Rapid isolation of apolipoprotein E from human plasma very low density lipoproteins by molecular sieve high performance liquid chromatography

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Abstract A rapid (less than 1 hour) and sensitive technique was developed for the isolation of apolipoprotein E from the other main components of human plasma very low density lipoproteins using molecular sieve high performance liquid chromatography with an approximate 80% recovery. The properties of the pure apoprotein were those reported in the literature for products isolated by conventional chromatographic and/or electrophoretic procedures.—**Pfaffinger, D., C. Edelstein, and A. M. Scanu.** Rapid isolation of apolipoprotein E from human plasma very low density lipoproteins by molecular sieve high performance liquid chromatography. *J. Lipid Res.* 1983. **24:** 796–800.

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Since the early recognition of apolipoprotein E (apoE) by Shore and Shore (1), several procedures for the isolation of this apoprotein have been reported starting from delipidated preparations of plasma very low density lipoprotein (VLDL) mainly using column chromatography techniques: Sephadex G-200 in 4 M GdmCl (2), Sephadex G-200 in 6 M urea followed by DEAE anion exchange chromatography in 6 M urea (3), DEAE anion exchange chromatography (1), Bio-Gel A5M in 6 M GdmCl (4), Sepharose 4B or 6B in 6 M GdmCl (5), Sepharose G-200 in 0.1% SDS (6), and Sephadex G-75 in 6 M urea followed by a heparin-Sepharose step (7). Preparative polyacrylamide gel electrophoresis in the presence of SDS (8, 9) and more recently the technique of chromatofocusing have been used to isolate apoE (10). All of these techniques, although capable of yielding a pure product, are lengthy and demanding in manpower. We wish here to report on a rapid and reproducible method for isolating apoE from human plasma VLDL using the technique of high performance liquid chromatography (HPLC), which in its various modes has already seen useful application in the separation of other apolipoproteins in our laboratory (11) and by other investigators (12–15).

MATERIALS AND METHODS

Preparation of sample

The VLDL fraction of d < 1.006 g/ml was isolated from the plasma of pooled human normal donors by preparative ultracentrifugation. In order to avoid potential apoprotein degradation during processing, the blood was collected into a solution containing EDTA, final concentration 1.5 g/liter, sodium azide 0.1 g/liter, chloramphenicol, 50 mg/liter, gentamycin sulphate, 0.1 g/liter, and aprotinin, 10,000 U/liter. The plasma, separated by centrifugation at 4°C (4,500 *g*, 10 min), was spun in a Ti 60 rotor (363,000 *g*, 18 hr) at 10°C. The top fraction was washed twice under the same ultracentrifugal conditions in 0.15 M NaCl, pH 7.0, 10^{-3} M DFP. Two ml (2–4 mg/ml) of the final preparation was delipidated with 50 ml of a 2:1 chloroform–methanol mixture at room temperature followed by five peroxide-free ethyl ether washes at 4°C according to Scanu and Edelstein (16), except that the chloroform–methanol extraction was carried out at room temperature and the ether washes at 4°C. The final preparation (apoVLDL) was dried under nitrogen and the powder was stored at −70°C, usually no longer than 2 days. Before use, 10 mg of apoVLDL was dissolved at room temperature in 1 ml of 0.01 M Tris HCL, 0.01 M dithiothreitol (DTT), 6 M GdmCl solution, pH 7.0, containing 1 mM phenylmethylsulfonylfluoride (PMSF). A clear solution was obtained within 2–3 hr. The sample was then filtered through a 0.2-μm polycarbonate filter, (Nucleopore, Pleasanton, CA). Based on protein deter-

Abbreviations: VLDL, very low density lipoproteins of d < 1.006 g/ml; apoVLDL, lipid-free VLDL apoprotein; apoB, apolipoprotein B; apoE, apolipoprotein E; apoC, apolipoprotein C; HPLC, high performance liquid chromatography; GdmCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; DFP, diisopropyl fluorophosphate; DTT, dithiothreitol; PMSF, phenylmethylsulfonylfluoride.

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minations before and after polycarbonate filtration, there was a mass loss of about 10%. The SDS-PAGE patterns were comparable (**Fig. 1**). No changes in the intensity of the stained bands corresponding to apoE and apoC were noted supporting that the loss was attributed to apoB. Two hundred-μl aliquots containing 2 mg of apoVLDL were used for each chromatographic injection. An optimum loading mass of 2 mg of apoVLDL was found to give the best resolution between the three main peaks.

Fractionation procedure

After several preliminary trials, the column system that proved successful was composed of a Bio-Gel TSK Guard column, 75 mm $\times$ 7.5 mm, (Bio-Rad, Richmond, CA) followed by a Bio-Gel TSK 50, 300 mm $\times$ 7.5 mm, column (Bio-Rad), a Bio-Sil TSK 400, 300 mm $\times$ 7.5 mm column (Bio-Rad), and a Spherogel TSK 3000, 300 mm $\times$ 7.5 mm column (Altex, Berkeley, CA), all connected in series. Bio-Sil TSK 400 and Spherogel TSK 3000 are composed of spherical porous silica gel. The Bio-Gel TSK 50 is a hydroxylated polyether-based material. We used this column system in order to cover the wide molecular weight range (250 K to 10 K) of the protein components of apoVLDL. The Bio-Gel TSK 50 column support can separate water-soluble polymers in the molecular weight range of apoB. Bio-Sil 400 and the TSK 3000 spherogel columns have exclusion limits of 7×10^6 and 5×10^5, respectively, and are suited for the fractionation of apoE and the C peptides. This column system was pre-equilibrated and eluted with a 4 M GdmCl solution in 0.01 M Tris, 0.01 M DTT, pH 7.0. The inclusion of DTT in the mobile phase was to promote the solubilization of apoB and the dissociation of the apoE which was disulfide-linked to apoA-II. The use of GdmCl as a denaturant was based on the results of the work on apoB by Smith, Dawson, and Tanford (17). The pH 7.0 was chosen both to avoid chemical degradation of the proteins and to increase the lifetime of the columns, since very low or very high pH ranges tend to degrade the silica-based supports. The HPLC system was protected from corrosion by thorough rinsing (overnight) with distilled water at the end of the working day at a flow rate of 0.5 ml/min. All parts that came in contact with GdmCl, i.e., solvent reservoir filters, pump, loop, and pump valve, were also separately flushed with water. When the columns were subjected to a large number of injections, e.g., 25–30, at 2 mg of protein per injection, an increase in pressure from 40 atm to 80 atm was observed due to obstruction of the frit of the guard column. This was remedied by removing the top of the column that holds the frit and rinsing it with 6 M nitric acid (5–10 min) then flushing with distilled water. This step prolonged the life of the column from 6 to 12 months. The instrument used was a Varian Model 5000 Liquid Chromatograph (Varian Co., Walnut Creek, CA). The flow rate was 0.5 ml/min at 23°C. The back pressure with the three column system was 40 atm. The effluents were continuously monitored at 280 nm in a Varian UV-50 variable wave length detector. Fractions, 0.25 ml/tube, were collected in a fraction collector (Frac-100 Pharmacia, Piscataway, NJ). Samples from each peak were pooled and aliquots were either desalted immediately on a Bio-Gel P-2 (Bio-Rad) column or stored at −70°C until use (for more details see Results). The relative area of each peak was determined with the aid of a Hewlett-Packard Integrator model 3390A (Hewlett Packard, Avondale, PA).

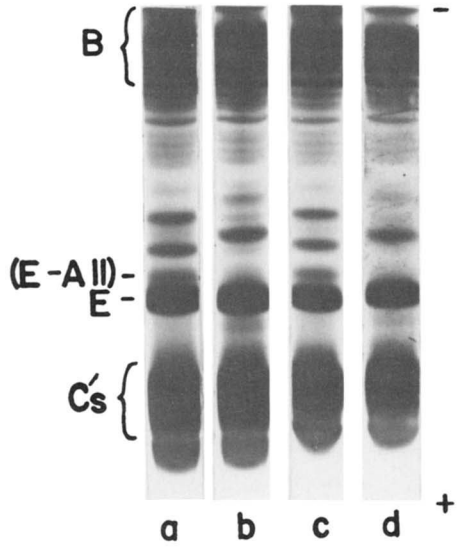

Fig. 1. SDS-PAGE patterns of apoVLDL in the absence (a, c) and presence (b, d) of β-mercaptoethanol and before (a, b) and after (c, d) polycarbonate filtrations a, b (240 μg applied), and c, d, (230 μg applied), respectively. The acrylamide concentration was 3.5% and the gels were stained with Coomassie blue. Identification of the bands was based on apolipoprotein standards.

Gel electrophoresis

Purity of samples and molecular weight determinations were carried out on 10% polyacrylamide gels in the presence of 0.1% SDS (18). Isoelectric focusing in 6% acrylamide was performed in tube gels as previously described (18) in the presence of 6 M urea. The pH gradient of each gel was between 4 and 6 and was measured using a Bio-Rad pH Profiler (Bio-Rad, Ca). Two-dimensional gel electrophoresis was performed according to O'Farrel (19) with the modifications as described by Lester et al. (20). The first dimension was run in a pH gradient between 4 and 6 and the second dimension in an acrylamide gradient between 10 and 15% and a urea gradient between 0 and 4 M.

Cysteamine modification of apoE

In order to better define the isoforms of apoE, charge modification with cysteamine was conducted according to Weisgraber, Rall, and Mahley (2). Briefly, 50–60 μg of purified apoE was dissolved in 0.1 M NH_4HCO_3, and 0.3 mg of cysteamine (Sigma, St. Louis, MO) was added. The mixture was incubated for 4 hr at 37°C and then lyophilized. Before analysis, the lyophilized sample was dissolved in 0.01 M NH_4HCO_3.

Other analyses

Protein content was determined by a modification of the Lowry procedure (21). For amino acid analyses, proteins were hydrolyzed in glass-distilled 6 N HCl at 110°C for 24 hr and analyzed in a Durrum Model 502 Amino Acid analyzer (Dionex Co. Palo Alto, CA).

Reagents

All the general chemicals were of reagent grade purity. The protein markers, thyroglobulin, bovine serum albumin, and ovalbumin were obtained from Sigma Chemical Co., St. Louis, MO. GdmCl (ultrapure grade) was obtained from Heico, Inc., Delaware Water Gap, PA. All buffers for HPLC use were filtered through a 0.4-μm millipore filter. Apolipoproteins A-I and A-II were prepared as previously described. (11)

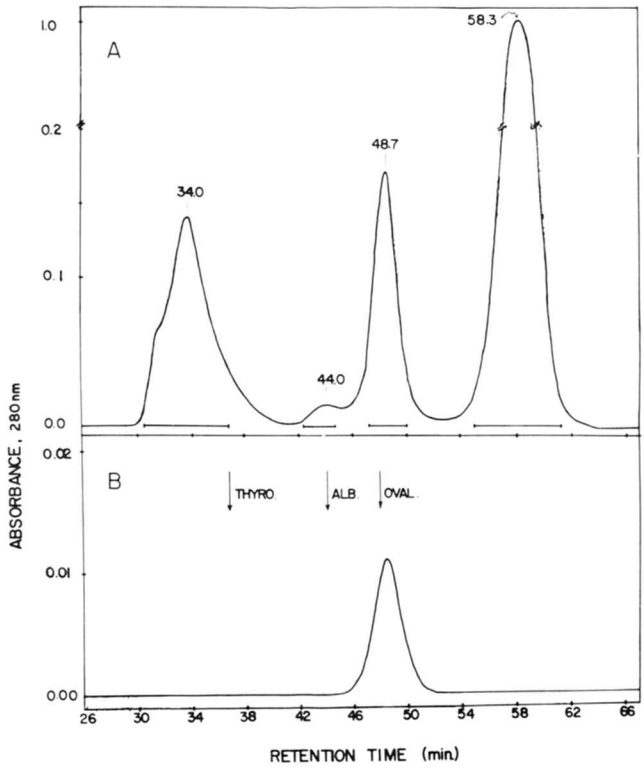

Fig. 2. A, HPLC elution profile of apoVLDL (2 mg, injection volume, 200 μl). Eluting buffer 4 M GdmCl solution in 0.01 M Tris, 0.01 M DTT, pH 7.0, flow rate, 0.5 ml/min. The effluent was continuously monitored at 280 nm. The number of each peak maximum indicates the retention times. Temperature of fractionation, 23°C. Chart speed 1 cm/min. The horizontal bars indicate the fractions pooled for analysis. B, Rechromatography of the 48.7-min fraction in Fig. 2A. Identical column conditions were used except that 40 μg of protein was injected. The arrows represent the elution position of standards (100 μg each) run separately under identical column conditions.

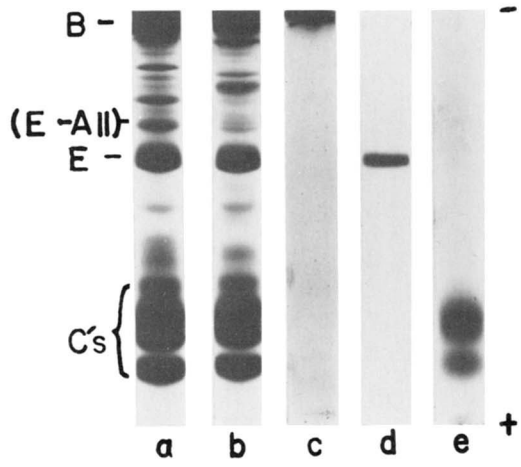

Fig. 3. SDS-PAGE patterns of apoVLDL and HPLC fractions named according to their retention times in Fig. 1A; (a) apoVLDL, 250 μg; (b) apoVLDL + β-mercaptoethanol, 250 μg; (c) 34-min fraction, 40 μg; (d) 48.7-min fraction, 15 μg; (e) 58.3-min fraction, 75 μg. Identification of the bands was based on the mobility of apolipoprotein standards.

RESULTS

A typical HPLC profile of a pooled apoVLDL sample is shown in **Fig. 2A.** Four components eluting at 34, 44, 48.7, and 58.3 min (peak maxima) were observed. Based on absorbance recordings, the percent area relationship among these peaks was 18, 1, 10, and 71, respectively. The appearance of the shoulder at 44 min was dependent upon the length of incubation of the apoVLDL in the GdmCl solubilizing buffer. A 3-hr incubation time was sufficient to eliminate this shoulder, suggesting that the shoulder at 44 min corresponds to the apoE–A-II disulfide linked complex. The aliquots corresponding to each peak area were pooled and either immediately used for analyses or stored at −70°C. Before use, the samples were desalted either by dialysis or in 0.5 cm × 30 cm columns packed with Bio-Gel P-2 (Bio-Rad) followed by lyophilization. Based on mass determinations of applied sample and of the total pool of the eluted fractions, the column recovery for apoVLDL was 93%. From the mass determinations and electrophoretic SDS gels of the samples after polycarbonate filtration (see Methods) and recoveries after HPLC fractionation, the total protein losses ranged between 15 and 20% and affected primarily apoB. By SDS-PAGE, **Fig. 3,** using known human apolipoproteins as stan-

dards, the 34-, 48.7-, and 58.3-min components corresponded to apoB, apoE, and apoC, respectively. This identification was supported by molecular weight estimates based on a calibration curve (**Fig. 4**). Based on these preliminary identifications, we elected to focus our attention on verifying the purity of apoE and comparing its properties to those in the literature. When the 48.7-min component, which we shall refer to as apoE, was re-run under the same column conditions as in Fig. 2A, a single symmetrical peak was observed with an identical retention time (Fig. 2B). A recovery of 80% was calculated based on the total absorbancy value of the applied and of the eluted sample. Its amino acid composition was compatible with that reported for human apoE by Rall, Weisgraber, and Mahley (22). By unidimensional isoelectric focusing, two major and at least two minor bands were observed. The apparent pI corresponding to the two major bands was 5.35 and 5.25, respectively (**Fig. 5A**). After cysteamine treatment (Fig. 5B), both major bands exhibited a shift in charge compatible with the existence of E_3/E_s isoforms according to the recently proposed nomenclature (23). By two-dimensional isoelectric focusing, the pattern obtained (**Fig. 6**) corresponded to that reported for human apoE.

DISCUSSION

We have developed a rapid (less than 1 hour) and reproducible method for a single-step isolation of apoE

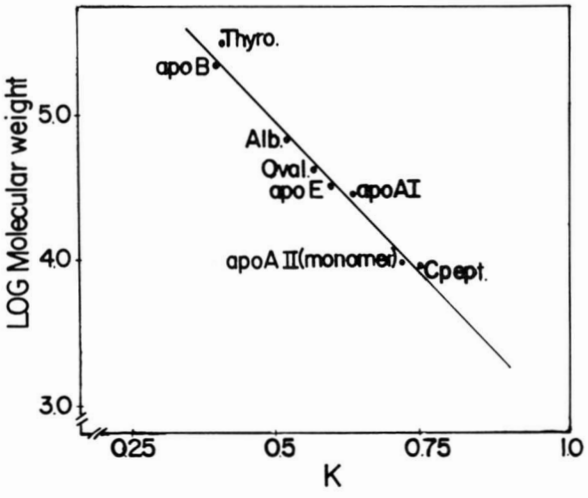

Fig. 4. Plot of the log of the molecular weight of standard proteins as a function of their retention times. The parameter K is defined as $\frac{T_{sample}}{T_{DNP\text{-}serine}}$, where T_{sample} is the retention time for a sample protein and $T_{DNP\text{-}serine}$ is the retention time (76 min) of dinitrophenyl serine which, because of its low molecular weight, is a good indicator of the column volume. The solid line is the best fit to the data obtained by linear regression analysis. The parameter K for apoB, E, and C was calculated and plotted on the line from which an apparent molecular weight was calculated.

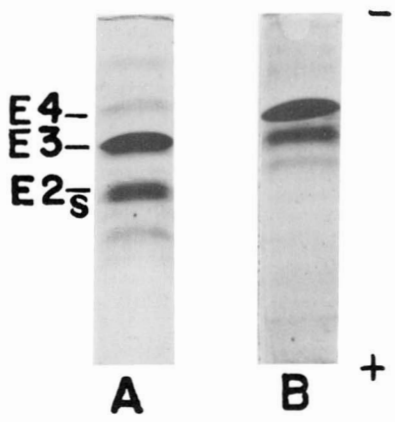

Fig. 5. One-dimensional isoelectric focusing patterns of A, 42 μg of apoE (HPLC 48.7-min fraction); B, same, but after treatment with cysteamine. The pH gradient was 4–6. The top of the gel is basic and the bottom is acid.

in good recoveries starting from delipidated apoVLDL from normolipidemic human donors. We find that the apoE so obtained has the same amino acid composition and electrophoretic properties as products obtained previously using, however, comparatively longer procedures (2, 6, 9). Our HPLC profile of apoVLDL resembles that observed by Sparks and Marsh (24) using SDS-gel filtration chromatography requiring at least 24 hr. A definite advantage of our technique is the rapidity whereby sufficient amounts of apoE can be prepared for physico-chemical and immunological studies. Among the positive points are the sensitivity of the method, which permits the processing of limited amounts of apoproteins, and its excellent recoveries. Moreover, pure samples of apoB and apoC can be obtained. Overall, we believe that our technical development should

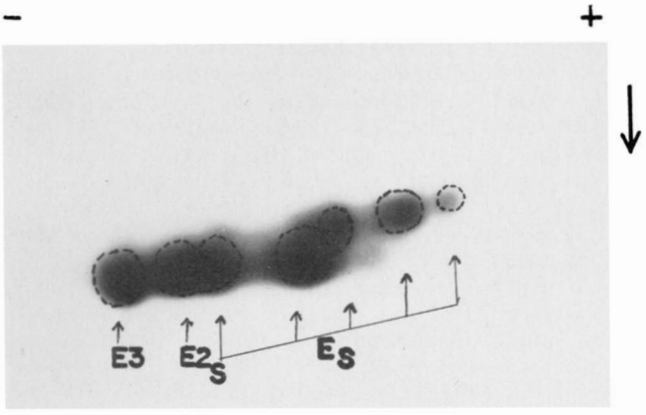

Fig. 6. Two-dimensional (isoelectric focusing, SDS electrophoresis) isodalt pattern of apoE isolated by HPLC, 60 μg. The pH gradient for the first dimension was 4–6. Only the portion corresponding to the stainable material is shown. The left is the basic and the right is the acid side. The arrow shows the direction of migration in the second dimension, from high to low molecular weight. A tentative identification for the E isoforms is sketched below the photograph. The nomenclature is according to reference 22.

facilitate the study of the properties of apoE and of the VLDL apoproteins in general. The anticipated availability of new HPLC columns now in the phase of development, invites the prediction that both resolution and separation time for the various apoproteins will be considerably improved.

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