apoCII) has an apparent molecular weight of ca. 9000 (Fig. 2), in agreement with the expected value [22], and differs from sequence-derived composition by 10.4% (Table II). Peaks 3' and 4' have amino acid compositions expected for apoCIII [23]. They agree with the expected values within 9.5% and 10.2%, respectively (Table II). The apparent molecular weight by SDS-PAGE is ca. 10,000 (Fig. 2), which is the expected weight for apoCIII's

TABLE II

AMINO ACID COMPOSITION OF VLDL APOLIPOPROTEINS

Single determination of peak material isolated by HPLC on an AX 300 anion-exchange column. Values are expressed as mol per 10^3 mol of amino acids.

Amino acid	CI peak 1	CI expected [21]	CII peak 2'	CII expected [22]	$CIII_1$ peak 3'	$CIII_2$ peak 4'	CIII expected [23]
Lys	115	140	69	76	70	75	76
His	3	0	0	0	15	23	13
Arg	54	58	15	13	24	25	25
Asp	87	88	72	64	94	90	89
Thr	45	53	107	103	55	56	63
Ser	113	88	115	103	131	128	139
Glu	171	105	187	180	138	139	127
Pro	26	17	54	51	29	28	25
Gly	58	17	37	26	44	49	38
Ala	63	53	78	77	124	121	127
Val	44	35	54	38	72	71	76
Met	13	18	16	26	22	20	25
Ile	43	53	7	13	0	0	0
Leu	110	105	104	103	74	70	63
Tyr	8	0	62	51	26	26	25
Phe	48	53	27	26	55	54	50
Trp	16	17	10	13	43	41	25

with carbohydrate moieties [23]. PAGIF analysis of peaks 3′ and 4′ places the major bands at p*I* = 4.8 and 4.6, respectively. These agree fairly well with the thin-layer assessments of isoelectric points of 4.9 and 4.8 for apoCIII$_1$ and apoCIII$_2$ respectively [24]. On the basis of these isoelectric points and the amounts of the species present we have assigned peak 3′ as apoCIII, and peak 4′ as apoCIII$_2$. The amounts of material isolated from the remaining peaks were insufficient for identification.

VLDL apolipoproteins and HDL apolipoproteins were cochromatographed in a mixing experiment (Fig. 6). Comparison of chromatograms of apoVLDL, apoHDL, and a mixture of the two solutions (4:1, v/v) confirms the placement of apoC species on the apoHDL chromatogram. ApoCI and contaminating species run near column volume in all cases. ApoCII, which elutes at ca. 35 min, appears on the trailing edge of the apoAII peak. It is not well resolved in 45-min chromatograms. The apoCIII species that elute at ca. 47 and 50 min co-chromatograph with species eluting at those times from apoHDL. Thus, the shorter run resolves the apoC's. A 120-min run with a shallower gradient is needed to resolve a mixture of apoA's and apoC's.

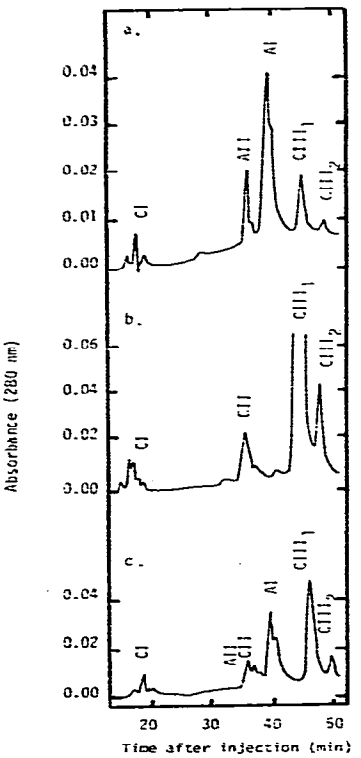

Fig. 6. Semipreparative-scale AX 300 chromatography of urea-soluble VLDL and HDL apolipoproteins. Apolipoproteins were eluted from a 250 × 9 mm AX 300 column with a linear 45-min gradient of 0.02—0.15 *M* Tris · HCl in 6 *M* urea at pH 7.9 and a flow-rate of 1 ml/min. The following samples were run: (a) 250 μg HDL apolipoprotein; (b) 650 μg urea-soluble VLDL apolipoprotein; (c) 130 μg urea-soluble VLDL apolipoprotein + 200 μg HDL apolipoprotein.

DISCUSSION

The preparative isolation and analytical determination of apolipoproteins have been approached by a variety of methods: anion-exchange chromatography [7, 8], gel-exclusion chromatography [25], combinations thereof and thin-layer isoelectric focusing [20, 24] for preparative purposes; urea-PAGE [13], SDS-PAGE [14], PAGIF [26], and a variety of immunological techniques [27] for qualitative and quantitative analysis. The rapid high-resolution methods developed for separation of proteins by HPLC [28, 29] have potential utility for these applications. Separation of apolipoproteins by gel-permeation HPLC with 300 × 22.5 mm columns of TSK-3000 SW (Toyo Soda Manufacturing Co., Tokyo, Japan) has been achieved for < 15 mg inputs of apoHDL in 6 *M* urea [30]. The resolution of purified apoAI into variant forms and the partial separation of a mixture of apoCI, apoCII, and apoCIII (derived from apoVLDL by conventional chromatography) by reversed-phase HPLC on a radially compressed C_{18} microparticulate support (Radial-Pak A, Waters Assoc.) [31] suggests its potential for analytical and possibly preparative work. For the preparative application, the reversibility of the denaturing effects of the organic solvent needs to be established. Apolipoproteins exposed to the denaturing solvents used in ion-exchange and gel-permeation HPLC have been shown to regain enzymatic cofactor activity on dialysis. Anion-exchange HPLC allows resolution of the major urea-soluble apolipoproteins and we have demonstrated the necessary loading capacity and sensitivity to justify development of both analytical and preparative separations of apolipoproteins by this chromatographic technique.

As a preparative method, HPLC conserves time and reagents, and by greatly reducing the exposure to denaturing solvents, reduces the risk of irreversible structural changes in the proteins. The useful loading capacity, with presently available columns, is limited to 10—20 mg. By comparison ca. 30 mg protein can be separated with excellent resolution by DEAE-cellulose chromatography on a 40 × 0.9 cm column. Advances in HPLC with larger-diameter preparative columns and less expensive supports [28, 29] may obviate this limitation in the near future. The alternative is repeated injections of sample, either manually or with an automated HPLC preparative system [32]. The seven apolipoprotein components (AI_1, AI_2, AII, CI, CII, $CIII_1$, and $CIII_2$) separated by anion-exchange HPLC are similar to those isolated by DEAE-cellulose chromatography by SDS-PAGE, PAGIF, and amino acid analysis. The chromatographic profiles are quite similar, with the notable exception that the ratio AI_1/AI_2 is substantially higher for anion-exchange HPLC separations. The number of apoAI variants isolated, and their ratios, appear to be a function of the method of isolation. As many as five variants and as few as two have been reported for ion-exchange separations [33, 34] while four are found with thin-layer isoelectric focusing [20]. Anion-exchange HPLC resolves only two apoAI variants in significant quantity. These results suggest that apoAI variants are generated during long exposure to denaturing conditions. In our hands PAGIF analysis of purified $apoAI_1$ does reveal two additional minor bands while anion-exchange HPLC continues to produce a single species. While shorter exposure to solvent enhances the attractiveness of anion-exchange HPLC, the limitations in resolu-

tion are similar to those for DEAE-cellulose. Purification of some species may require a combination of gel permeation and ion-exchange separations.

The high resolution of apolipoproteins by HPLC indicates the potential for simultaneous qualitative analysis and quantitative determination of the various apolipoproteins in a lipoprotein sample. It offers some advantages over the two basic analytical procedures currently in use. Immunoassays analyze for a single protein with high sensitivity, but the variants of a given apolipoprotein are not distinguished and there are numerous technical constraints [27]. Simultaneous determination with high resolution of apolipoproteins is possible with SDS-PAGE, urea-PAGE, and particularly PAGIF, which resolves the variant forms of apoAI, apoCII, apoCIII, and apoE. The quantitative application of the gel methods has several disadvantages that are obviated by HPLC. These include the uncertainties associated with staining and destaining (which are generally slow and difficult to reproduce), differences in staining intensities of the various apolipoproteins, and the possibility of nonlinear response. In HPLC, peak area is directly related to the amount of protein and its absorption coefficient, which is a characteristic of a given apolipoprotein. HPLC analysis offers the advantages of 1—2 h analysis time and applicability of readily available automated instrumentation, which allows continuous operation. We have demonstrated sufficient resolution and sensitivity for the simultaneous analysis of several apolipoprotein species by anion-exchange HPLC.

Assays for apoAI_1, AI_2, AII, CII, $CIII_1$, and $CIII_2$ are feasible with sufficient input. Differences in peak width and extinction coefficient create some variation in sensitivity. While apoAI can be detected at < 1 μg, AII requires ca. 2 μg for detection (data not shown). Routine analysis of apoHDL will require ca. 50-μg inputs if reproducible analysis of apoC's is to be achieved.

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