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Short Communication

Application of reversed-phase high-performance liquid chromatography to the separation of apolipoproteins A-IV, A-I and E from rat high-density lipoprotein

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ABSTRACT

Apolipoproteins A-IV, A-I and E from rat high-density lipoprotein (HDL) were successfully purified by reversed-phase high-performance liquid chromatography (RP-HPLC), using a method which we have previously developed for the separation of apolipoproteins A-IV, A-I and E from human lymph chylomicrons [T. Tetaz, E. Kecorius, B. Grego and N. Fidge, *J. Chromatogr.*, 511 (1990) 147]. Since analytical-scale RP-HPLC indicated that the C apolipoproteins from rat HDL coeluted with both apo A-IV and apo A-I, delipidated rat HDL was first subjected to preparative-scale size-exclusion HPLC (HPSEC) on a Serva Si300 column, which effectively separated the C apolipoproteins from all but apolipoprotein E. Fractions from HPSEC which were enriched for apolipoproteins A-IV, A-I or E were directly applied to RP-HPLC on a TSK Phenyl-5PW column. This procedure yielded fractions containing apolipoproteins A-IV, A-I or E which were pure as assessed by N-terminal sequencing and silver staining of sodium dodecyl sulphate–polyacrylamide gels.

INTRODUCTION

In order to further investigate the metabolism of high-density lipoprotein (HDL) in rats, we required specific immunoassays for the quantitation of the medium-molecular-weight apolipoprotein constituents of rat HDL: apo A-IV, apo A-I and apo E. Production of monospecific antisera for these assays is often hindered by the presence of low concentrations of contaminating apolipoproteins which may be more antigenic than the principal immunogen, or by sodium dodecyl sulphate (SDS) which may change the epitopes usually recognised in the native peptide. A method which isolates highly purified apolipoproteins without exposure to SDS would have significant advantages over other methods for antibody production.

We have recently described a novel high-performance liquid chromatographic (HPLC) method by which apolipoproteins A-IV, A-I and E were purified from human lymph chylomicrons by reversed-phase (RP) HPLC, following preliminary size-

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exclusion chromatography (SEC) to remove the C apolipoproteins [1]. RP-HPLC was performed using a conventional hydrophobic interaction chromatography column (TSK Phenyl-5PW) in the unconventional reversed-phase mode; this procedure yielded a separation of apolipoproteins A-IV, A-I and E which was not possible using conventional C_8 or C_{18} reversed-phase columns. The following paper details the application of this same procedure to the purification of apolipoproteins A-IV, A-I and E from rat HDL.

EXPERIMENTAL

Materials

Serva Si300 Polyol and TSK Phenyl-5PW columns were purchased from Imbros (Australia) and Toyo Soda (Japan), respectively. High-purity (HPLC-grade) urea, water and acetonitrile were purchased from Mallinckrodt, while orthophosphoric acid (H_3PO_4) was obtained from Merck Chemicals.

Preparation and delipidation of HDL

Ten non-fasted, male Sprague-Dawley rats (350–400 g), anaesthetised with a mixture of ketamine (80 mg/kg) and rompun (12 mg/kg), were bled via the abdominal aorta and the blood collected into tubes containing Na_2EDTA (1 mg/ml) on ice. The plasma (40 ml) was separated by centrifugation at 4°C. HDL was separated according to the method of Havel *et al.* [2]. Using a 50 Ti rotor in a Beckman L5-65B ultracentrifuge, very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) of density $d < 1.055$ g/ml were separated by ultracentrifugation at 150 000 *g* for 24 h. HDL of d 1.055–1.21 g/ml was separated by ultracentrifugation at 150 000 *g* for 26 h. The HDL fraction was dialysed against 2 l of saline at a density of 1.006 g/ml (including three changes) over a 36-h period and then delipidated with ethanol–diethyl ether (3:1, v/v).

High-performance SEC (HPSEC)

Delipidated HDL (20 mg protein) was dissolved in 1 ml of denaturant buffer (0.1 *M* Tris (pH 7)–0.15 *M* NaCl–6 *M* urea) and applied to a Serva Si300 Polyol column (500 mm × 22 mm I.D., separation range 6000–500 000 dalton) which had been previously equilibrated with the same buffer. The column was run at a flow-rate of 1 ml/min at ambient temperature and 2-ml fractions were collected by a Cygnet fraction collector (Iscom). The HPLC system consisted of two Model 114M solvent delivery modules and a Model 421A controller (Beckman), and was equipped with a Model 210A injection valve (Altex) fitted with a 2-ml injection loop. Protein elution was monitored at 280 nm using a variable-wavelength detector (Shimadzu SPD-2A).

Reversed-phase HPLC

Reversed-phase separations were performed on a Hewlett-Packard HP1090 HPLC equipped with a Rheodyne sample injector (2-ml sample 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 60% acetonitrile. Samples were applied to a TSK Phenyl-5PW column (75 mm × 7.5 mm I.D.) at a flow-rate of 1 ml/min at 45°C. After the non-retained material had passed through the diode-array detector, the column was re-equilibrated

with solvent A and the proteins were eluted by a linear (50 min) gradient from solvent A to solvent B. Protein elution was monitored simultaneously at 215, 254 and 280 nm.

Analytical methods

Samples for the SDS–polyacrylamide gel electrophoresis (PAGE) were run on 8–25% polyacrylamide gels and stained with Coomassie blue followed by silver staining, using a Phast gel system (Pharmacia). Amino acid analysis and N-terminal sequencing of purified apolipoproteins were performed as previously described [1].

RESULTS AND DISCUSSION

Delipidated rat HDL (200 μg protein) was dissolved in 20 m*M* H_3PO_4 (1 ml) and applied to a TSK Phenyl-5PW column which had previously been equilibrated with the same buffer. Proteins were eluted using a gradient of acetonitrile from 0 to 60% over a 50-min period. Fractions were collected at 1 min intervals and subjected to SDS–PAGE, followed by Coomassie blue and silver staining. Although Coomassie blue staining indicated that all the major rat HDL apolipoproteins were separated effectively, silver staining revealed that a small proportion of the C apolipoproteins co-eluted with both apo A-IV and apo A-I, and a small proportion of apo A-I co-eluted with apo A-IV (Fig. 1). A similar observation was made with RP-HPLC separation of apolipoproteins from human lymph chylomicrons [1].

In order to separate the C apolipoproteins (< 10 000 dalton) from apo A-IV (42 000 dalton) and apo A-I (28 000 dalton), the mixture was first subjected to preparative-scale HPSEC (Fig. 2). Delipidated rat HDL (20 mg protein) was dissolved in 1 ml denaturant buffer (0.1 *M* Tris · HCl (pH 7.0)–0.15 *M* NaCl–6 *M* urea) and applied to a Serva Si300 column as described in the Experimental section. Fractions were collected at 2 min intervals and their apolipoprotein content was assessed by SDS-PAGE followed by Coomassie blue or silver staining. Apo A-IV and the bulk of apo A-I eluted earlier than the C apolipoproteins. Apo A-I eluted earlier than apo E

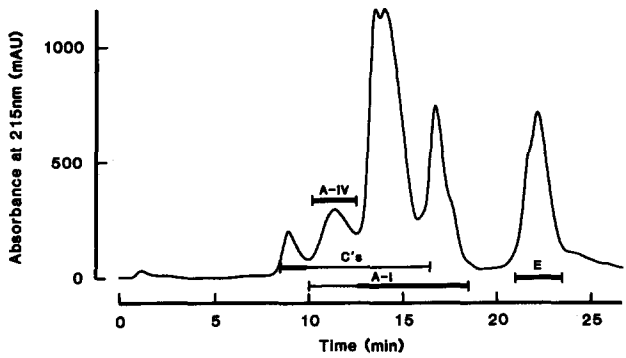

Fig. 1. RP-HPLC of delipidated rat HDL (200 μg protein) separation on a TSK Phenyl-5PW column. Gradient conditions were as described in the text. Fractions were analysed by SDS-PAGE and their apolipoprotein content as assessed by Coomassie blue staining (bold bars) and by silver staining (normal bars) are as indicated.

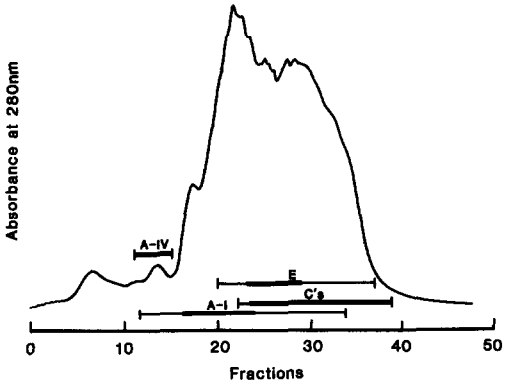

Fig. 2. HPSEC of delipidated rat HDL (20 mg protein) separation on a Serva Si300 Polyol column. Running conditions were as described in the text. Fractions were analysed by SDS-PAGE and their apolipoprotein content as assessed by Coomassie blue staining (bold bars) and by silver staining (normal bars) are as indicated.

(34 000 dalton) despite its lower molecular weight, presumably due to its tendency to aggregate even in the presence of urea [3].

Fractions from HPSEC, enriched for various apolipoproteins, were then applied directly to RP-HPLC on a TSK Phenyl-5PW column, as described in the Experimental section. Fractions which were enriched for apo A-IV (Fig. 2, fractions 11–15), and which contained only trace amounts of apo A-I as detected by silver staining of SDS-polyacrylamide gels, yielded pure apo A-IV after a single chromatographic step on the TSK Phenyl-5 PW column. The bulk of the apo A-IV was obtained pure in the non-retained fraction; this was in contrast to RP-HPLC of delipidated rat HDL where apo a-IV was retained (Fig. 1). We have also observed that denaturation of human apo A-IV with 6 *M* urea causes the bulk (>99%) of apo A-IV to be non-retained on a TSK Phenyl-5PW column [4]. However, urea-denatured apo A-IV (human or rat) is still retained on a standard C_8 column (RP300, Activon, 30 mm × 4.6 mm I.D.) under the same chromatographic conditions [4]. Fractions from HPSEC which were enriched for apo A-I (Fig. 2, fractions 17–21), but which also contained apo E, yielded pure apo A-I and apo E after one run on the TSK Phenyl-5PW column (Fig. 3A). Pure apo E was also obtained when HPSEC fractions enriched for apo E (Fig. 2, fractions 21–29), but which also contained C apolipoproteins plus some apo A-I, were applied to the TSK Phenyl-5PW column (Fig. 3B).

Apolipoproteins A-IV, A-I and E, obtained from single chromatographic runs on the TSK Phenyl-5PW column as described above, were assessed for purity by SDS-PAGE followed by Coomassie blue and silver staining (Fig. 4), and their identities were further confirmed by N-terminal sequencing of the first ten amino acid residues. Purified apo A-I and apo E both appear as doublets on SDS-PAGE (Fig. 4, lanes 3 and 4). The appearance of apo A-I as a doublet on SDS-PAGE presumably results from the removal of a signal peptide from a proportion of apo A-I precursor molecules, since sequencing revealed that the purified apo A-I was a mixture of pro A-I and mature A-I [5]. Only a single N-terminal sequence was obtained for pure apo E; the two bands seen on SDS-PAGE are possibly different glycosylated forms of apo E [6].

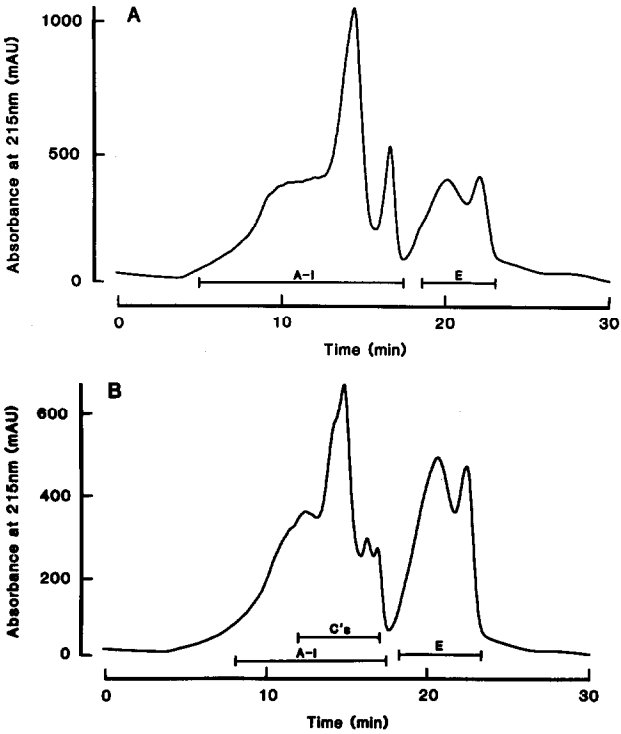

Fig. 3. RP-HPLC of fractions from HPSEC (Fig. 2), which were enriched for (A) apo A-I (fraction 21, Fig. 2) and (B) apo E (fraction 29, Fig. 2). Gradient conditions were as described in the text. Fractions were analysed by SDS-PAGE and their apolipoprotein content as assessed by silver staining are as indicated (bars).

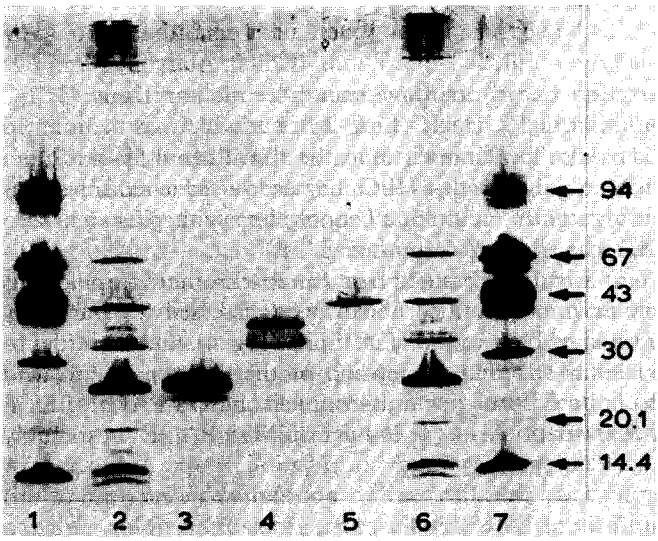

Fig. 4. Analysis of crude and purified apolipoproteins by SDS-PAGE followed by Coomassie blue and silver staining. Lanes 1 and 7 contain low-molecular-weight standards (mol. wt. $\times 10^{-3}$). Lanes 2 and 6 contain delipidated rat HDL. Lanes 3, 4 and 5 contain apo A-I, apo E and apo A-IV, respectively, after purification by HPSEC followed by RP-HPLC.

In conclusion, we have demonstrated that a method which we had originally developed for the purification of apolipoproteins A-IV, A-I and E from human lymph chylomicrons can be successfully applied to the purification of these apolipoproteins from rat HDL.

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