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Complementary use of counter-current chromatography and hydroxyapatite chromatography for the separation of three main classes of lipoproteins from human serum

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Abstract

High-density, low-density and very-low-density lipoproteins (HDLs, LDLs and VLDLs) were purified from human serum by the combined use of counter-current chromatography (CCC) and hydroxyapatite chromatography. Polymer-phase CCC of human serum using the cross-axis coil planet centrifuge yielded two lipoprotein fractions, one containing HDLs and LDLs and the other VLDLs and serum proteins. Each fraction was concentrated and subjected to hydroxyapatite chromatography to obtain three lipoprotein fractions, all free from serum proteins. Each lipoprotein was confirmed by agarose gel electrophoresis.

1. Introduction

Three main classes of human lipoproteins are known as high-density, low-density and very-low-density lipoproteins (HDLs, LDLs and VLDLs). Chromatographic separations of these three main classes of lipoproteins have been reported using several types of column packings, such as Bio-Gel A [1,2], Superose 6B (6HR) [3], TSK Gel [4–6] and hydroxyapatite [7]. However, all of these separation methods required a prior procedure such as ultracentrifugation for the preparation of the sample loaded on the column.

Recently, semi-preparative fractionation of LDLs and VLDLs was performed using a Bio-Gel HTP DNA-grade hydroxyapatite column by eluting with potassium phosphate buffers at pH 7.4 in four stepwise elutions [8]. In this chromatographic system, HDL fractions were always contaminated with serum proteins such as albumin and globulins because HDLs eluted from the hydroxyapatite column at the buffer concentration (75 m*M*) used for eluting serum proteins.

Counter-current chromatography (CCC) is a generic name for various liquid partition chromatographic methods which are used without solid support matrices [9–11]. Among all of the existing CCC instruments, the cross-axis coil planet centrifuge (X-axis CPC) is considered most advantageous for protein separations with aqueous–aqueous polymer phase systems [12].

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The X-axis CPC has been successfully used for the separation and purification of stable proteins [13], histones and serum proteins [14], recombinant uridine phosphorylase from *Escherichia coli* lysate [15] and human serum lipoproteins [16,17]; all employ aqueous polymer-phase systems.

Our previous studies have shown that a mixture of HDL and LDL fractions prepared by ultracentrifugation could be separated by the X-axis CPC [16]. The separation was performed with a polymer-phase system composed of 16% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate by eluting the lower phase at a flow-rate of 0.5 ml/min. HDLs and LDLs were resolved within 12 h. We also demonstrated the separation of an HDL–LDL fraction from a VLDL–serum protein mixture directly from human serum with the above polymer-phase system at pH 9.4 using the X-axis CPC [17]. However, using this CCC system alone, the purification of all lipoprotein fractions from each other was not realized.

This paper describes the separation of all three lipoprotein fractions by the combined use of polymer-phase CCC and hydroxyapatite chromatography.

2. Experimental

2.1. Apparatus

The detailed design of the X-axis CPC was described earlier [18,19]. The apparatus holds a pair of horizontal rotary shafts, symmetrically mounted one on each side of the rotary frame, at a distance of 10 cm from the centrifuge axis. A spool-shaped column holder is mounted on each rotary shaft at an off-centre position 10 cm from its mid-point. The large multi-layer coil separation column was prepared from 2.6 mm I.D. PTFE tubing (Zeus Industrial Products, Raritan, NJ, USA) by winding it on a 15.2 cm diameter holder hub, forming four layers of left-handed coils between a pair of flanges spaced 5 cm apart. A pair of columns mounted on the rotary frame were connected in series with a flow tube (PTFE, 0.85 mm I.D.) to give a total capacity of 340 ml. A small-capacity column consisting of a single layer of coil with 30-ml capacity was also prepared. Both inflow and outflow tubes leave together at the centre of the top plate of the centrifuge case where they are tightly supported with a pair of clamps equipped with silicone-rubber pads. The speed of the apparatus is regulated at 500 rpm with a speed control unit (Bodine Electric, Chicago, IL, USA).

2.2. Reagents

Polyethylene glycol (PEG) 1000 (average molecular mass, $M_r = 950–1050$) and potassium phosphate were obtained from Kanto Chemicals (Tokyo, Japan). Bio-Gel HTP DNA-grade hydroxyapatite (crystal size 10–170 μm, hexagonal prisms) was purchased from Bio-Rad Labs. (Richmond, CA, USA). Universal Gel/8 agarose gel for electrophoresis of lipoproteins was purchased from Ciba Corning Diagnostics (Palo Alto, CA, USA). Other chemicals were all of analytical reagent grade.

2.3. Preparation of human serum from peripheral blood

Human peripheral blood (ca. 10 ml) was collected from normolipidaemic males by venipuncture after 12–16 h of fasting. The blood was allowed to stand at room temperature until agglutination was completed. The serum was collected after centrifugation at 1000 *g* at 15°C for 15 min.

2.4. Preparation of PEG–potassium phosphate aqueous two-phase solvent system

A two-phase solvent system composed of 16% (w/w) PEG 1000 and 12.5% (w/w) anhydrous dibasic potassium phosphate solution was prepared by dissolving 320 g of PEG and 250 g of K_2HPO_4 in 1430 g of distilled water. The solution was thoroughly mixed in a separating funnel and allowed to settle into the clear layer before use.

2.5 Counter-current chromatography of human serum

In each experiment, the CCC column was first entirely filled with the PEG 1000-rich upper stationary phase and the sample solution (a mixture of 4 ml of human serum and 2 ml of each phase to which appropriate amounts of PEG 1000 and dibasic potassium phosphate were added to adjust the two-phase composition) was injected through the sample port. Then the potassium phosphate-rich lower mobile phase was eluted through the column at the desired flow-rate while the apparatus was rotated at 500 rpm. The effluent from the outlet of the column was continuously monitored with an ISCO UA-5 absorbance monitor (Instrumentation Specialities, Lincoln, NE, USA) at 280 nm and fractionated into a Bio-Rad Model 2110 fraction collector. After the desired peak had been collected, the column was eluted with the PEG 1000-rich upper phase (initially used as the stationary phase) in the opposite direction without stopping the centrifugation. This reversed mode of elution was continued until all the retained analytes were eluted from the column.

2.6. Analysis of CCC fractions

An aliquot of each fraction was diluted with three times its volume of distilled water and the absorbance was measured at 280 nm with a Shimadzu (Kyoto, Japan) UV-1200 spectrophotometer.

2.7. Hydroxyapatite chromatography of CCC fractions

Bio-Gel HTP DNA-grade hydroxyapatite was suspended in 75 m*M* potassium phosphate buffer (pH 7.4) and, after swelling, slurry-packed in the column (5.0 × 2.5 cm I.D.). Each of the two CCC fractions (ca. 60–100 ml) was separately concentrated and dialysed with an ultrafiltration membrane (Diaflo YM-10) (molecular mass cut-off = 10 000) (Amicon, Beverly, MA, USA), and the contents were concentrated to 1.0–1.5 ml. Each concentrate was loaded on the hydroxyapatite column, which was then eluted stepwise with two different concentrations of potassium phosphate (pH 7.4) at a flow-rate of 1.0 ml/min. The fractions were collected and concentrated with an ultrafiltration membrane for the characterization of the lipoproteins and serum proteins by electrophoresis.

2.8. Characterization of human lipoproteins and serum proteins by electrophoresis

Lipoproteins in both the counter-current and hydroxyapatite chromatographic fractions were characterized using agarose gel electrophoresis with Oil Red 7B staining [20]. The eluate (ca. 60–100 ml), consisting of a potassium phosphate-rich lower phase (normal elution mode) or PEG-rich upper phase (reversed elution mode), was concentrated and dialysed with distilled water until the contents were concentrated to 1 ml. A 1-μl aliquot of the concentrate was loaded on the 1% agarose gel. The gel was immersed in the running buffer, composed of 43 m*M* sodium barbital and 7 m*M* barbital, then the run was performed at 90 V for about 40 min.

Serium proteins and apoproteins in both the CCC and hydroxyapatite chromatographic fractions were also characterized by sodium dodecylsulfate polyacrylamide slab gel electrophoresis (SDS-PAGE) according to the method of Laemmli [21]. Gels containing 3% (w/v) (stacking gel) and 10% (w/v) (separation gel) of acrylamide were prepared from stock solutions of 30% (w/v) acrylamide and 0.8% (w/v) N,N'-methylenebisacrylamide. A 10 × 5.5 cm separation gel and a 10 × 1.0 cm stacking gel, each 0.75 mm thick, were prepared between glass plates. A 5-μl volume of concentrated eluate was mixed with 95 μl of sample solution [a mixture of 0.025 *M* tris(hydroxymethyl)aminomethane, 2% (w/v) SDS, 5% (w/v) 2-mercaptoethanol, 4% (w/v) glycerol and 0.01% (w/v) bromophenol blue (BPB)] and 10–20 μl of the sample solution were loaded on the stacking gel. Electrophoresis proceeded at 10 mA until the BPB marker reached the separation gel. Thereafter, the current was increased to 20 mA and the electrophoresis continued until the BPB marker

reached the bottom of the separation gel. The migrated proteins were stained for 5 min at room temperature with a staining solution composed of 0.25% (w/v) Coomassie Brilliant Blue, 50% (v/v) methanol and 10% (v/v) acetic acid. The gel was destained by washing in a mixture of 7.5% (v/v) acetic acid and 2.5% (v/v) methanol.

3. Results and discussion

3.1. Stationary phase retention

CCC is a liquid–liquid partition chromatographic method utilizing no solid matrix in the column space where the liquid stationary phase is retained in the column by the aid of a centrifugal force field [9–11]. Therefore, good retention of the stationary phase in the column is critical to the success of the method and this, in turn, is greatly affected by the flow-rate of the mobile phase. The percentage retention of the polymer-phase system composed of 16% (w/w) PEG 1000–12.5% (w/w) potassium phosphate buffer (pH 9.4) in two coiled columns was examined at three different flow-rates (Table 1). In the high-capacity column, the retention of the stationary phase was over 40% even at a flow-rate of 2.0 ml/min. However, in the lower capacity column, consisting of a single layer of coil, the same retention was attained only by using a flow-rate of 0.5 ml/min.

3.2. CCC of human serum

The two lipoprotein fractions (HDL–LDL and VLDL–serum proteins) from human serum were obtained in earlier work [17] with a polymer-phase system using the X-axis CPC equipped with a large-capacity column (340 ml). In these studies, we employed a small-capacity column (60 ml) in the same apparatus to shorten the separation time. Fig. 1 shows a chromatogram of human serum (4 ml) obtained with the X-axis CPC using 16% (w/w) PEG 1000–12.5% (w/w) dibasic potassium phosphate (pH 9.4). The separation was performed at 500 rpm and at a flow-rate of 0.5 ml/min using the lower phase as the mobile phase where both HDLs and LDLs were eluted together near the solvent front (SF) while other proteins were retained in the column much longer. After collecting the HDL–LDL fraction (CCC-fr. 1), VLDL was eluted together with serum proteins (CCC-fr. 2) by pumping the upper phase in the reverse direction (marked UP in Fig. 1). The separation was completed within 4.5 h. The lipoprotein(s) in each peak was confirmed by agarose gel electrophoresis with Oil Red 7B stained and the serum proteins were also detected by SDS-PAGE with Coomasie Brilliant Blue protein staining (Fig. 2). The first peak (CCC-fr. 1) contained HDLs and LDLs but no serum proteins of M_r ca. 60 000 (Fig. 2B) and the second peak (CCC-fr. 2) contained VLDLs and serum proteins.

Table 1
Comparison of column dimensions and the stationary upper phase retention with different flow-rates

Column	Length (m)	I.D. (mm)	Layers	Flow-rate[a] (ml/min)	Retention[b] (%)
Large (340 ml)	64	2.6	4	0.5	51.3
				1.0	41.8
				2.0	40.1
Small (60 ml)	11	2.6	1	0.5	41.1
				1.0	29.4
				2.0	–

Solvent system: 16% PEG 1000–12.5% dibasic potassium phosphate (pH 9.4).
[a] Lower phase mobile.
[b] PEG 1000-rich upper stationary phase retention.

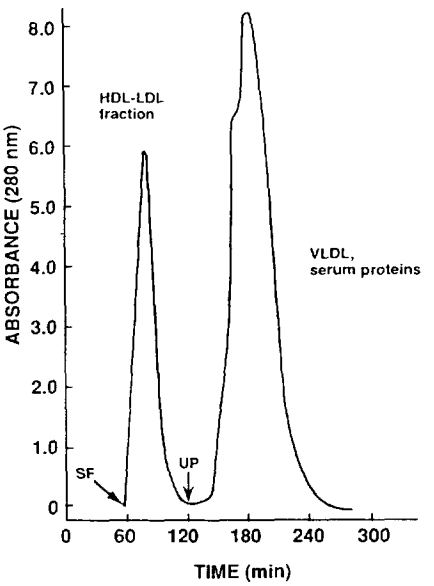

Fig. 1. Counter-current chromatographic separation of HDL–LDL fractions from human serum with aqueous polymer-phase system. Column 2.6 mm I.D. PTFE single-layer coil (×2) with 60-ml capacity; sample, 4 ml of human serum in which 0.9 g of PEG 1000 and 0.7 g of dibasic potassium phosphate were dissolved; solvent system, 16% (w/w) PEG 1000–12.5% (w/w) K_2HPO_4 at pH 9.4; mobile phase, lower phase; flow-rate, 0.5 ml/min; rotation speed, 500 rpm. SF = solvent front; UP = starting point of the reversed elution mode with the upper mobile phase.

3.3. Hydroxyapatite chromatography of CCC fractions

In previous studies, we separated LDLs and VLDLs from human serum by hydroxyapatite chromatography with stepwise elution using potassium phosphate buffers of two different concentrations at pH 7.4 [8]. Four-step elution with 75, 200, 300 and 650 m*M* potassium phosphate resulted in the elution of HDLs–serum proteins, other serum proteins, LDLs and VLDLs, respectively. In this hydroxyapatite chromatographic system, the HDL fraction is always contaminated by serum proteins because these two protein groups elute together with 75 m*M* potassium phosphate (pH 7.4).

In the present study, CCC fractions 1 (HDL–LDL) and 2 (VLDL–serum proteins) were each separately dialysed against distilled water until the concentration of the potassium buffer was decreased to that in the starting buffer used for the hydroxyapatite chromatography. This process required a long time for the CCC-fr. 2, which contained a high concentration of PEG. The concentrates of both fractions were chromatographed separately on the hydroxyapatite column.

Fig. 3 shows the elution profile on hydroxyapatite obtained from CCC-fr. 1. A 1.4-ml volume of the concentrate was loaded on the Bio-Gel HTP DNA-grade column (5.0 × 2.5 cm I.D.) and eluted at 1.0 ml/min with 75 and 290 m*M* potassium phosphate buffers (KPi) at pH 7.4. Two lipoprotein peaks were eluted; the first peak (HA-fr. 1) contained HDLs and the second peak (HA-fr. 2) LDLs.

The concentrate (1.5 ml) of CCC-fr. 2, which contained VLDLs and serum proteins, was similarly chromatographed (Fig. 4). The separation was performed with two-step elution with 290 and 650 m*M* potassium phosphate buffer (KPi) at pH 7.4. Most of the serum proteins, including albumin and globulins, were eluted with 290 m*M* potassium phosphate buffer (HA-fr. 3) at pH 7.4. The VLDLs, on the other hand, were retained in the column for a much longer time and were eluted with 650 m*M* potassium phosphate buffer (HA-fr. 4).

Lipoproteins in the hydroxyapatite chromatographic fractions (HA-frs. 1–4) were confirmed by agarose gel electrophoresis with Oil Red 7B staining (Fig. 2A). The results of agarose gel electrophoresis indicated that HDLs, LDLs and VLDLs were present in HA-fr. 1, HA-fr. 2 and HA-fr. 4, respectively. The serum proteins were detected by SDS-PAGE with Coomassie Brilliant Blue staining, which demonstrates that CCC-fr. 1, HA-fr. 2 and HA-fr. 4 are free from serum proteins except for apoproteins corresponding to each lipoprotein (Fig. 2B). HA-fr. 3 contained only serum proteins.

The overall results of the above studies indicate that the complementary use of counter-current and hydroxyapatite chromatography is a very useful method for the separation of three main classes of lipoproteins from human serum without prior ultracentrifugation.

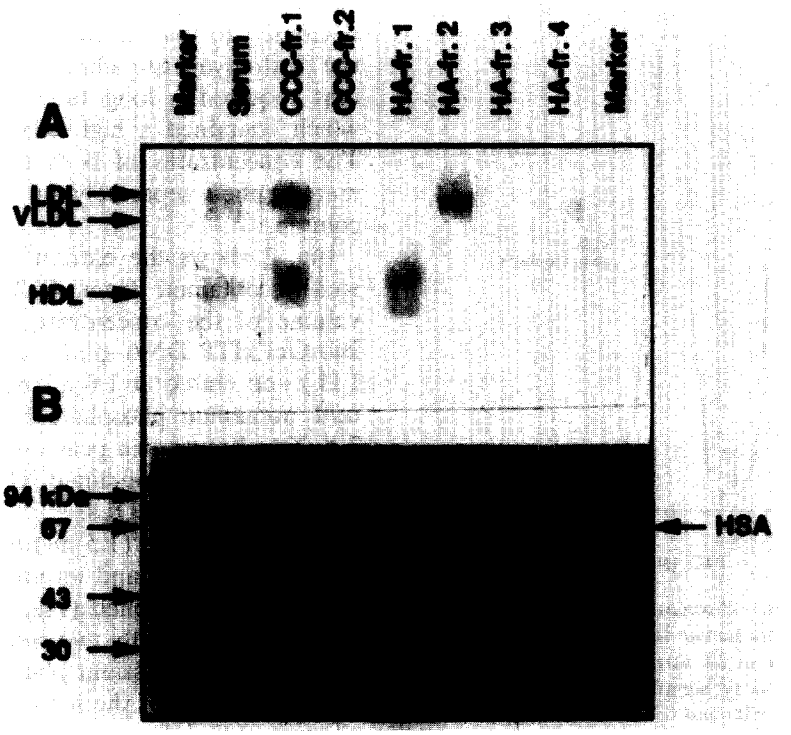

Fig. 2. (A) Agarose gel electrophoresis and (B) SDS-PAGE of CCC and hydroxyapatite chromatographic fractions.

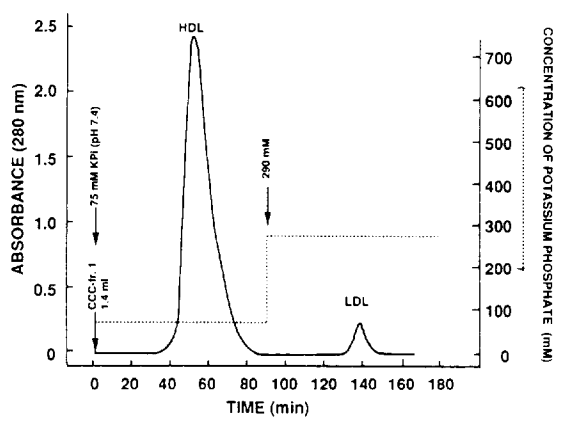

Fig. 3. Stepwise elution profile of HDLs and LDLs by hydroxyapatite chromatography. Column, Bio-Gel HTP DNA-grade hydroxapatite (5.0 × 2.5 cm I.D.); eluent, 75 and 290 m*M* potassium phosphate buffers at pH 7.4; flow-rate, 1.0 ml/min; sample, 1.4 ml concentrate of HDL–LDL CCC fraction containing 13.9 mg total proteins (CCC fr. 1).

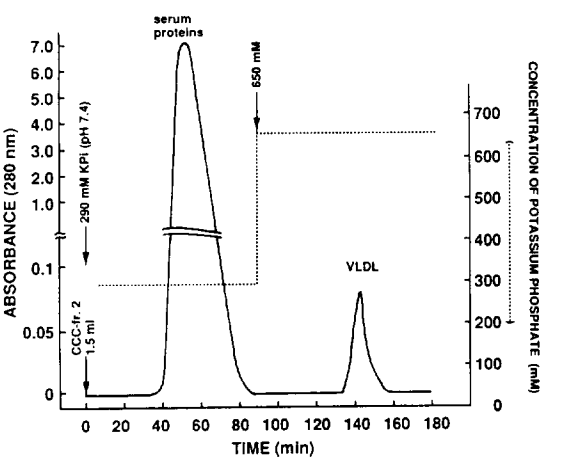

Fig. 4. Stepwise elution profile of serum proteins and VLDLs. Column, Bio-Gel HTP DNA-grade hydroxyapatite (5.0 × 2.5 cm I.D.); eluent, 290 and 650 m*M* potassium phosphate buffer at pH 7.4; flow-rate 1.0 ml/min; sample, 1.5 ml concentrate of serum proteins–VLDL fraction containing 41.8 mg total proteins (CCC-fr. 2).

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