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ANION-EXCHANGE FAST PROTEIN LIQUID CHROMATOGRAPHIC CHARACTERIZATION AND PURIFICATION OF APOLIPOPROTEINS A-I, A-II, C-I, C-II, C-III$_0$, C-III$_1$, C-III$_2$ AND E FROM HUMAN PLASMA

HAFID MEZDOUR, VÉRONIQUE CLAVEY, IBRAHIM KORA, MONIQUE KOFFIGAN, AHMED BARKIA and JEAN-CHARLES FRUCHART*

Serlia, Institut Pasteur, 1, Rue du Professeur Calmette, 59019 Lille Cédex (France) and INSERM U279, Faculté de Pharmacie, 3, Rue du Professeur Laguesse, 59045 Lille Cédex (France)*

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SUMMARY

This paper describes a procedure for the rapid isolation of urea-soluble apolipoproteins (apo) from delipidated human very-low- and high-density lipoproteins using anion-exchange fast protein liquid chromatography. The separation was complete within 30 min and peaks corresponding to apolipoproteins A-I, A-II, C-I, C-II, C-III$_0$, C-III$_1$, C-III$_2$ and E were identified by comparing their chromatographic, electrophoretic and immunological behaviour with that of purified standards of each protein. A second purification step is necessary to obtain pure apolipoproteins. Apo E, which is difficult to purify by conventional chromatography, has been obtained in a good yield. The apo C-II that was obtained produced a symmetrical peak on chromatography but three bands in isoelectric focusing. The method can be upgraded to a preparative scale and offers the possibility of direct purification of apolipoproteins both from high-density lipoproteins and (following preliminary gel chromatography) from very-low-density lipoproteins.

INTRODUCTION

The elucidation of lipoprotein metabolism, structure and function requires routine isolation of pure apolipoproteins (apo) and analytical determination of the apolipoproteins of both high-density lipoproteins (HDL) and very-low-density lipoproteins (VLDL). This purification is generally performed using successive chromatographic techniques [1], including gel chromatography (Sephadex or Sephacryl S-200), ion-exchange chromatography (DEAE–Trisacryl or DEAE–cellulose) and, more recently, chromatofocusing [2–4] and isoelectric focusing [5–7]. All these techniques, although able to yield a pure product, are time-consuming. Recently, some authors have reported the separation of apo E

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[8] and of apo HDL [9, 10] by high-performance liquid chromatography (HPLC) on gel chromatography columns. In addition, C apolipoproteins have been purified on a reversed-phase system [11,12], apo HDL and apo VLDL have been prepared by anion-exchange HPLC [13].

With gel chromatography or the reversed-phase system it is not possible to purify all the apolipoproteins on the same column and it is often difficult to obtain large yields. We describe here the analytical determination and preparation of apolipoproteins from VLDL and HDL with a fast protein liquid chromatography (FPLC) system (Pharmacia) using Mono Q HR 5/5 semi-preparative and Mono Q HR 10/10 preparative anion-exchange columns.

EXPERIMENTAL

Preparation of VLDL and HDL apolipoproteins

VLDL were isolated from hypertriglyceridaemic plasma by ultracentrifugation at density 1.006 g/ml for 25 h at 100 000 *g* in a Beckman L8.70 ultracentrifuge with a 50.2Ti rotor (4°C). HDL were isolated by sequential ultracentrifugation in the density range 1.12–1.21 g/ml under the same ultracentrifugation conditions, and were then dialysed against a solution of 0.15 *M* sodium chloride and 0.1 g/l EDTA (pH 7.4). Both lipoproteins fractions were lyophilized and delipidated with ethanol–diethyl ether [14]. The VLDL proteins were solubilized in 0.01 *M* Tris–HCl–6 *M* urea–0.01 *M* dithiothreitol (pH 8.2). Apo HDL were solubilized in the same buffer, but without dithiothreitol. Samples were either filtered through a 0.22-μm Millipore membrane filter or spun at 100 000 *g* for 30 min to remove particulate material prior to dialysis against the same buffer using PM10 Amicon membranes for apo HDL and YM2 Amicon membranes for apo VLDL (there were some losses of apo C on PM10 membranes).

Apo VLDL gel chromatography

Up to 60–80 mg of soluble apo VLDL fraction were chromatographed on a 100×2.6 cm I.D. column of Sephacryl S-200 (Pharmacia) gel, equilibrated with 0.01 *M* Tris–HCl–6 *M* urea buffer (pH 8.2). Four peaks were obtained. The second peak contained apo E, the third the C apolipoproteins and the fourth was rich in apo C-I.

Ion-exchange chromatography of the C apolipoproteins and apo HDL

Up to 60–80 mg of apo C (peak III from Sephacryl S-200 gel filtration) were applied to a 30×2.5 cm I.D. DEAE–Trisacryl (IBF) column. Apo C-I, C-II, C-III_0, C-III_1 and C-III_2 were eluted successively using a 2×1 l linear gradient of 0.028 *M* to 0.1 *M* Tris–HCl in 6 *M* urea (pH 8.2). 100 mg of apo HDL were chromatographed on the same column to obtain apo A-I and A-II. In this instance a 2×1 l linear gradient of 0 to 0.06 *M* sodium chloride in 0.01 *M* Tris–HCl–6 *M* urea (pH 8.2) was used.

Anion-exchange FPLC

An FPLC system (Pharmacia) was used to purify apo VLDL and apo HDL. The system consisted of two P500 high-pressure pumps, an LCC-500 solvent con-

troller to programme the gradients, an MV-7 injection valve and an MV-8 distributor valve. The system was connected to a single-path UV monitor, a fraction collector (FRAC-100) and a chart recorder with two channels (UV absorbance and programmed gradient).

All buffers were prepared with freshly deionized urea solution, filtered through a 0.22-μm Millipore membrane filter and degassed before use. Elution was achieved using a linear gradient of 0 to 0.15 *M* sodium chloride in 0.01 *M* Tris–HCl–6 *M* urea (pH 8.2). After each run the column was washed for 2 min with 1 *M* sodium chloride in starting buffer. Then 0.05–5 mg of proteins were applied to a Mono Q HR 5/5 (5×0.5 cm I.D.) column and separated at a flow-rate of 0.5–1 ml/min. When larger amounts of protein (5–100 mg) were fractionated we used a Mono Q HR 10/10 (10×1 cm I.D.) column at a flow-rate of 1.5–2 ml/min. Semi-preparative chromatography was carried out using a Mono Q HR 5/5 column set to automatic repetitive model. Buffers, samples and elution fractions were stored at 4–10°C to minimize carbamylations, but FPLC separation was performed at room temperature. Urea was removed from the FPLC peak fractions by PD-10 column chromatography (G-25 coarse Pharmacia gel filtration columns) equilibrated with $5 \cdot 10^{-3}$ *M* ammonium hydrogen carbonate. The isolated proteins were then concentrated or freeze-dried.

Protein characterization

Protein quantification was performed by the procedure of Lowry et al. [15], with serum albumin used as standard. Ouchterlony double immunodiffusion [16] was performed in 1% agarose using monospecific rabbit antibodies against A-I, A-II, B, C-III, C-II, C-I, D and E apolipoproteins.

Isoelectric focusing was conducted at 4°C for 1 h in 7% acrylamide–6 *M* urea containing 3% ampholine with a pH range of 4–6, as previously described [6]. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) was performed in discontinuous gel: 3% stacking gel and 10% polyacrylamide separating gel (pH 8.8) [17], or in gradient gels of 10–30% polyacrylamide. Electrophoresis in alkaline urea gels (urea PAGE) was performed as described by Kane [18].

Amino acid analysis with a Biotronic analyser was performed on 0.5 mg of purified apolipoprotein after 72 h of total acid hydrolysis.

Enzyme immunoassay (ELISA) was used to quantify apolipoproteins in purified fractions and performed as previously described [19, 20].

Immunoblotting

The isoforms of apo E were separated from purified apo E by isoelectric focusing on polyacrylamide gel slabs containing ampholines with pH range 4–6. Immunoblotting was performed as described by Kittler [21] with an anti-E antibody peroxidase conjugate prepared as previously described [20].

RESULTS

Fast anion-exchange chromatography of HDL apolipoproteins A-I and A-II

The semi-preparative Mono Q HR 5/5 anion-exchange column was first used with the same gradients that were employed in the conventional DEAE–Trisacryl

column chromatography. The profiles of apo A-I and apo A-II from apo HDL prepurified on DEAE–Trisacryl column are shown in Fig. 1A. Elution was achieved in 30 min. The position of apo A-I and A-II are the same as when apo HDL are injected, and if apo A-II and apo A-I are added to the sample, they coelute with peaks 1 and 2, respectively (Fig. 1B). After this analysis, traces of apo A-II and apo D are found in apo A-I (Fig. 1C). Traces of apo C are also found in apo A-II.

Resolution in the region between the A-II and A-I peaks can be enhanced by modifying the gradient slope; for example, the resolution was improved further by inserting two pauses just before the apo A-I and A-II elution and prolonging the total elution time to 50 min.

Fast anion-exchange chromatography of VLDL apolipoproteins C and E

The elution positions of apo C-I, C-II, C-III_1 and C-III_2 were identified by injection of prepurified apolipoproteins. Apo VLDL were first chromatographed on Sephacryl S-200 gels. The second peak was "apo E rich" and the third peak contains apoliproteins C. These C proteins were separated by DEAE–Trisacryl ion-exchange chromatography. Fig. 2A shows the elution pattern of each purified C protein and Fig. 2B indicates the profile obtained when total apo C from gel filtration were injected. The elution position is the same for each C protein. Peak III was identified as apo C-III_0. Occasionally, smaller unidentified peaks were present between C-I and C-II. The apolipoproteins were also characterized by alkaline urea PAGE (Fig. 2C), isoelectric focusing and double immunodiffusion. In order to obtain pure peptides, it was necessary to rechromatograph each fraction.

Purification of apolipoprotein E is more complex because of the low apo E protein content of apo VLDL. The "apo E rich" peak of gel chromatography (peak II) was rechromatographed on a Mono Q column, and apo E was eluted in two peaks near the elution position of C-III_2 (Fig. 3B). When a sample of apo VLDL was injected with the same gradient (Fig. 3A), the apo E was included in the leading shoulder of the apo C-III_2 peak. We checked for the presence of albumin, apo C, apo A-I, apo A-II, apo B and D in the purified apo E peak. There was no contamination on the basis of double immunodiffusion and less than 0.5% as evaluated by ELISA. The major peak of apo E was subjected to isoelectric focusing in 6 *M* urea and gave bands that all reacted with an immunoserum anti-apo E on immunoblotting. The second peak probably corresponds to aggregation of apo E, since only one peak was observed when $5 \cdot 10^{-3}$ *M* was added to the sample and to elution buffer. A small amount of apo E (2–3% of the total E content of the sample as evaluated by ELISA) eluted when the column was given a final wash with 1 *M* sodium chloride.

Semi-preparative chromatography of apo A-I

Apo A-I (30 mg) was purified on a DEAE–Trisacryl column as outlined above. This apo A-I was contaminated by apo A-II and apo C-III. We rechromatographed the fraction on the Mono Q HR 5/5 column. The optimum injection was 3 mg of prepurified apo A-I in 0.5 ml of starting buffer. Peaks were collected. Repetitive injections were programmed and the gradient used is shown in Fig. 1. Pure apo A-I (26 mg) was obtained in ten successive cycles (86.5%). The puri-

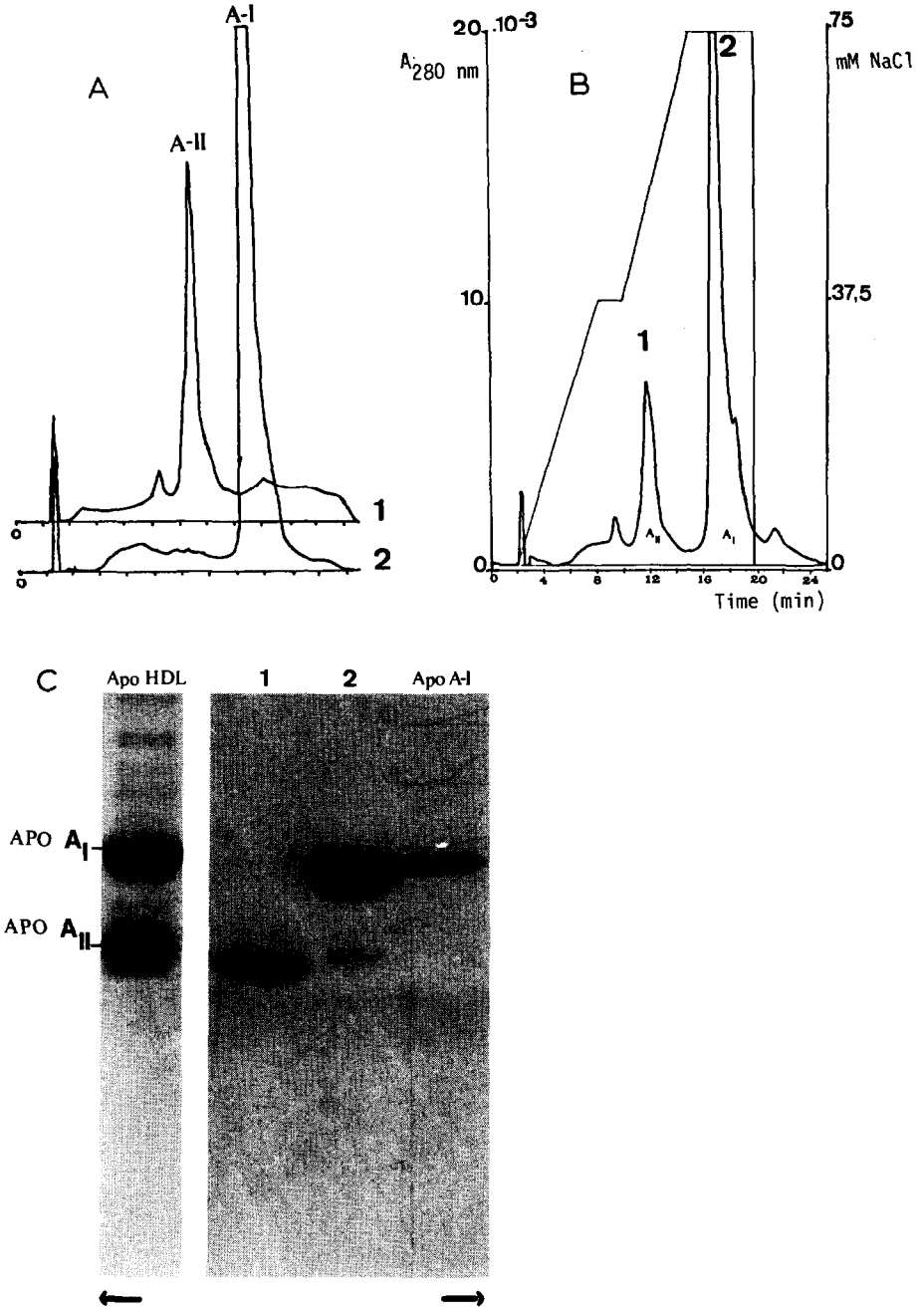
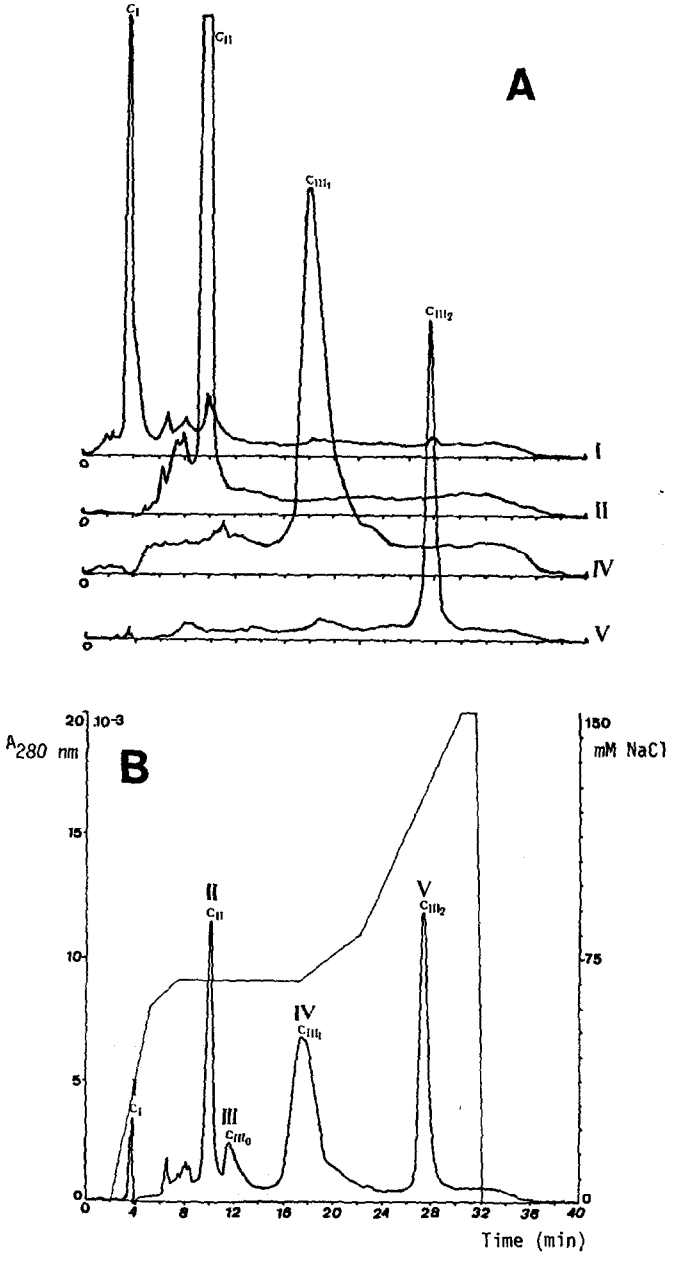


Fig. 1. Elution profiles of apo A-I and A-II from delipidated HDL on Mono Q HR 5/5. Flow-rate, 0.5 ml/min; starting buffer, 0.01 *M* Tris-HCl-6 *M* urea (pH 7.5). (A) Successive injections of DEAE-Trisacryl prepurified apo A-I (0.1 mg) and apo A-II (0.1 mg). (B) Injection of 0.2 mg of apo HDL. (C) SDS-PAGE identification of peak 1 and peak 2 (10% acrylamide gel). The theoretical gradient slope is shown in (B).

fied protein did not react with antibodies against apo A-II, C-II, C-III, C-I, E and D, and ran as a single band on SDS–PAGE. The amino acid composition of this fraction is very similar to that reported in the literature [22].

The same purity of apo A-I can be obtained from apo HDL after two successive FPLC assays on Mono Q HR 5/5. The apo A-I peaks and apo A-II from repetitive



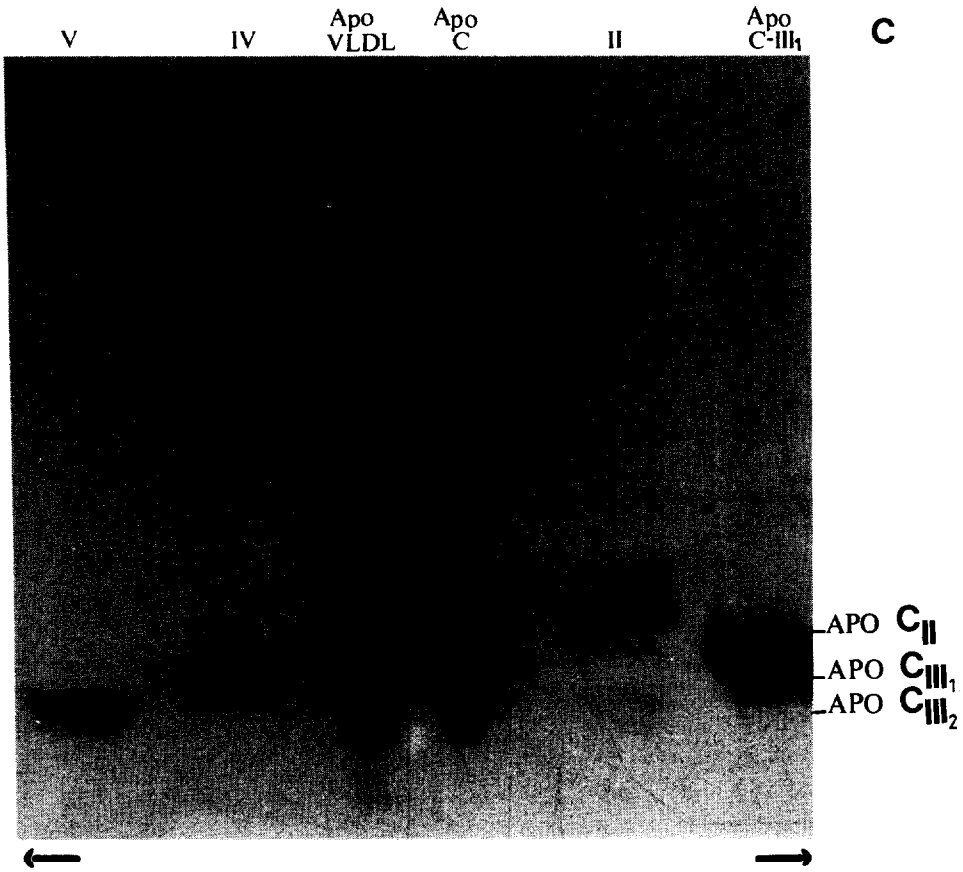


Fig. 2. Elution profiles of apo C from delipidated VLDL on Mono Q HR 5/5. Flow-rate, 1 ml/min; starting buffer, 0.01 *M* Tris–HCl–6 *M* urea (pH 8.2). (A) Successive injections of 0.1 mg of apo C-I, 0.25 mg of apo C-II, 0.25 mg of apo C-III_1 and 0.1 mg of apo C-III_2 prepurified on Sephacryl S-200 gel chromatography then DEAE–Trisacryl. (B) Injection of 0.25 mg of the third peak of Sephacryl S-200 gel filtration of apo VLDL. (C) Alkaline urea PAGE of apo VLDL, apo C from Sephacryl S-200 gel filtration and of peaks II, IV and V from FPLC chromatography shown in B.

analyses of apo HDL purification were respectively pooled and dialysed against the initial buffer prior to a final repurification on the column, with the same gradient.

Semi-preparative purification of apo C-I, C-II, C-III_0, C-III_1 and C-III_2

A 17-mg amount of the apo C peak (free of apo B, E and A-I) from Sephacryl S-200 gel chromatography of apo VLDL was injected into the FPLC column in cycles of 1.7 mg in 1 ml of starting buffer, as previously described for apo A-I. The gradient used was the same as in Fig. 2B. The total yield after ten analyses was 1 mg of apo C-II, 0.3 mg of apo C-III_0, 5 mg of apo C-III_1 and 2.5 mg of apo C-III_2, as estimated by the Lowry technique. High purity was obtained for apo C-III_1 and C-III_2, both giving one band in urea PAGE and on isoelectric focusing and one precipitin line against the anti-apo C-III immunserum. The amino acid compo-

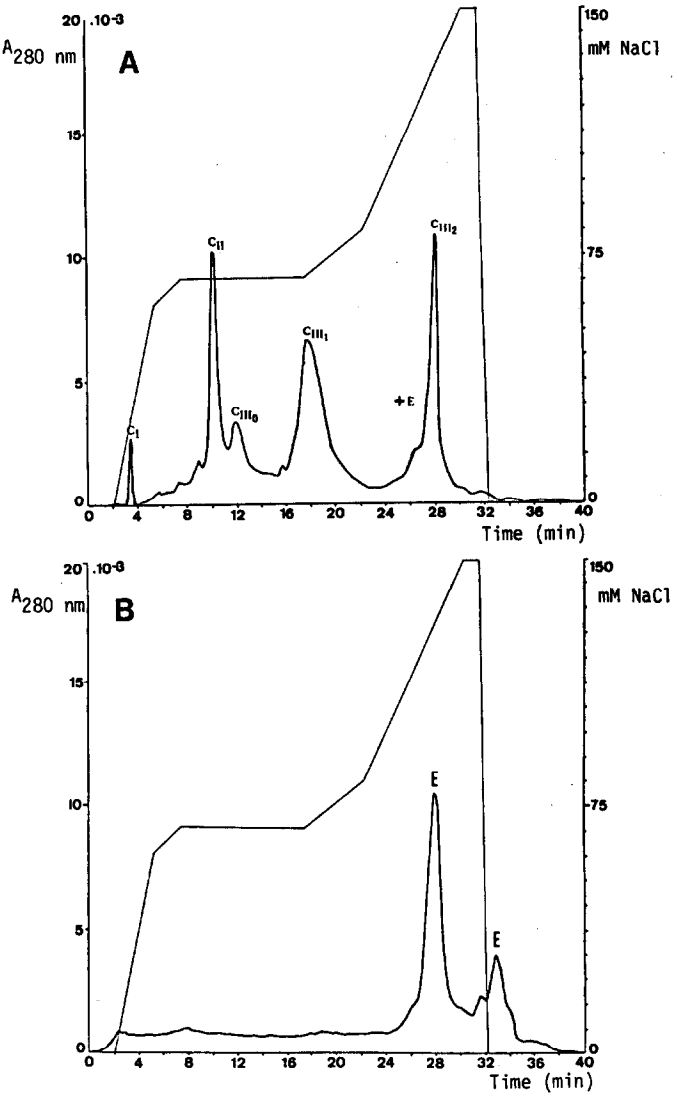


Fig. 3. Elution profile of apo E from delipidated VLDL on Mono Q HR 5/5. Flow-rate, 1 ml/min; starting buffer, 0.01 *M* Tris-HCl–6 *M* urea (pH 8.2). (A) Injection of 0.2 mg of apo VLDL. (B) Injection of 0.25 mg of the second peak of Sephacryl S-200 gel chromatography of apo VLDL. Apo E is found in the two peaks.

sition of the proteins is very similar to that reported in the literature [23].

Apo C-II was contaminated by apo $C\text{-}III_0$ and $C\text{-}III_1$, as assessed in isoelectric focusing and double immunodiffusion. Accordingly, 9.6 mg of prepurified apo C-II was chromatographed with a modified gradient. The amino acid composition of the fraction obtained (6.15 mg in six successive analyses) was the same as that of an analogous fraction repurified by chromatofocusing [2] or reported by Jackson et al. [24]. On isoelectric focusing the apo C-II contained a trace of apo $C\text{-}III_0$ and a band that focused as apo $C\text{-}III_2$.

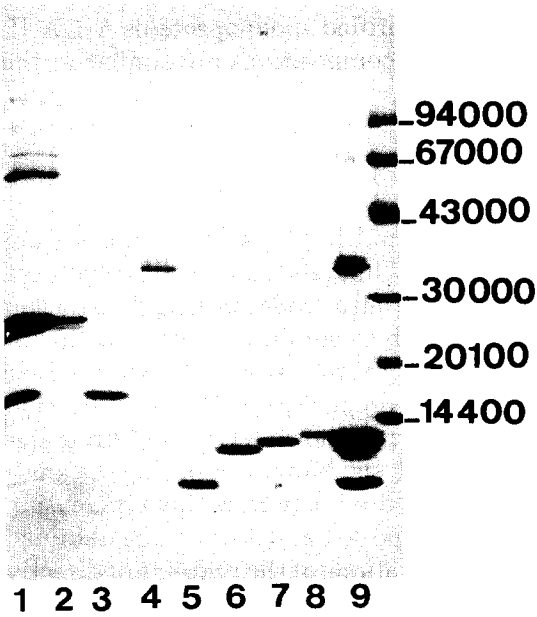


Fig. 4. A 10-30% gradient SDS-PAGE of apo HDL (1), apo A-I (2), apo A-II (3), apo E (4), apo C-I (5), apo C-II (6), apo C-III_1 (7), apo C-III_2 (8) and apo VLDL (9). The pure apolipoproteins were obtained from two successive analyses on Mono Q HR 10/10.

The amino acid composition of apo C-III_0 purified under the same conditions is not different from the amino acid composition of apo C-III_1 and C-III_2.

Apo C-I purification was performed from the fourth peak of Sephacryl S-200 gel chromatography: 3 mg of protein were purified on Mono Q HR 5/5 in two runs and 2.5 mg of pure apo C-I was obtained. The amino acid composition is similar to that reported in the literature [25]. No trace of either apo C-II or C-III was found by immunological or electrophoretic methods.

Apo E purification

The "apo E rich peak" from Sephacryl S 200 gel filtration containing 15 mg of proteins (apo E, albumin and traces of apo B and apo A-I) was fractionated by successive injection of 1.5 mg of apolipoproteins and gave the gradient profile shown in Fig. 3. From the pooled peak 1 we obtained 4.2 mg of pure apo E, which is about 80% of the apo E injected as estimated by ELISA. Double immuodiffusion and ELISA were used to assess the purity of the fraction. The amino acid composition of the purified apo E was similar to published values [26] and the same as that of an analogous fraction repurified by chromatofocusing.

Mono Q HR 10/10 purification of apo HDL and apo VLDL

A Mono Q HR 10/10 column with higher loading capacity was tested with 50 mg of apo HDL, 25 mg of total apo C or 10 mg of prepurified gel filtered apo E. The flow-rate was 2.0 ml/min and the gradient was modified by doubling the elution time. Two successive analyses are necessary to obtain pure apolipopro-

teins. Fig. 4 shows the gradient SDS–PAGE of purified apolipoproteins A-I, A-II, E, C-I, C-II, C-III_1 and C-III_2. The amino acid compositions are similar to the published values [22–27].

DISCUSSION

This study examines the use of FLPC for the rapid, high-resolution preparation of apolipoproteins. The capacity of the gel used is limited to 2.5 mg but repetitive automatic injections allowed us to purify 30 mg from a crude starting preparation of apo A-I in 5 h. The system is sensitive enough to permit the identification of apo A-I, A-II, C-I, C-II, C-III_0, C-III_1 and C-III_2 from 50–100 μg of apo HDL or apo VLDL. For apo E isolation it is necessary to prepurify apo E by gel filtration on Sephacryl S-200 or FPLC Superose-12 (data not shown). Gradient programming is easy and can be modified during the analysis. Moreover, during the analysis it is possible to retard the gradient to obtain pure fractions without increasing the elution time substantially. Highly purified apo A-I and A-II can be obtained easily and in good yield, either from crude preparations of the proteins or directly from apo HDL in two successive analyses. Apo C and E from VLDL must be separated on gel chromatography columns before the preparative step. The purity of apo E, C-III_1 and C-III_2 is good following one chromatographic cycle.

The amino acid composition of FPLC-purified apo C-II does not differ very much from the expected value but the protein contains three bands in isoelectric focusing, which may be traces of apo C-III_0 or isoforms apo C-II_1 and apo C-II_0, as described by Sprecher et al. [28].

Multiple peaks are obtained for apo A-I (Fig. 1B) and apo E (Fig. 3B). Further analyses will show whether these represent apo E and A-I variants identified by this system. The use of a Mono Q HR 10/10 column with higher loading capacity (200 mg of proteins) has been tested with apo HDL, 25 mg of total apo C or 10 mg of prepurified, gel chromatographed apo E. Separation can be achieved in 60–90 min and the resolution is the same as that of the analytical HR 5/5 column. This method should be a useful technique for the preparation of large amounts of plasma apolipoproteins.

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