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Review

Lipoproteins: comparison of different separation strategies

Yoichi Shibusawa

School of Pharmacy, Tokyo University of Pharmacy and Life Science 1432-1, Horinouchi, Hachioji, Tokyo 192-03, Japan

Abstract

This review describes two chromatographic techniques for the separation of three main classes of lipoproteins (HDLs, LDLs and VLDLs) from human serum: hydroxyapatite chromatography and counter-current chromatography. The HDLs, LDLs and VLDLs were purified by the combined use of the two chromatographic techniques without prior ultracentrifugation.

Keywords: Reviews; Lipoproteins

Contents

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1. Introduction

Serum lipoproteins are molecular assemblies of lipids and proteins. They consist of hydrophobic core molecules, such as triglycerides and esterified cholesterol, and surface amphiphilic molecules composed of apoproteins, phospholipids and free cholesterol. They are classically defined according to their hydrated density differences and isolated by flotation in sequential preparative ultracentrifugation. The three main classes of lipoproteins are known as high density, low density and very low density lipoproteins (HDLs, LDLs and VLDLs, respectively). The density gradient ultracentrifugal procedure [1] requires ca. two days for the separation of HDLs, LDLs and VLDLs. Many technical difficulties arise in the otherwise perfect separation of the three main classes by using tedious sequential gradient centrifugation method.

The chromatographic separation of lipoproteins into the three main classes has been reported using several types of column packings, such as Bio-Gel A [2,3], Superose 6B (6HR) [4] and TSK Gel [5–7]. The high-performance size-exclusion chromatography has involved separation of the eluate and additional post-chromatographic reactions, such as on-line determination of cholesterol and other lipids [8,9]. However, relatively costly columns and complex equipment are required.

Hydroxyapatite has been widely used for the fractionation of proteins and nucleic acids by column liquid chromatography since its introduction by Tiselius et al. [10]. It has been reported previously that human serum HDLs fraction is divided into five ($HDLs_2$) to six ($HDLs_3$) subclasses [11], and LDLs has been separated from other proteins [12] by using hydroxyapatite chromatography. Semi-preparative fractionation of human serum lipoproteins into three major classes was investigated by hydroxyapatite chromatography with stepwise elution using commercially available hydroxyapatite [13]. 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 4 stepwise elutions [14]. In this chromatographic system, HDLs 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–liquid partition chromatographic methods which are used without solid support matrices [15–17]. The stationary phase is retained in the column by the aid of a gravity or centrifugal force. The system eliminates all complications arising from the solid support. The cross axis coil planet centrifuge (X-axis CPC) has been remarkably improved in terms of retention of the stationary phase. As reported elsewhere [18,19], this type-XLL X-axis CPC has a unique capability of retaining large amounts of stationary phase of low-interfacial-tension, viscous solvent systems effectively used for separation of polar compounds. Recently, some coil planet centrifuge (CPC) apparatus have been modified for performing CCC with polymer phase systems [20]. The X-axis CPC has been successfully used for the separation and purification of stable proteins [21], histones and serum proteins [22] and recombinant uridine phosphorylase from *Escherichia coli* lysate [23]. The method was applied for the separation and fractionation of human serum lipoproteins [24,25]. The studies have shown that a mixture of HDLs and LDLs fractions prepared by ultracentrifugation could be separated by the X-axis CPC [24]. 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. The separation of a HDLs–LDLs fraction from a VLDLs–serum protein mixture directly from human serum was also demonstrated with above polymer phase system at pH 9.2 using the X-axis CPC [25]. However, using this CCC system alone, the purification of all lipoprotein fractions from each other was not realized. The complementary use of counter-current chromatography and hydroxyapatite chromatography was attempted for the separation of three main classes of lipoproteins. Consequently, the fractionation of HLDs, LDLs and VLDLs were performed by combined use of polymer phase CCC and hydroxyapatite chromatography without prior ultracentrifugation [26].

The details of the lipoprotein separations by two chromatographic separation techniques, hydroxyapatite chromatography and counter-current chromatography and combined use of these two separation techniques will be described in this chapter.

2. Preparation of lipoprotein samples from human serum

The lipoprotein fractions, three main classes lipoprotein fractions and human serum for the samples of hydroxyapatite chromatography and counter-current chromatography are prepared as follows.

2.1. Preparation of lipoprotein fractions

The lipoprotein fraction floated by centrifugation was prepared by a procedure modified from that of Rudel et al. [3]. Human blood (ca. 20–30 ml) was collected from normolipidemic males by venepuncture after 12–16 h of fasting. The blood was allowed to stand for 2–3 h at room temperature until agglutination was complete. The plasma was withdrawn after centrifugation at 1000 *g* at 15°C for 15 min. The plasma density was adjusted to 1.225 g/ml by adding solid potassium bromide (0.3517 g of KBr per ml of plasma). Plasma (1.225 g/ml, ca. 3–5 ml) was then placed in ultracentrifuge tubes, which were centrifuged in a swinging-bucket rotor at 200 000 *g* at 10°C for 40 h. The lipoprotein fraction prepared by this procedure did not contain serum protein, except for a small amount of albumin. The lipoprotein fraction in the KBr solution was dialysed against 0.154 *M* sodium chloride solution.

2.2. Preparation of standard HDLs, LDLs and VLDLs fractions

Each main class of lipoproteins was collected by ultracentrifugation using a multiple discontinuous density gradient, as proposed by Sclavons et al. [27]. Human blood was collected from fasting normolipidemic healthy males in tubes containing 0.15% ethylenediamine tetraacetic acid (EDTA). The plasma was separated by centrifugation at 700 rpm at 7°C for 20 min. A discontinuous (NaCl/KBr) density gradient (total volume 18.5 ml) was formed by adjusting the density of plasma to 1.30 g/ml with solid KBr and sequentially layering on the adjusted plasma salt (NaCl/KBr) solutions with densities of 1.240, 1.063, 1.019 and 1.006 g/ml, and 0.5 ml of distilled water. Tubes loaded with the discontinuous density gradient were placed in a RPV 50T vertical rotor and centrifuged at 313 500 *g* at 7°C for 80 min.

2.3. Preparation of human serum

Human blood (ca. 10 ml) was collected from normolipidemic males by venepunctures after 12–16 h of fasting. The blood was allowed to stand for 2–3 h at room temperature until agglutination was completed. The serum was collected after centrifugation at 1000 *g* at 15°C for 15 min.

3. Characterization of human lipoproteins and serum proteins by electrophoresis

3.1. Polyacrylamide gel disk electrophoresis

Three main classes of lipoproteins in an eluate were characterized by polyacrylamide gel disk electrophoresis, modified from the method of Frings et. al. [28]. The eluates (ca. 5–10 ml) with the different phosphate concentrations were placed in dialysis bags (molecular mass cut-off values 3500), which were immersed in aqueous 30% (w/v) PEG 6000 solution. After 5–6 h of dialysis, each eluate was concentrated to 0.1–0.2 ml. If necessary, two or

three granules of Lyphogel were added to the concentrate, and further concentration was performed. Polyacrylamide gel disk electrophoresis was performed in 3.1% (w/v) separation gel and in 2.5% (w/v) concentration gel.

A 30-μl aliquot of concentrated eluate was mixed with 15 μl of Sudan Black B staining solution, and 450 μl of the sample gel solution were added. The mixture was placed on the polymerized concentration gel, and allowed to stand under a daylight fluorescent lamp for ca. 30 min. When photopolymerization was complete, the gel tubes were inserted into the electrophoretic cell. A few drops of 0.01% (w/v) bromophenol blue were added to the upper running buffer as an electrophoretic marker. The electrophoresis was completed in ca. 1 h, at which time the marker had migrated 5 mm from the end of the tube at 3 mA per gel tube.

3.2. Agarose gel electrophoresis

Lipoproteins in both the counter-current and hydroxyapatite chromatographic fractions were also characterized using agarose gel electrophoresis with Oil Red 7B staining [29]. The eluates (ca. 60–100 ml) were 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.

3.3. Sodium dodecyl sulfate polyacrylamide slab gel electrophoresis

Serum proteins and apoproteins in the chromatographic fractions were also characterized by sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (SDS-PAGE) according to the method of Laemmli [30]. Gels containing 3% (w/v) (stacking gel) and 10% (w/v) (separation gel) of acrylamide and 0.8% (w/v) *N,N'*-methylene bisacrylamide. 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.

4. Hydroxyapatite chromatography (HAC)

4.1. Hydroxyapatite column packings

Fast flow-type hydroxyapatite (crystal size 75–150 μm) with plate-like crystalline lamellae (Taihei Chemical Industry, Osaka, Japan) and Hiber Hydroxyapatite-MP column (10×0.8 cm I. D.) prepacked with microporous spherical hydroxyapatite beads, beads size 1–10 μm, average size 6 μm (Kanto Chemical, Tokyo, Japan) were purchased from commercial sources. HA-Ultrogel (particle size 60–180 μm), a microcrystal hydroxyapatite coated with 4% (w/v) cross-linked agarose (IBF Parmindustri, Villeneuve-La-Garenne, France), Nihon Chemical hydroxyapatite (particle size 50–100 μm), a powder crystal (Nihon Chemical Co. Ltd., Tokyo, Japan), Bio-Gel HT (crystal size 10–250 μm) and Bio-Gel HTP DNA grade (crystal size 10–170 μm), both of which are hexagonal prisms of hydroxyapatite (Bio-Rad Labs, Richmond, CA, USA) were commercially available. These four types of hydroxyapatite were suspended and swelled with 1 m*M* potassium phosphate buffer at desired pH and slurry-packed in the columns (10×1.0 cm I.D., or 25×1.0 cm I.D.).

4.2. Stepwise elution

After swelling in the starting potassium phosphate buffer, several types of hydroxyapatite were packed into the column and throughly equilibrated with the starting buffers. Lipoprotein fraction (10–100 μl) or human serum (0.3–2.0 ml) were loaded, then eluted

stepwise with several concentrations (25–650 m*M*) of potassium phosphate buffers at 12.0 ml/min.

4.3. Gradient elution

Several kinds of hydroxyapatites were suspended and swelled with 1 m*M* potassium phosphate buffer and slurry-packed in the columns. After the columns were thoroughly equilibrated with 1 m*M* potassium phosphate buffer, 1.5–3.0 ml of the human serum was loaded, then eluted with a linear gradient of potassium phosphate buffer (1–700 m*M*) at 12.0 ml/h.

4.4. Semi-preparative separation of human lipoproteins

4.4.1. Effect of salt composition in the mobile phase on the retention of lipoproteins on the hydroxyapatite columns

In general, proteins and nucleic acids have been separated on conventional hydroxyapatite columns by a stepwise elution of potassium phosphate buffer at pH 6.8.

To evaluate the peak elution molarity for the three main classes of lipoprotein fraction from normolipidemic male serum prepared by centrifugal flotation at 1.225 g/ml, the lipoproteins were eluted with 100, 300 and 500 m*M* potassium phosphate buffers from the Tiselius-type hydroxyapatite (Bio-Gel HTP DNA grade) column (10×1.0 cm I.D.) at pH 6.8 (Fig. 1). The absorbance of the column eluate was monitored at 280 nm, which corresponds to the absorption maximum of lipoproteins. Alternatively, the light-scattering intensity at right angles caused by lipoprotein particle was monitored with a fluorescence detector at 580 nm. Then the eluates were fractionated and identified by polyacrylamide disk electrophoresis, the modified method of Frings et. al. [28]. HDLs were eluted with 100 m*M* potassium phosphate buffer, and LDLs and VLDLs were eluted with 300 m*M* potassium phosphate buffer. Even when the molarity of the buffer concentration was increased to 500 m*M*, the lipoproteins were no longer eluted. It has been already noted that the peak elution molarity for LDLs and VLDLs is higher than that for HDLs on hydroxyapatite columns [11,12]. The same elution order was observed on this chromatogram shown in Fig. 1.

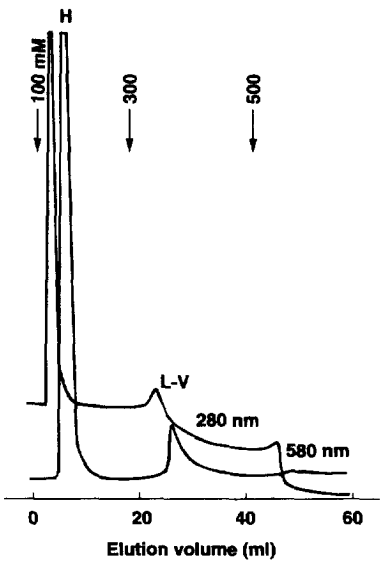

Fig. 1. Stepwise elution profile of human serum high (H), low (L) and very low (V) density lipoproteins. Column: Bio-Gel HTP DNA grade (10×1.0 cm I.D.); eluents: 100, 300 and 500 m*M* potassium phosphate buffer at pH 6.8; flow-rate: 7.4 ml/h. A 100-μl volume of lipoprotein fraction was loaded on the column.

However, it was desirable to increase the retention of LDLs and VLDLs, and so we used ammonium phosphate buffer at pH 6.8 instead of potassium salts. Fig. 2 shows the elution pattern of the three main classes of lipoproteins at four buffer concentrations (100, 300, 400 and 500 m*M*) from the Tiselius-type hydroxyapatite column. HDLs were completely eluted with 100 and 300 m*M* ammonium phosphate buffer. LDLs were mainly eluted with the ammonium phosphate buffer at 400 m*M*, and the part of them are remainded on the column and eluted with VLDLs at 500 m*M*, respectively. Thus LDLs and VLDLs are retained more strongly in the ammonium phosphate buffer than in the potassium phosphate. It seemed advantageous to use the ammonium phosphate eluent for separation of the LDLs-fraction from the VLDLs–LDLs fractions.

4.4.2. Effect of pH of potassium phosphate buffer and of hydroxyapatite morphology on elution behavior of lipoproteins

The pH value of the potassium phosphate buffer affects the retention of proteins in hydroxyapatite chromatography. The effect on the peak elution molarity for standard lipoprotein fractions isolated by

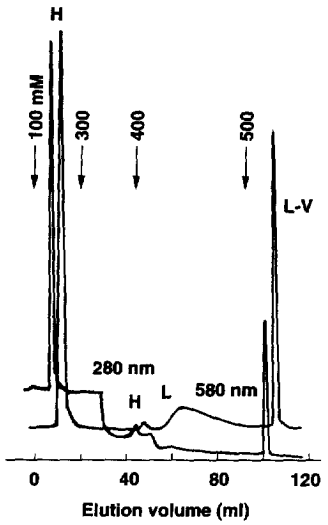

Fig. 2. Stepwise elution profile of human serum high (H), low (L) and very low (V) density lipoproteins. Column: Bio-Gel HTP DNA grade (10×1.0 cm I.D.); eluents: 100, 300, 400 and 500 m*M* ammonium phosphate buffer at pH 6.8; flow-rate: 15.2 ml/h. A 50-μl volume of lipoprotein fraction was loaded on the column.

density gradient ultracentrifugation was examined on the Tiselius-type hydroxyapatite (Bio-Gel HTP DNA grade) column at pH 5.6, 6.2, 6.8 and 7.4. Fig. 3 shows the total elution molarity of monobasic and dibasic potassium phosphates for the elution of the three main classes of lipoproteins at differant pH values. The longer retention time was observed for

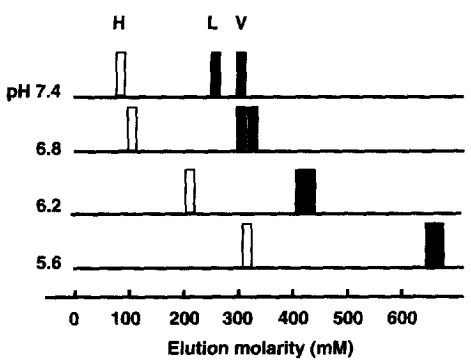

Fig. 3. Effect of pH of potassium phosphate buffer on elution molarity of high (H), low (L) and very low (V) density lipoproteins. Column: BioGel HTP DNA grade (10×1.0 cm I.D.); eluents: potassium phosphate buffers at pH ranging from 5.6 to 7.4; flow-rate: 15.2–16.8 ml/h; 50 μl of each lipoprotein was loaded on the column.

three main classes of lipoproteins at pH 5.6 and 6.2 compared with pH 6.8. The elution molarity of LDLs and VLDLs were the same at pH 5.6, 6.2 and 6.8, respectively. At pH 7.4, LDLs were retained less strongly than at pH 6.8 and VLDLs were eluted with 300 m*M* potassium phosphate buffer. This indicates that it is preferable to use potassium phosphate buffer at pH 7.4 for the separation of LDLs into VLDLs.

The human serum lipoprotein fraction was separated into three main classes by three-step elution of potassium phosphate buffer molarity at pH 7.4, as expected from the elution behavior of standard lipoproteins (Fig. 3). HDLs, LDLs and VLDLs were eluted from the column with 75, 250 and 300 m*M* potassium phosphate buffer, respectively (Fig. 4). The eluates were identified by polyacrylamide disk electrophoresis on 3.1% separation gel (Fig. 4). Some 85–90% of the loaded lipoprotein fraction was recovered, when the total lipoprotein was eluted isocratically from the column with 500 m*M* potassium phosphate buffer at pH 7.4.

Serum HDLs, LDLs and VLDLs are separable on

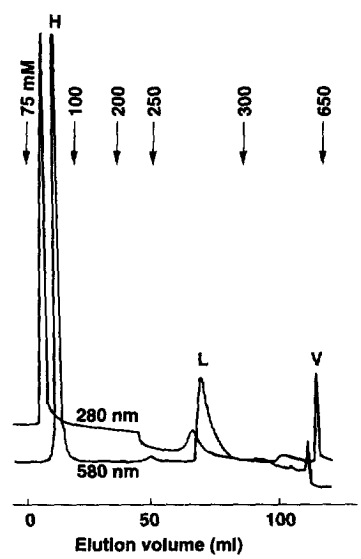

Fig. 4. Stepwise elution profile of human serum high (H), low (L) and very low (V) density lipoproteins. Column: Bio-Gel HTP DNA grade (10×1.0 cm I.D.); eluents: 75, 100, 200, 250, 300 and 650 m*M* potassium phosphate buffers at pH 7.4; flow-rate: 15.6 ml/h. A 50-μl volume of lipoprotein fraction was loaded on the column.

the Tiselius type hydroxyapatite (Bio-Gel HTP DNA grade) column by a three-step elution of phosphate molarity at pH 7.4. It was also found that the crystalline morphology of commercial hydroxyapatite packings, including the crystal size and the Ca/P molar ratio, affects the separation of three main classes of human serum lipoproteins.

4.5. Preparative separation of LDLs and VLDLs from human serum

In the previous section, the lipoprotein fractions collected by ultracentrifugation method [3] were loaded on the columns packed with Bio-Gel HTP DNA grade (10×1.0 cm I.D.) and separated into three major classes of lipoproteins (HDLs, LDLs and VLDLs) by means of stepwise elutions with 75, 250 and 300 m*M* potassium phosphate buffers at pH 7.4. In practice, however, it reqires about 40 h to prepare the lipoprotein samples for chromatography by ultracentrifugation. Then it took about 45 h for purification of VLDLs from human serum. The rapid purification and isolation of the lipoproteins from human serum has been required for a long time.

In this section, we have demonstrated the separation and fractionation of the lipoproteins from human serum directly on hydroxyapatite columns, without prior procedures such as ultracentrifugation. The chromatographic separations of lipoproteins from the serum proteins, such as albumin, α-, γ-globulins in human serum are investigated using four types of hydroxyapatites by gradient and stepwise elution with potassium phosphate buffers at pH 7.4.

4.5.1. Gradient elution of human serum lipoproteins

The chromatograms of the human serum lipoproteins eluted with a gradient from four kinds of hydroxyapatite columns (10×1.0 cm I. D.) are shown in Fig. 5A–D. The gradient elution proceeded at a flow-rate of 12 ml/h of potassium phosphate buffer at pH 7.4. The absorbance of the column eluate was monitored at 280 nm, which corresponds to the absorption maximum of lipoproteins. Using HA-Ultrogel coated by agarose, LDLs and VLDLs were eluted with potassium phosphate buffer not exceeding a concentration of 50 m*M* (Fig. 5A). It appears that these LDLs and VLDLs passed through

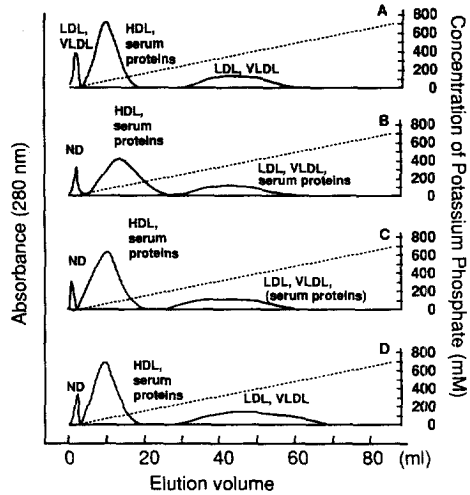

Fig. 5. Chromatograms of human serum HDLs, LDLs and VLDLs on four kinds of hydroxyapatite by gradient elution. ND=not detectable. Columns: HA-Ultrogel (A); Nihon Chemical (B); Bio-Rad HT (C) and Bio-Rad HTP DNA grade (D) hydroxyapatite columns (10×1.0 cm I.D.); eluents: 1–700 m*M* potassium phosphate buffer at pH 7.4; flow-rate: 12.0 ml/h; sample: 0.3 ml human serum.

the column, because they do not penetrate the surface cross-linked 4% (w/v) agarose layer of the hydroxyapatite. Another LDLs–(VLDLs) fraction was eluted at 250 to 500 m*M* potassium phosphate buffer concentration. The retained LDLs and VLDLs have smaller particle size than those eluted with 50 m*M* potassium phosphate buffer because they could penetrate into the surface cross-linked agarose and interact with the core hydroxyapatite. The HDLs were eluted with 50 to 200 m*M* potassium phosphate buffer and separated from the LDLs–VLDLs fractions. However, some serum proteins, such as albumin, α- and γ-globulins were contaminated in the first and second peaks. It showed that a part of LDLs and VLDLs in the human serum were separated from the serum proteins using HA-Ultrogel. The packings are coated by cross-linked agarose, and provide two separation mechanisms of the lipoproteins. One is the gel permeation mode of the surface agarose gel and the other is adsorption of the lipoproteins to the core hydroxyapatite.

We used three other kinds of hydroxyapatites which have only the adsorptive separation mode for the lipoproteins. Fig. 5B and Fig. 5C show the

elution patterns of the three main classes of lipoproteins and serum proteins eluted with a gradient from Nihon Chemical and Bio-Gel HT hydroxyapatites, respectively. In both chromatograms, HDLs and the serum proteins were eluted at concentrations ranging from 50 to 200 m*M* potassium phosphate buffer and the LDLs–VLDLs fractions eluted at the concentrations above 200 m*M* together with serum proteins. It has become apparent that the HDLs and LDLs–VLDLs fractions could not be separated from serum proteins by these two types of hydroxyapatite packings with gradient elution.

The packings which provide the best separation of HDLs, LDLs–VLDLs fractions by stepwise elution in the previous section, Bio-Gel HTP DNA grade, were also used to separate the main classes of lipoproteins from human serum with gradient elution (Fig. 5D). HDLs were eluted at the concentration ranging from 100 to 150 m*M* potassium phosphate buffer at pH 7.4 together with the serum proteins, and LDLs–VLDLs fractions were eluted at 250 to 600 m*M* potassium phosphate buffer. Each fraction was confirmed by 12% SDS polyacrylamide gel electrophoresis and the LDLs–VLDLs fractions were not contaminated with serum proteins. However, it can be seen that LDLs and VLDLs were not separated from each other by a gradient elution from 250 to 650 m*M* of potassium phosphate buffer.

For the purpose of separating LDLs from VLDLs, the combination of the gradient and the stepwise elution were attemped using Bio-Gel HTP DNA grade hydroxyapatite (Fig. 6). The absorbance of the eluate was monitored at 280 nm and the light-scattering intensity at right angles caused by the lipoprotein particles was also monitored with a fluorescence detector at 580 nm. In order to load a large amount of human serum, we used a longer column (25×1.0 cm I.D.). Human serum (1.5 ml) was loaded onto the column and eluted by the gradient elution of the potassium phosphate buffer concentration from 1 to 200 m*M*. After the concentration of potassium phosphate buffer reached at 200 m*M*, the concentration of potassium phosphate buffer was increased to 300 and 650 m*M* stepwise, respectively. Five peaks were detected on this chromatogram. The first peak contained serum proteins which eluted at 1 m*M* potassium phosphate buffer (pH 7.4). As the concentration of the potassium phosphate buffer gradient increased from 1 to 200 m*M*, the HDLs–serum proteins fractions eluted at about 150 m*M*. After the serum proteins eluted, a sharp peak of LDLs–(VLDLs) fractions were eluted immediately upon increasing the potassium phosphate buffer concentration to 300 m*M*. Increasing the concentration to 650 m*M* resulted in elution of the VLDLs fractions. The LDLs–(VLDLs) fractions can be separated from human serum protein using the combination of the gradient and the stepwise elutions, and the VLDLs fractions were purified from human serum within 11 h.

It is considered that the gradient elution is useful to determine the optimum potassium phosphate buffer concentration to elute the three main classes of lipoproteins from hydroxyapatite. The LDLs–VLDLs fractions can be separated from human serum proteins, such as albumin, α- and γ-globulins etc., using Bio-Gel HTP DNA grade. The combination of gradient and stepwise elution allows the

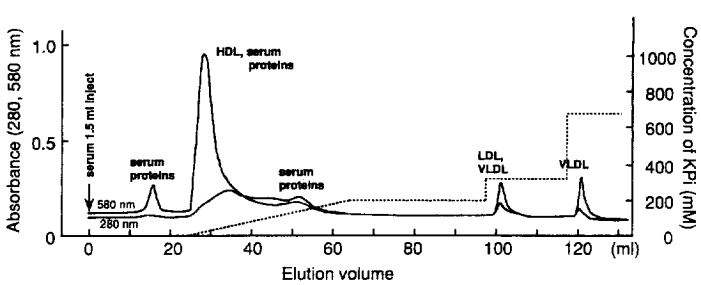

Fig. 6. Elution profile of human lipoproteins by a combination of gradient and stepwise elution. Column: Bio-Gel HTP DNA grade (25×1.0 cm I.D.); eluents: 1–200 m*M* for gradient and 300, 650 m*M* potassium phosphate (KPi) at pH 7.4 for stepwise elution; flow-rate: 12.0 ml/h; sample: 1.5 ml human serum.

separation of the LDLs–VLDLs and VLDLs fractions from human serum proteins.

4.5.2. Stepwise elution of human serum lipoproteins

In general, proteins have been separated by hydroxyapatite chromatography with a stepwise elution of the potassium phosphate buffer concentration. This elution mode is favorable for collecting small fractions, because it results in sharp protein peaks which are retained longer on the column. Human serum was eluted from hydroxyapatite with potassium phosphate buffer at pH 7.4. The optimum concentration of potassium phosphate buffer for stepwise elution were obtained from the results of the gradient elution.

To separate a large amount of LDLs and VLDLs from the human serum, we used Bio-Gel HTP DNA grade column (25×1.0 cm I.D.) and carried out the four stepwise elutions. A 2-ml volume of human serum was loaded on the column and eluted with 75, 200, 300 and 650 m*M* potassium phosphate buffer at pH 7.4. Fig. 7 shows the elution profile of human serum and four peaks were detected. The first one contained HDLs and serum proteins (fr. 1). Further, the serum proteins were eluted from the column by a potassium phosphate buffer concentration of 200 m*M* (fr. 2). The fractions eluted at 300 m*M* potassium phosphate buffer were mainly LDLs (fr. 3) and VLDLs were eluted at 650 m*M* potassium phosphate buffer (fr. 4). The fractions 3 and 4 were not contaminated with serum proteins. The amount of the lipoproteins in both fractions were 4.49 mg (2.25 mg/ml serum) for LDLs and 0.68 mg (0.34 mg/ml serum) for VLDLs, respectively. The lipoproteins and the serum proteins in the fractions were identified by electrophoresis. Disk polyacrylamide gel electrophoresis (Disk PAGE) patterns of the fractions are shown in Fig. 8A. The lipid moiety of the lipoproteins was stained by Sudan Black B. The fractions eluted by 75, 300 and 650 m*M* potassium phosphate buffer (fr. 1, 3 and 4), corresponding to the center cuts of the first, third and forth peaks in the chromatogram, contained HDLs, LDLs and VLDLs. The second peak may represent serum protein, because the fraction showed no lipid staining (Fig. 8A). The serum proteins contained in the fraction were identified by the slub SDS PAGE patterns with Coomasie Briliant Blue protein staining (Fig. 8B). The SDS PAGE analysis revealed that the fractions 1 and 2 contained most of the serum proteins. The fractions 3 and 4 show no protein band except for the apoprotein B in LDLs and VLDLs. It was shown that the LDLs and VLDLs were separated from the human serum proteins by hydroxyapatite chromatography of Bio-Gel HTP DNA grade column (25×1.0 cm I. D.) within 10 h.

The hydroxyapatite developed by Tiselius et al. [10] for chromatography is a crystallized form of calcium phosphate. It has been suggested that the crystal size is an effective criterion of the binding strength of hydroxyapatite packing. It is considered that the separation of the HDLs from the serum proteins is difficult on several types of hydroxyapatites. It will be necessary to use another chromatographic technique for separation of HDLs from serum proteins.

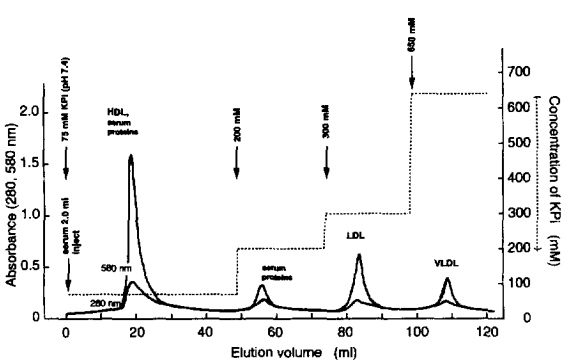

Fig. 7. Stepwise elution profile of human serum lipoproteins. Column: Bio-Gel HTP DNA grade (25×1.0 cm I.D.); eluents: 25, 200, 300 and 650 m*M* potassium phosphate buffer (KPi) at pH 7.4; flow-rate: 12.0 ml/h; sample: 2.0 ml human serum.

5. Counter-current chromatography (CCC)

5.1. Apparatus

Counter-current chromatographic separation of lipoproteins were performed with the cross-axis coil planet centrifuges (CPCs). The cross-axis CPCs used in the lipoprotein separations are modified versions of the high-speed CCC centrifuge. The cross-axis CPC has a unique feature among the CPC systems in

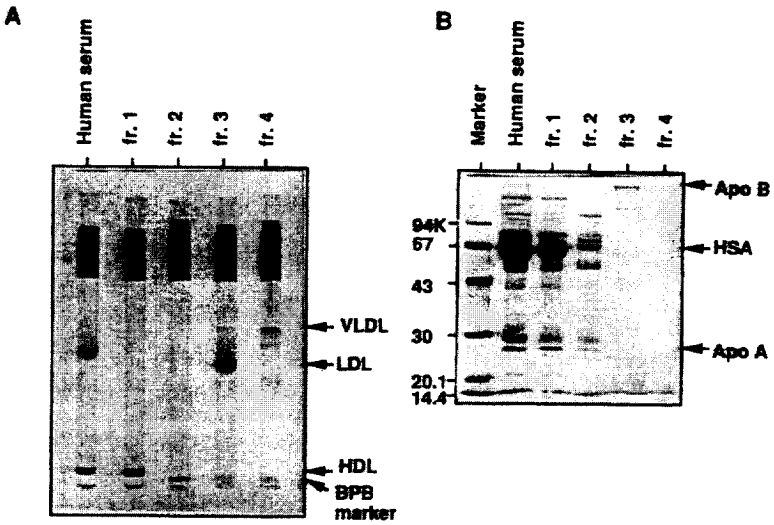

Fig. 8. (A) 3% Polyacrylamide disk and (B) 12% SDS polyacrylamide slab gel electrophoretic profiles of the fractions collected from hydroxyapatite chromatography of human serum.

that the system provides reliable retention of the stationary phase for viscous polymer phase systems. Fig. 9 shows the orientation and motion of the coil holder in the cross-axis CPC systems [31]. R is the radius of revolution; ω is the angular velocity; $\beta = r/R$ where r is the distance from the holder axis to the coil and L is the lateral disposition of the coil expressed by the distance from the center of the holder shaft to the coil holder. A series of studies has

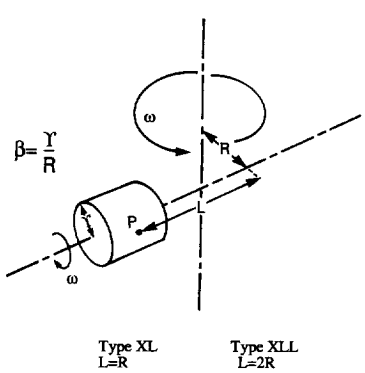

Fig. 9. Planetary motion of the coil holder in the cross-axis coil planet centrifuge.

shown that the stationary-phase retention is enhanced by laterally shifting the position of the coil holder along the holder shaft, apparently due to the asymmetry of the laterally acting force field between the upper and the lower halves of the rotating coil. The degree of the lateral shift of the column holder may be conveniently expressed by L/R, where L is the distance from the center of the holder axis to the coil holder and R is the distance from the centrifuge axis to the holder axis. Among those, XL and XLL cross-axis CPCs have been successfully used for lipoprotein separation with polymer phase systems composed of poly(ethylene glycol) 1000 and potassium phosphate buffer. Figs. 10 and 11 show the photograph and the horizontal cross-section of the XL ($L/R=1$) cross-axis CPC equipped with a pair of multilayer coil separation columns. The apparatus holds a symmetrically placed pair of horizontal rotary shafts, 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-center position 10 cm from the midpoint. Each multilayer coil separation column was prepared from 2.6 mm I.D. polytetrafluoroethylene (PTFE) tubing by winding it onto a 15.2 cm

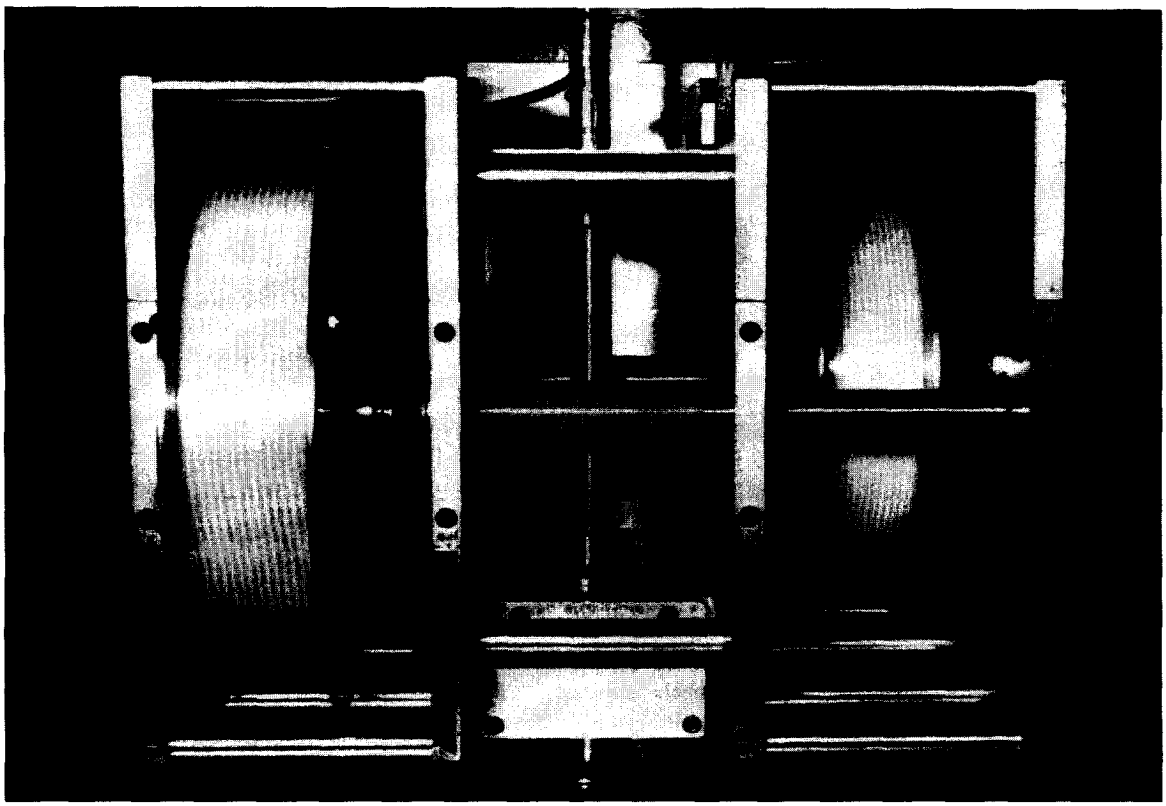

Fig. 10. Photograph of the type XL cross-axis coil planet centrifuge.

diameter holder, forming multiple layers of left-handed coils between a pair of flanges spaced 5 cm apart. The column consisted of 4 layers of coil with a 170-ml capacity. A pair of columns mounted on the rotary frame was connected in series to make up a total capacity of 340 ml.

The XLL apparatus holds a pair of horizontal rotary shafts symmetrically at 7.6 cm from the central axis of the centrifuge. A spool-shaped coil holder is mounted on each rotary shaft at a lateral position 15 cm away from its midpoint. Each coil holder measures 3.8 cm in hub diameter and 5 cm in width between the pair of flanges. A separation column was mounted on each holder by winding a 2.6 mm I.D. PTFE tube directly onto the holder hub making 8 layers of left-handed coils with a 125-ml capacity. A pair of columns mounted on the rotary frame was connected in series to make up a total capacity of 250 ml. The speed of the columns of type XL and XLL CPCs are regulated at 500 and 750 rpm, respectively, with a speed control unit.

5.2. Polymer phase systems for lipoprotein separation

Two-phase solvent systems composed of 16% (w/w) poly(ethylene glycol) (PEG) 600, 1000, 2000 or 4000 and 12.5% (w/w) potassium phosphate (KPi) aqueous solutions were prepared by dissolving 320 g of PEG and 250 g of potassium phosphate buffer (mixture of anhydrous KH_2PO_4 and K_2HPO_4) in 1430 g of distilled water, and the solvent systems containing 25% (w/w) PEG and 12.5% (w/w) potassium phosphate buffer were similarly prepared

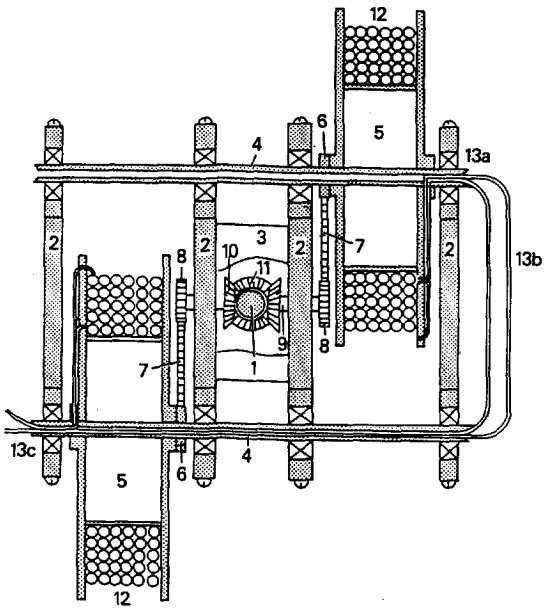

Fig. 11. Cross-sectional view of the type XL coil planet centrifuge. 1=Central shaft; 2=side plates; 3=bottom plate; 4=rotary shafts; 5=column holders; 6 and 8=toothed pulleys; 7=toothed belt; 9=countershafts; 10=planetary miter gears; 11=stationary miter gear; 12=multilayer coil separation columns; 13a–c=flow tubes.

by dissolving 500 g of PEG and 250 g of potassium phosphate buffer in 1250 g of distilled water.

Fig. 12 shows the composition of eight different polymer phase systems composed of PEG 1000 and potassium phosphate buffer. The pH values of the solvent systems were adjusted to 6.8, 7.3, 8.0 and 9.2 by changing the ratio between monobasic and dibasic

PEG 1000	KPi KH_2PO_4	K_2HPO_4	pH	Volume Ratio
	(w/w %)			UP/LP
25% PEG 1000-12.5% KPI systems				
25.0	0	12.5	9.2	1.31
25.0	2.1	10.4	8.0	1.40
25.0	4.2	8.3	7.3	1.61
25.0	6.25	6.25	6.8	1.73
16% PEG 1000-12.5% KPI systems				
16.0	0	12.5	9.2	0.82
16.0	2.1	10.4	8.0	0.88
16.0	4.2	8.3	7.3	1.00
16.0	6.25	6.25	6.8	1.22

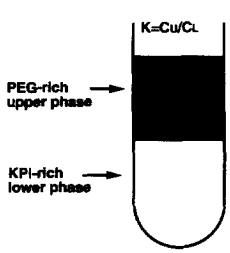

Fig. 12. Composition of various aqueous polymer phase systems used for counter-current chromatography of human serum lipoproteins.

potassium phosphates in the two-phase system. When the concentration of monobasic to dibasic potassium phosphate slightly exceeds a one to one ratio, the solvent mixture forms a single phase.

5.3. Partition coefficient of lipoproteins and serum proteins

Counter-current chromatography is a two-phase procedure where the separation is based on the difference in the partition coefficient of solutes within the phases. To achieve efficient separation of lipoproteins from human serum, it is essential to optimize the partition coefficient of each component by selecting a proper pH of the polymer phase system.

Fig. 13 shows the partition coefficients of three lipoproteins and three serum proteins plotted on a logarithmic scale against the pH of two different polymer phase systems: 16% (w/w) PEG 1000–12.5% (w/w) potassium phosphate buffer and 25% (w/w) PEG 1000–12.5% (w/w) potassium phosphate buffer. In the 16% (w/w) PEG 1000 systems (Fig. 13 left), human serum albumin, α- and γ-globulins and VLDLs show an increase of their partition coefficients with pH from 6.8 to 9.2, while the partition coefficients of HDLs and LDLs display quite different trends. At the highest pH of 9.2, the partition coefficients of both HDLs an LDLs are less than 1.0 indicating that these lipoproteins are mainly distributed to the potassium phosphate buffer-rich

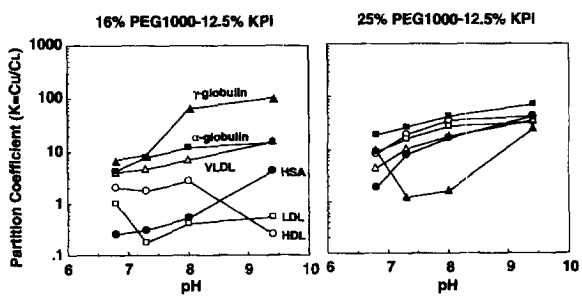

Fig. 13. Partition coefficients (K) of HDLs (○), LDLs (□), VLDLs (△), human serum albumin (●), α-globulin (■) and γ-globulin (▲) in various polymer phase systems composed of PEG 1000 and potassium phosphate buffer. K is solute concentration in the upper phase divided by that in the lower phase.

lower phase. On the other hand, VLDLs are not expected to separate from α-globulin at any pH.

In the 25% (w/w) PEG 1000 system (Fig. 13, right), all proteins except γ-globulin show similar trends for their K-pH curves indicating that the system is unsuitable for the desired separation.

5.4. Separation of artificial HDLs and LDLs mixture by CCC

In order to demonstrate the capability of type XLL cross-axis CPC for the separation of lipoproteins from other serum proteins, a mixture of LDLs and HDLs fractions from human serum was eluted using the polymer phase system composed of 16% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate at pH 9.2. As shown in Fig. 14A, the HDLs and LDLs fractions were eluted from the column in the order of their partition coefficient values and also partially separated from other serum proteins. The experiment was initiated by filling the entire column with the stationary upper phase. This was followed by sample injection through the sample port. The apparatus rotated at 750 rpm while the mobile lower phase was pumped into the column at a flow-rate of 0.5 ml/min in the proper elution mode [18]. The separation was completed within 12 h and the volume of the upper stationary phase retained in the column was 45.0% of the total column capacity (250 ml). Fig. 14B shows the agarose gel electrophoretic patterns of each peak. The lipid moiety of the lipoproteins was stained by Oil Red 7B. The fractions 43–50 and 60–70, corresponding to the center cuts of the first and second peaks in the chromatogram, contained HDLs and LDLs, which migrated to the respective positions in lanes 1 and 2. The third peak in the chromatogram may represent serum protein, because the fraction showed no lipid staining. No lipoprotein was detected in either the upper or lower phase collected from the column after the completion of the separation.

5.5. Isolation of HDLs–LDLs fractions from human serum by CCC

In the previous Section 5.4, we have succeeded in separating HDLs and LDLs lipoproteins with an aqueous polymer phase system using a type-XLL cross-axis CPC. In this experiment, the human serum was first processed by ultracentrifugation to eliminate serum proteins such as albumins and globulins from the CCC samples. In this section, this step will also be done by CCC using a type-XL cross-axis CPC with a polymer phase system composed of different molecular masses of PEG and potassium phosphate buffers over a broad range of pH.

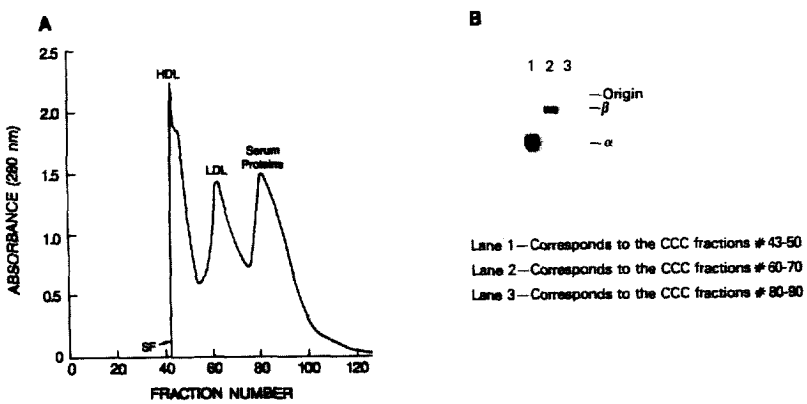

Fig. 14. Separation of lipoproteins by the cross-axis coil planet centrifuge (A) and 0.6% agarose gel electrophoretic profile of the fractions (B). Columns: a pair of multilayer coils connected in series, 2.6 mm I.D. and 250 ml capacity; solvent system: 16.0% (w/w) PEG 1000–12.5% (w/w) dibasic potassium phosphate at pH 9.2; mobile phase: phosphate-rich lower phase; flow-rate: 0.5 ml/min; revolution speed: 750 rpm; maximum column pressure: 20 psi. SF(A)=solvent front. Lanes (B): 1=CCC fractions 43–50; 2=CCC fractions 60–70; 3=CCC fractions 80–90.

5.5.1. CCC of human serum with different molecular masses of PEG in the aqueous polymer solvent systems

The effect of the molecular mass of the PEG was studied with the 16% (w/w) PEG–12.5% (w/w) potassium phosphate systems. Fig. 15 shows the chromatograms of human serum (4 ml) obtained from four solvent systems each containing different molecular mass PEGs (M_n=600, 1000, 2000 and 4000).

In each experiment, the CCC column was first entirely filled with the PEG-rich upper stationary phase and the sample solution (a mixture of 4 ml human serum and 2 ml each of upper and lower phases to which proper amounts of PEG and inorganic phosphate were added to adjust the two-phase composition) was injected through the sample port. The potassium phosphate buffer-rich lower mobile phase was eluted through the column at various flow rates ranging from 0.5 to 2.0 ml/min while the columns of the XL cross-axis CPC were rotated at 500 rpm.

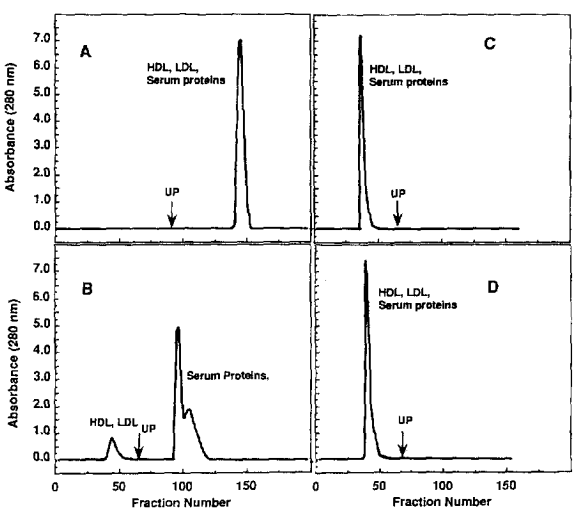

Fig. 15. Counter-current chromatographic fractionation of HDLs–LDLs fractions from human serum with four different aqueous polymer phase systems containing PEG 600 (A), PEG 1000 (B), PEG 2000 (C) and PEG 4000 (D). Experimental conditions: column, 2.6 mm I.D. PTFE multilayer coils (×2) with 340 ml capacity; sample, a mixture of 4-ml volume of human serum, 2 ml of upper phase and 2 ml of lower phase in which 0.9 g of PEG and 0.7 g of dibasic potassium phosphate were dissolved; solvent systems, 16% (w/w) PEG 1000–12.5% (w/w) dibasic potassium phosphate at pH 9.2; mobile phase, lower phase; flow-rate, 2.0 ml/min; revolution, 500 rpm; SF=solvent front; UP=starting point of reversed elution mode with the upper phase mobile.

The lipoproteins in the CCC fractions were characterized using polyacrylamide gel disk electrophoresis (disk PAGE) with a method modified from that of Frings et al. [28]. Serum proteins in the CCC fractions were also characterized by SDS-PAGE according to the method of Laemmli [29].

In the PEG 600 system (Fig. 15A), all proteins including HDLs, LDLs and serum proteins were strongly retained in the PEG-rich upper stationary phase and eluted together when the column was eluted in a reversed elution mode with the PEG-rich upper phase. Similarly, when PEGs with molecular masses higher than 2000 were used in the solvent system, all proteins including HDLs, LDLs and serum proteins were distributed to the potassium phosphate buffer-rich lower phase and eluted together at the solvent front (SF) of the chromatogram (Fig. 15C and Fig. 15D). Successful separation of the combined HDLs and LDLs fraction were achieved with the 16% (w/w) PEG 1000–12.5% (w/w) potassium phosphate buffer solvent system at pH 9.2, where both HDLs and LDLs were eluted together near the solvent front while other proteins were retained in the column for a much longer period of time. The separation time of these two lipoproteins was 3 h. From the partition behavior of the VLDLs (Fig. 14, left), we assume that these lipoproteins are eluted by the PEG-rich upper phase in the second peak or its shoulder (Fig. 15B).

5.5.2. Effect of the flow-rate on the CCC separation of lipoprotein fractionation

The effect of the flow-rate of the mobile phase on the lipoprotein fractionation was next investigated using the 16% (w/w) PEG 1000–12.5% (w/w) potassium phosphate buffer (pH 9.2) system by eluting the potassium phosphate buffer-rich lower phase. Fig. 16A–C show chromatograms of human serum at a flow-rate of 2.0 (A), 1.0 (B) and 0.5 ml/min (C) according to the applied flow-rates. It was evident from these chromatograms that HDLs and LDLs were not resolved even at the lowest flow-rate of 0.5 ml/min.

Fig. 17 shows both the disk and slab polyacrylamide gel electrophoresis of the CCC fractions (Fig. 15B). The first peak corresponding to the

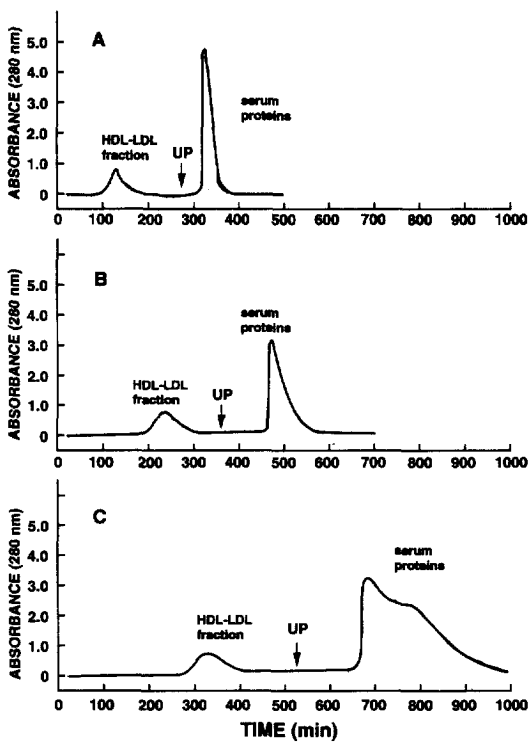

Fig. 16. Counter-current chromatographic fractionation of HDLs–LDLs fractions from human serum at different flow-rate of the lower phase. Experimental conditions are the same as those described in Fig. 14 except the following: flow-rate: 0.5 ml/min (A), 1.0 ml/min (B) and 2.0 ml/min (C). SF=solvent front; UP=the starting point of the reversed elution mode with the upper phase mobile.

fractions 40–55 contained both HDLs and LDLs. The second peak, which was produced by eluting with the PEG-rich upper phase, consisted mostly of human serum proteins such as HSA, α- and γ-globulins. The 3.1% disk-PAGE (Fig. 17B, left) shows that the CCC fractions 40–55 contained both HDLs and LDLs, while the other fractions (92–100, 101–110 and 111–120) show only BPB bands used as a marker. The 12% SDS PAGE (Fig. 17B, right) revealed that the serum proteins including HSA were eluted in fractions 92–120. The HDLs–LDLs fractions corresponding to CCC fractions 40–55 were free of serum proteins, but contained ApoA and ApoB proteins, which are the apolipoproteins of HDLs and LDLs, respectively.

The results show that the HDLs–LDLs fractions were fractionated within 3 h by CCC with a polymer phase system composed of 16% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate at a relatively high flow-rate of 2.0 ml/min. The amounts of phospholipids, cholesterols and triglycerides in the CCC fractions, 44–55 and 92–120, were determined by enzymatic analysis. The triglycerides in the serum protein fractions (92–120) eluted with the PEG-rich upper phase were as high as 0.74 mg/ml. It is most likely that the VLDLs were eluted in the same fractions, because they contain more triglycerides than other lipoproteins.

The overall results of this section are as follows: The CCC fractionation of the HDLs–LDLs fractions by the cross-axis CPC is greatly influenced by the molecular mass of PEG in the solvent system. A polymer system composed of 16% (w/w) PEG 1000 and 12.5% (w/w) dibasic potassium phosphate (pH 9.2) was found to be most suitable since it provides a large difference in K values between the serum proteins and major lipoproteins (HDLs and LDLs). The solvent system allows CCC fractionation of these two lipoproteins directly from the human serum in 3 h. Since the partition system (with PEGs) is very mild, there may be less breakdown of the lipoproteins than with column centrifugation in high salt media. Direct comparison of the samples with the above mentioned techniques would be interesting. With further refinement we hope to fractionate HDLs and LDLs.

6. Complementary use of CCC and HAC for the separation of three main classes of lipoproteins from human serum

6.1. Separation of HDLs–LDLs fractions and VLDLs–serum protein fractions by CCC

The two lipoprotein fractions (HDLs–LDLs and VLDLs–serum proteins) from human serum were obtained in the previous section with a polymer phase system using the type XL cross-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. 18 shows a chromatogram of human serum (4 ml) obtained with the cross-axis CPC using 16% (w/w) PEG 1000–12.5% (w/w) dibasic potas-

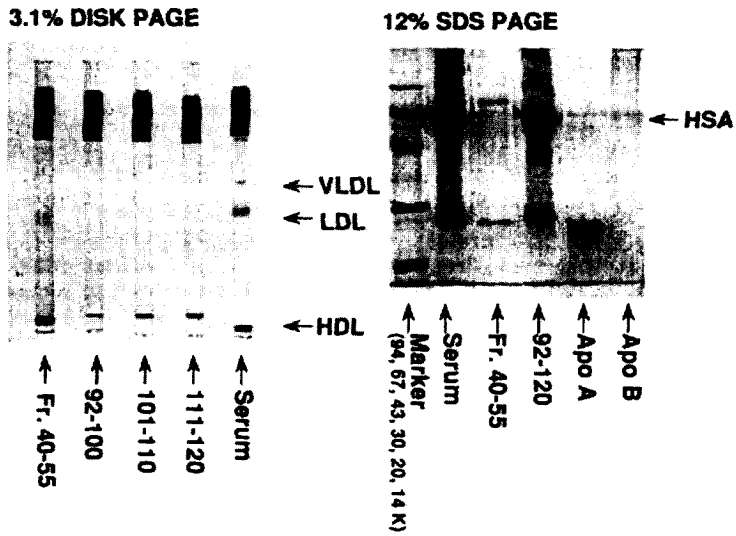

Fig. 17. 3.1% polyacrylamide gel electrophoresis of lipoproteins and 12% SDS PAGE of serum proteins.

sium phosphate (pH 9.2). 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 HDLs–LDLs fraction (CCC–fr. 1), VLDLs were eluted together with serum proteins (CCC–fr. 2) by pumping the upper phase in the reverse direction (marked UP in Fig. 18). The separation was completed within 4.5 h. The lipoproteins in each peak was confirmed by 1% agarose gel electrophoresis with Oil Red 7B stained and the serum proteins were also detected by 10% SDS-PAGE with Coomasie Brilliant Blue protein staining (Fig. 19). The first peak (CCC–fr. 1) contained HDLs and LDLs but no serum proteins of M_n ca. 60 000 (Fig. 19B) and the second peak (CCC–fr. 2) contained VLDLs and serum proteins.

6.2. Separation of HDLs, LDLs and VLDLs from CCC fractions by HAC

In this section, the CCC fractions 1 (HDLs–LDLs) and 2 (VLDLs–serum proteins) obtained in the previous section were each separately dialysed against distilled water until the concentration of the potassium phosphate 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 containted a high concentration of PEG. The concentrates of both fractions were chromatographed separately on the hydroxyapatite column.

Fig. 20 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 buffer at pH 7.4. Two lipoprotein peaks were eluted; the first peak (HA–fr. 1) contained HDLs and the second peak (HA–fr. 2) contained LDLs.

The concentrate (1.5 ml) of CCC–fr. 2, which contained VLDLs and serum proteins, was similary chromatographed (Fig. 21). The separation was performed with two-step elution with 290 and 650 m*M* potassium phosphate buffer 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

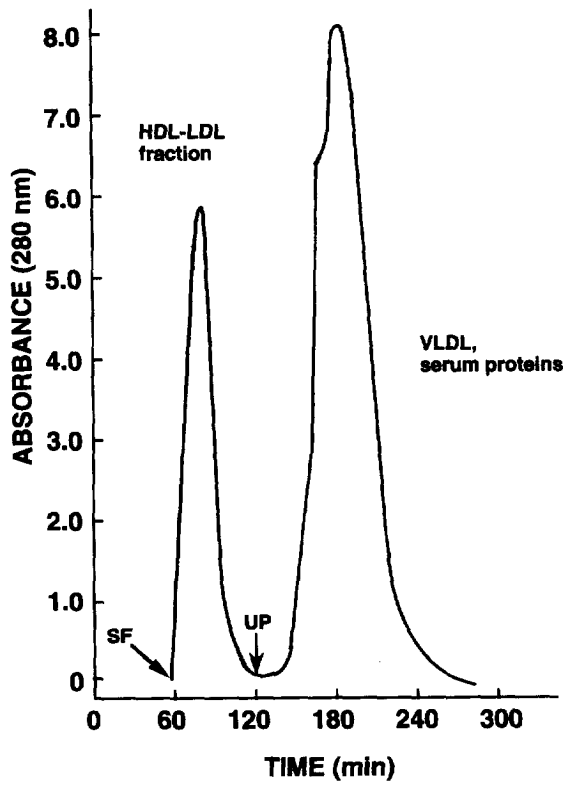

Fig. 18. Counter-current chromatographic separation of HDLs–LDLs fractions from human serum with aqueous polymer phase system. Column: 2.6 mm I.D. PTFE single-layer coil (×2) with 60-ml capacity; sample: 4 ml of human serum in which 0.9 g of PEG 1000 and 0.7 g of dibasic potassium phosphate were dissolved; solvent system: 16% (w/w) PEG 1000–12.5% (w/w) dibasic potassium phosphate at pH 9.2; 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.

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. 19A). 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

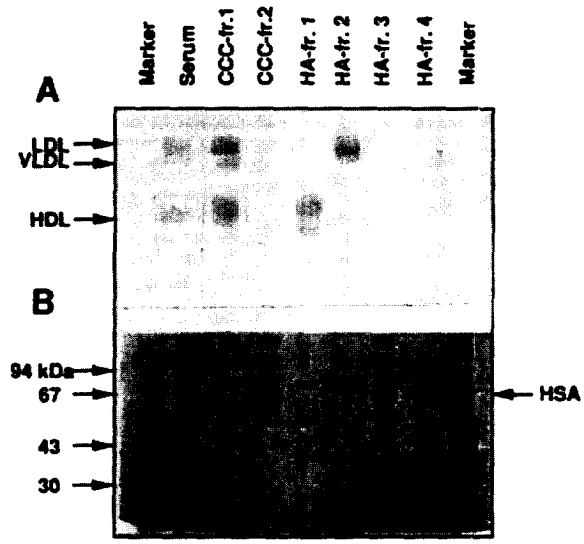

Fig. 19. Agarose gel electrophoresis (A) and SDS-PAGE of CCC and hydroxyapatite chromatographic fractions.

proteins except for apoproteins corresponding to each lipoprotein (Fig. 19B). HA–fr. 3 contained only serum proteins.

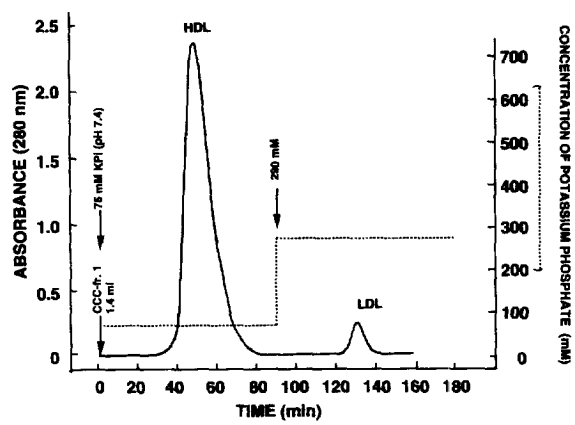

Fig. 20. Stepwise elution profile of HDLs and LDLs by hydroxyapatite chromatography. Column: Bio-Gel HTP DNA-grade hydroxyapatite (5.0×2.5 cm I.D.); eluents: 75 and 290 m*M* potassium phosphate buffers at pH 7.4; flow-rate: 1.0 ml/min; sample: 1.4 ml concentrated of HLDs–LDLs CCC fraction containing 13.9 mg total proteins (CCC–fr. 1).

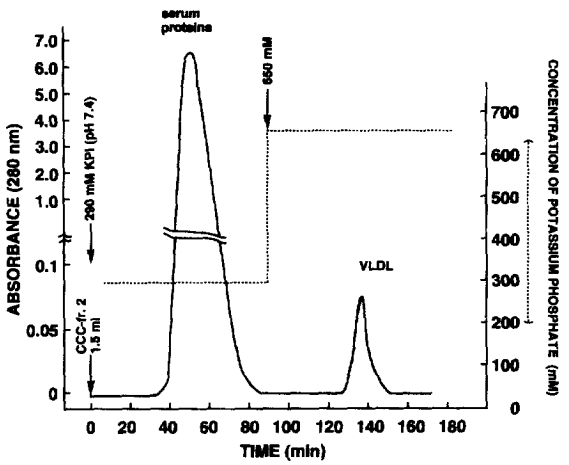

Fig. 21. Stepwise elution profile of serum proteins and VLDLs by hydroxyapatite chromatography. Column: Bio-Gel HTP DNA-grade hydroxyapatite (5.0×2.5 cm I.D.); eluents: 290 and 650 m*M* potassium phosphate buffers at pH 7.4; flow-rate:1.0 ml/min; sample:1.5 ml concentrated of serum proteins–VLDLs CCC fraction containing 41.8 mg total proteins (CCC–fr. 2).

7. Conclusion

In conclusion, serum HDLs, LDLs and VLDLs are separable on the Tiselius-type hydroxyapatite (Bio-Gel HTP DNA grade) column by a three-step elution of phosphate molarity at pH 7.4. It was also found that the crystalline morphology of commercial hydroxyapatite packings, including the crystal size and the Ca/P molar ratio, affects the separation. However, it was impossible to separate HDLs from serum protein, such as albumin and globulin by the hydroxyapatite chromatography.

The other separation technique, counter-current chromatography, is a very useful method for the separation and fractionation of human serum lipoproteins. The HDLs–LDLs and VLDLs–serum proteins fractions were directly obtained from human serum by counter-current chromatography using the solvent system composed of 16% (w/w) PEG 1000–12.5% (w/w) potassium phosphate at pH 9.2. The separated HDLs–LDLs and VLDLs–serum proteins fraction were loaded on the hydroxyapatite columns and separated into HDLs, LDLs, VLDLs and serum proteins, respectively, and eluted with stepwise elution of potassium phosphate buffers. As shown in the Section 6.2, the complementary use of counter-current chromatography and hydroxyapatite chromatography is a very useful method for the separation of three main classes of lipoproteins from human serum without prior ultracentrifugation.

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