Journal of Chromatography, 345 (1985) 145—149
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2763

Note

Characterization of human apolipoprotein A-I by reversed-phase high-performance liquid chromatography

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(First received October 18th, 1984; revised manuscript received July 10th, 1985)

Human apolipoprotein A-I (apo A-I), the major protein constituent of high-density lipoproteins (HDLs), contains 243 amino acids and is secreted primarily by the liver and small intestine [1—3]. In analytical isoelectric focusing (IEF), mature serum apo A-I can be separated into several isoforms, the main ones being designated apo $A\text{-}I_0$, apo $A\text{-}I_{-1}$ and apo $A\text{-}I_{-2}$ [4, 5]. These isoforms have identical amino acid compositions, peptide maps and antigenicity, and the bands are not caused by variable amounts of phosphate or carbohydrates or by interaction with the ampholyte [6—8].

To obtain further information on the nature of these isoforms, we characterized apo A-I by reversed-phase high-performance liquid chromatography (HPLC). This technique has been successfully applied for the separation of microheterogeneous proteins and peptides [9—12]. The development of reversed-phase supports with large pore size (> 300 Å), resulting in high resolution and recovery of high-molecular-weight solutes such as polypeptides and proteins [13, 14], allowed the application of reversed-phase HPLC for the separation of apo A-I (molecular weight: 28 000).

EXPERIMENTAL

Blood was obtained from normolipidaemic volunteers after an overnight fast. From serum, HDLs were isolated after precipitation of other lipoproteins [15] by ultracentrifugation at $d = 1.21$ g/ml in a Beckman 50 Ti rotor at 4°C for 24 h [16]. After lyophilization, HDLs were delipidated with ethanol—diethyl ether (3:1), and dried under a gentle stream of nitrogen. From these

0378-4347/85/$03.30

apolipoproteins, apo A-I was isolated by gel permeation chromatography, as described previously [17], to homogeneity in polyacrylamide gel electrophoresis in urea [18] and sodium dodecyl sulphate [19].

HPLC experiments were performed with two Altex solvent delivery units 110 A controlled by an Altex programmer 420 and connected to a Uvicon LC 725 variable-wavelength UV detector (Kontron, Eching, F.R.G.). Two reversed-phase columns with large pore size were used for the separations: an Aquapore RP 300 column (25 × 0.42 cm I.D.) from Kontron and a PNP I (polyamide) column (15 × 0.4 cm I.D.) from Hamilton (Darmstadt, F.R.G.).

Separations were performed at ambient temperature at a flow-rate of 1 ml/min; detection at 225 nm; buffer A, 0.1 mol/l sodium phosphate (pH 2.1); buffer B (eluent), acetonitrile. A linear gradient from 30 to 45% buffer B was applied in 60 min. In some experiments, 0.01 mol/l ammonium acetate (pH 8.6) was used as buffer A, and resolution with a gradient from 45 to 60% buffer B in 120 min. Apo A-I was dissolved either in 0.1 mol/l sodium phosphate (pH 2.1), 0.01 mol/l ammonium bicarbonate (pH 8.6) or at neutral pH in 6 mol/l urea.

The collected peaks were desalted either by gel permeation chromatography or dialysis against double-distilled water, and identified by amino acid analysis [8] and IEF (pH range 4—6) in the presence of urea [20]. The ability to activate lecithin—cholesterol acyltransferase (LCAT) was assayed as described previously [21].

RESULTS AND DISCUSSION

The HPLC experiments with silica and polyamide support, respectively, resulted in identical chromatograms. With the acidic buffer system, apo A-I was eluted in one main peak. Identical results were obtained, whether apo A-I was pretreated under acidic (pH 2.1) or alkaline conditions (pH 8.6), or at a neutral pH in the presence of 6 mol/l urea (Fig. 1A—C). The yield of the main

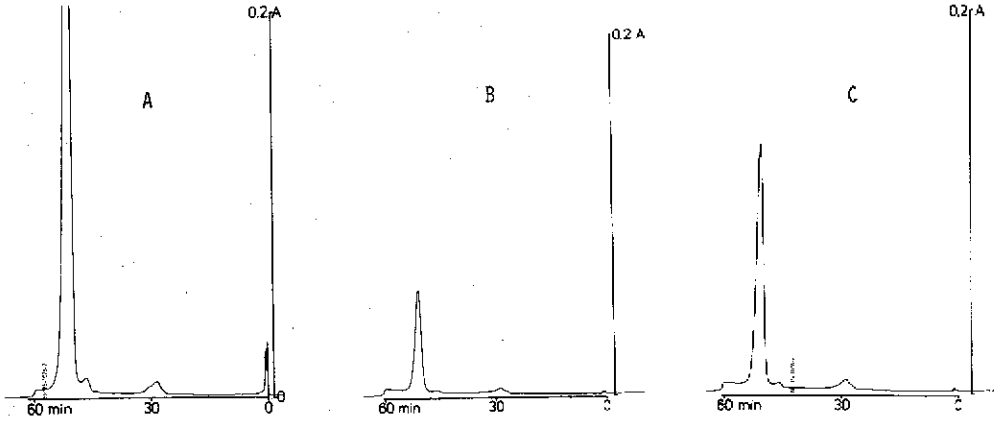

Fig. 1. Separation of apo A-I by reversed-phase HPLC in acidic conditions. Aquapore RP 300 column; conditions as described in the text. A = absorbance. (A) Dissolution in 0.1 mol/l sodium phosphate (pH 2.1); (B) dissolution in 0.01 mol/l ammonium bicarbonate (pH 8.6); (C) dissolution in 6 mol/l urea at neutral pH.

peak from the column was estimated by plotting the peak areas at different concentrations: 1 μg per 0.21 cm^3, 2.8 μg per 0.55 cm^3, 8.4 μg per 1.67 cm^3 and 50 μg per 10.72 cm^3. These data show only a minimal loss of apo A-I during the HPLC procedure. The identity of this peak was proved by the amino acid composition, which was identical with apo A-I, and by IEF, showing all isoforms (Fig. 2a). The ability to stimulate the enzyme LCAT remained unchanged: 729 pmol/h ml prior to HPLC and 747 pmol/h ml after HPLC. The two small peaks eluting with retention times of 28 and 46.5 min, respectively, were also identified as apo A-I due to their characteristics in the amino acid composition, in IEF and in the ability to stimulate the LCAT activity (669 and 651 pmol/h ml, respectively). Until now, further structure-elucidating work was not possible because of the shortage of this peak material.

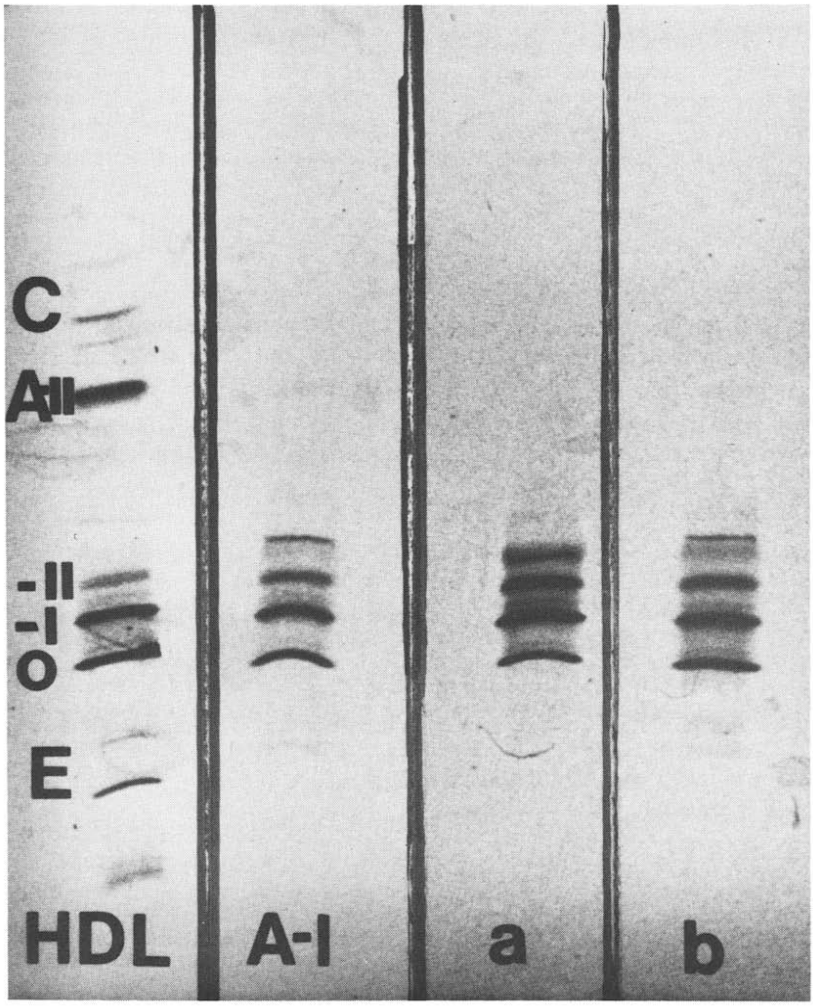

Fig. 2. Isoelectric focusing in urea, pH 4.0—6.0. From left to right: HDL apolipoproteins; apo A-I after gel permeation; main peak after HPLC at pH 2.1 (a); main peak after HPLC at pH 8.6 (b).

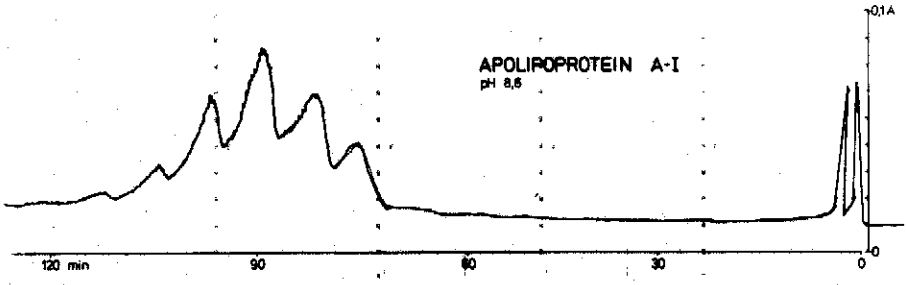

Fig. 3. Separation of apo A-I by reversed-phase HPLC in alkaline conditions. Aquapore RP 300 column; conditions as described in the text. Dissolution in 0.01 mol/l ammonium acetate (pH 8.6).

When the conditions for the separation of apo A-I were changed from pH 2.1 to 8.6, a subfragmentation of the apo A-I material was obtained (Fig. 3). The amino acid compositions of the different peaks were identical and characteristic for apo A-I. In IEF, all isoforms of apo A-I were present (Fig. 2b = main peak). The rechromatography of these peaks under acidic conditions showed one peak with the retention time of the main apo A-I peak. These data suggest that experimental conditions, especially treatment in alkaline conditions during HPLC, may be responsible for the fragmentation, although the reason for the heterogeneity has not yet been identified.

In conclusion, reversed-phase HPLC was not able to separate the isoforms of mature apo A-I [5]. On the other hand, HPLC at low pH is suitable for the preparation of highly purified apo A-I with high yield, which is also supported by the fact that the ability to stimulate LCAT activity is unaffected by the HPLC procedure. Furthermore, this procedure allows the quantitation of apo A-I in the microgram range, which is of advantage for the standardization of apo A-I in immunochemical assays [22].

ACKNOWLEDGEMENTS

The technical assistance of Miss Christine Friedl and Beate Schalk is greatly acknowledged. This work was supported by Deutsche Forschungsgemeinschaft (We 955/1-3).

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