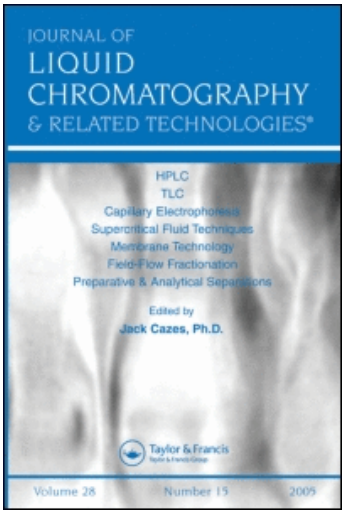

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Separation of Low Density and Very Low Density Lipoproteins from Human Serum by Hydroxyapatite Chromatography

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SEPARATION OF LOW DENSITY AND VERY LOW DENSITY LIPOPROTEINS FROM HUMAN SERUM BY HYDROXYAPATITE CHROMATOGRAPHY

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ABSTRACT

The separation of human serum lipoproteins were studied by hydroxyapatite chromatograhy with gradient or stepwise elution using potassium phosphate (KPi) buffers at pH 7.4.

The low-density (LDL) and very low-density (VLDL) lipoproteins were separated from human serum on Tiselius-type hydroxyapatite (Bio-Gel HTP DNA grade) column (25 x 1.0 cm) by four stepwise elutions with 75, 200, 300 and 650 mM KPi buffers. The fractions eluted by 300 and 650 mM KPi contained 4.49 mg LDL and 0.68 mg VLDL, respectively. High-density (HDL) lipoprotein was eluted by 75 mM KPi together with the serum proteins such as albumin, globulin *etc*..

INTRODUCTION

The chromatographic separations of lipoproteins into three main classes, high-density (HDL), low-density (LDL) and very low-

density (VLDL) lipoproteins, have been reported using several types of column packing materials such as Bio-Gel [1, 2], Superose [3] and TSK GEL [4-6].

In previous studies, human serum HDL, LDL and VLDL were separated using several types of commercially available hydroxyapatites [7]. The lipoprotein fractions collected by ultracentrifugation method [8] were loaded on the columns packed with some hydroxyapatites and separated into three major classes lipoproteins (HDL, LDL and VLDL) by means of stepwise elutions with several concentrations of phosphate buffers at various pH values. The retention of the lipoproteins on the hydroxyapatite packings depended both upon cations, for example, sodium, potassium and ammonium ions in the phosphate buffers, and the pH value of the mobile phases. Pre-packed hydroxyapatite, which consists of microporous spherical beads used for high-performance liquid chromatography could not separate LDL from VLDL. This suggested that both the particle size and the crystal form of the hydroxyapatite play an important role in the retention and the separation of the human serum lipoproteins.

The best separation into three main classes (HDL, LDL and VLDL) of human serum lipoproteins was performed on Tiselius-type hydroxyapatite (Bio-Gel HTP DNA grade), which consists of hexagonal prisms with a wide range of crystalline sizes (10-170 μm) packed in a column (10 x 1.0 cm I.D.) by three stepwise elutions with potassium phosphate (KPi) buffers at pH 7.4. It requires about 5 h to elute the VLDL, which was retained the longest on the

column, with 650 mM KPi buffer. In practice, however, it reqires about 40 h to prepare the lipoprotein samples for the chromatography by the ultracentrifugation. Then it took about 45 h for purification of VLDL from human serum. The rapid purification and isolation of the lipoproteins from human serum has been essential for quite some time.

In this work, we have studied the separation and fractionation of the lipoproteins from human serum 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 KPi buffers at pH 7.4.

EXPERIMENTAL

Materials

HA-Ultrogel (particle size 60-180 μm), a microcrystal hydroxapatite coated with 4% (w/v) cross-linked agarose (IBF Parmindustrie 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, U.S.A.) were commercially available. Lyphogel, polyacrylamide gel granules (Gelman Sciences, Ann Arbor, MI,

U.S.A.) and Spectrapor 3 membranes (Spectrum Medical Industries, Los Angeles, CA, U.S.A.) were used for concentration and dialysis of the eluted fractions. Other reagents were of analytical-reagent grade.

Instruments

Human sera were prepared from blood using a Kokusan Type H103N centrifuge (Kokusan Enshinki, Tokyo, Japan). An LKB 2120 Varioperpex II peristaltic pump (LKB, Bromma, Sweden) was used to elute the serum lipoproteins. A Soma Model S-310A UV detector (Soma Optics, Tokyo, Japan) and a JASCO Model 820-FP fluorescence spectrophotometric detector (Japan Spectroscopic, Tokyo, Japan) were used for detecting the absorbance of the eluate at 280 nm and for monitoring the light scattering of the eluate at 580 nm, respectively. The electric conductivity of the eluate was detected with a Bio-Rad Model 1710 Gradient Monitor (Bio-Rad Labs, Richmond CA, U.S.A.) . The lipoproteins and the serum proteins in the fractions were identified and characterized using a disk electrophoresis aparatus (Atto, Tokyo, Japan) and Bio-Rad Mini Protean II slab electrophoresis apparatus (Bio-Rad Labs).

Preparation of human serum from peripheral blood

Human blood (*ca.* 10 ml) was collected from normolipidemic males by venipuncture 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.

Hydroxyapatite chromatography of human serum

Gradient elution

Four kinds of hydroxyapatite were suspended and swelled with 1 mM KPi (pH 7.4) and slurry-packed in columns (10 x 1.0 cm I.D.), (25 x 1.0 cm I.D.). After the columns were thoroughly equilibrated with 1 mM KPi, 0.3-1.5 ml of the human serum was loaded, then eluted with a linear gradient of KPi (1-700 mM) at pH 7.4. The flow-rate was 12.0 ml/h .

Stepwise elution

After swelling in starting KPi buffer, four types of hydroxyapatite were packed into columns (10 x 1.0 cm I.D.), (25 x 1.0 cm I.D.) and thoroughly equilibrated with the starting buffers. Human serum (0.3-2.0 ml) was loaded, then eluted stepwise with several concentrations (25-650 mM) of KPi (pH 7.4). The flow-rate was 12.0 ml/h. The amount of the proteins in the fraction was determined by the modified Lowry method [9].

Characterization of human lipoproteins and serum proteins by polyacrylamide gel electrophoresis

The lipoproteins in the eluates were characterized by polyacrylamide gel disk electrophoresis (disk PAGE), modified from the method of Frings *et al.* [10]. The eluates (*ca.* 5-10 ml) at various KPi were placed in dialysis bags (molecular mass cut-off values 3500), which were immersed in aqueous 30%(w/v) polyethylene glycol (PEG) 8000. After 5-6h of dialysis, the eluates

were concentrated to 0.1-0.2 ml. If necessary, two or three granules of Lyphogel were added to the concentrates, which were concentrated further. Disk PAGE was performed in a 3.1% (w/v) separation gel and in 2.5% (w/v) sample gel in glass tubes (10 x 0.5 cm I.D. gel bed).

A 30 µl aliquot of concentrated eluate was mixed with 15 µl of 0.25%(w/v) Sudan black B in 30%(w/v) ethanol, and 450 µl of the sample gel solution was added. The mixture was placed on the polymerized 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. Bromophenol blue, 0.01%(w/v), was added to the upper running buffer as a marker. The electrophoresis was completed in about 1 h, at which time the marker had migrated 5 mm from the end of the tube at 3 mA per gel.

Serum proteins in the fractions were also characterized by SDS polyacrylamide slab gel electrophoresis (SDS-PAGE), according to the method of Laemmli [11]. Gels containing 3% (w/v) (stacking gel) and 10% (w/v) (separation gel) acrylamide were prepared from a stock solution of 30% (w/v) acrylamide and 0.8% (w/v) N, N′-methylene-bis acrylamide. The 5.5 x 10 cm separation gel and the 1.0 x 10 cm stacking gel were prepared between glass plates (gel is 0.75 mm thick). 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) sodium dodecyl sulphate (SDS), 5%(w/v) 2-mercaptoethanol, 4%(w/v) glycerol and

0.01%(w/v) bromophenol blue (BPB)] and 10-20 µl of it was loaded onto the stacking gel. Electrophoresis proceeded at a current of 10 mA until the BPB marker reached the stacking gel. Thereafter, the current of the apparatus was increased to 20 mA and the electrophoresis continued untile the BPB marker reached the bottom of the separation gel. The migrated proteins were stained for 5 min at room temperature with the 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.

RESULTS AND DISCUSSION

Hydroxyapatite chromatography of human serum lipoproteins

The chromatograms of the human serum lipoproteins eluted with a gradient from four kinds of hydroxyapatite columns (10 x 1.0 cm I.D.) are shown in Fig. 1. The gradient elution proceeded at a flow-rate of 12 ml/h of KPi 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, LDL and VLDL were eluted with KPi buffer not exceeding a concentration of 50 mM (Fig. 1A). It appears that these LDL and VLDL passed through the column, because they do not penetrate the surface cross-linked 4% (w/v) agarose layer of the hydroxyapatite. Another LDL-(VLDL) fraction was eluted at 250 to 500 mM KPi concentration. These retained LDL and VLDL have smaller

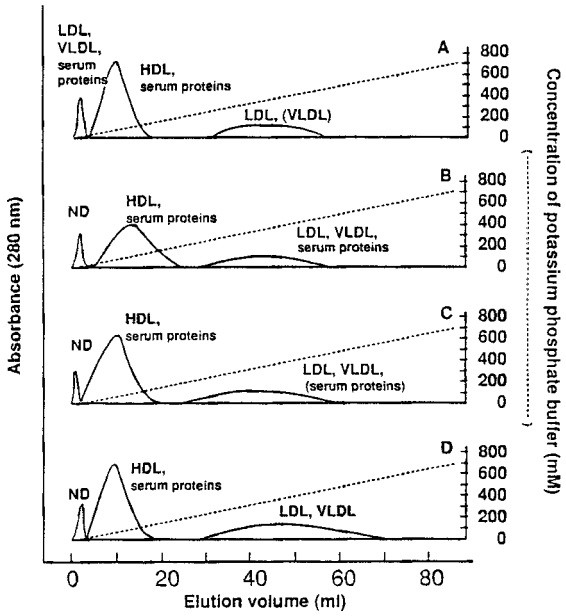

Fig. 1 Chromatograms of human serum HDL, LDL and VLDL on four kinds of hydroxyapatite by gradient elution. ND=not detectable. Columns: HA-Ultrogel (A); Nihon Chemical (B); Bio-Rad HT (C) and Bio-Rad HTP DNA grade (D) hydroxyapatite (10 x 1.0 cm I.D.); eluents: 1-700 mM potassium phosphate (KPi) buffer (pH 7.4); flow-rate: 12.0 ml/h; sample: 0.3 ml human serum.

particles size than those eluted with 50 mM KPi because they penetrated the surface cross-linked agarose and interacted with the core hydroxyapatite. The HDL was eluted with 50 to 200 mM KPi and separated from the LDL-VLDL fractions. However, some serum proteins, such as albumin, α-, γ-globulins were contaminated in the first and second peaks. It showed that a part of LDL and VLDL in the human serum were separated from serum proteins using HA-Ultrogel. This hydroxyapatite is coated by cross-linked

agarose, and provides 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 are not modified on the surface and have only the adsorptive separation mode. Fig. 1B and C shows 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 the both chromatograms, the HDL and the serum proteins were eluted at concentrations ranging from 50 to 200 mM KPi and the LDL-VLDL fractions eluted at the concentrations above 200 mM together with serum proteins. It has become apparent that HDL and LDL-VLDL fractions could not be separated from serum proteins by these two types of hydroxyapatites with gradient elution.

The hydroxyapatite which best separated the HDL, LDL-VLDL fractions by stepwise elution in the previous study [7], Bio-Gel HTP DNA grade was also used to separate the lipoproteins from human serum with gradient elution (Fig. 1D). HDL was eluted at the 100-150 mM KPi at pH 7.4 together with the serum proteins and the LDL-VLDL fractions were eluted at 250 to 600 mM KPi. The LDL-VLDL fractions include no serum proteins which confirmed by SDS-PAGE. The LDL-VLDL fractions were separated from the HDL-serum protein fractions. However, it can be seen that the LDL and VLDL were not separated each other by a gradient elution from 250 mM to 650 mM KPi.

For the purpose of separating the LDL from VLDL, the combination of the gradient and the stepwise elution were attempted (Fig. 2) using Bio-Gel HTP DNA grade hydroxyapatite. 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 x 1.0 cm I.D.). Human serum (1.5 ml) was loaded onto the column and eluted by the gradient elution of the KPi concentration from 1 to 200 mM. After the concentration of KPi buffer reached at 200mM, the concentration of KPi was increased to 300 and 650 mM with stepwise, respectively. Five peaks were detected on this chromatography. The first peak contained serum proteins which eluted at 1 mM KPi (pH 7.4). As the concentration of the KPi gradient increased from 1 to 200 mM, the HDL-serum proteins fractions eluted at about 150 mM. After these serum proteins eluted, a sharp peak of LDL-(VLDL) was eluted immediately upon increasing the KPi concentration to 300 mM. Increasing the concentration to 650 mM resulted in elution of the VLDL fraction. LDL-(VLDL) fractions can be separated from human serum protein using the combination of the gradient and the stepwise elutions, and the VLDL fraction was purified from human serum within 11 h.

It is considered that gradient elution is useful to determine the optimum the KPi concentration to elute the three main classes of lipoproteins from hydroxyapatite. LDL-VLDL fractions can be separated from human serum proteins, such as albumin, α-, γ-

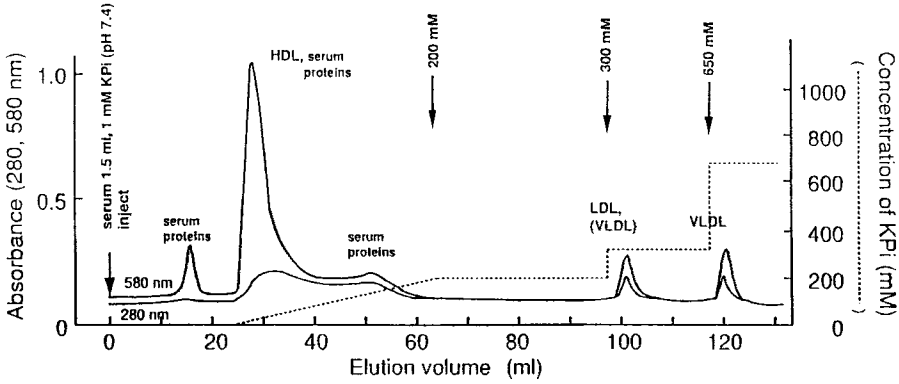

Fig. 2 Elution profile of human lipoproteins by a combination of gradient and stepwise elution. Columns: Bio-Gel HTP DNA grade (25 x 1.0 cm I.D.); eluents: 1-200 mM for gradient and 300, 650 mM KPi (pH 7.4) for stepwise elution; flow-rate: 12.0 ml/h; sample: 1.5 ml human serum.

globulins etc., using Bio-Gel HTP DNA grade. The combination of gradient and stepwise elution allows the separation of the LDL-VLDL and VLDL fractions from human serum proteins.

In general, proteins have been separated by hydroxyapatite chromatography with a stepwise elution of the KPi 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 KPi at pH 7.4. The optimum concentrations of KPi for stepwise elution were obtained from the results of the gradient elution.

To separate a large amount of LDL and VLDL from the human serum, we used Bio-Gel HTP DNA grade column (25 x 1.0 cm I.D.) and carried out the four stepwise elutions. A 2 ml-volume

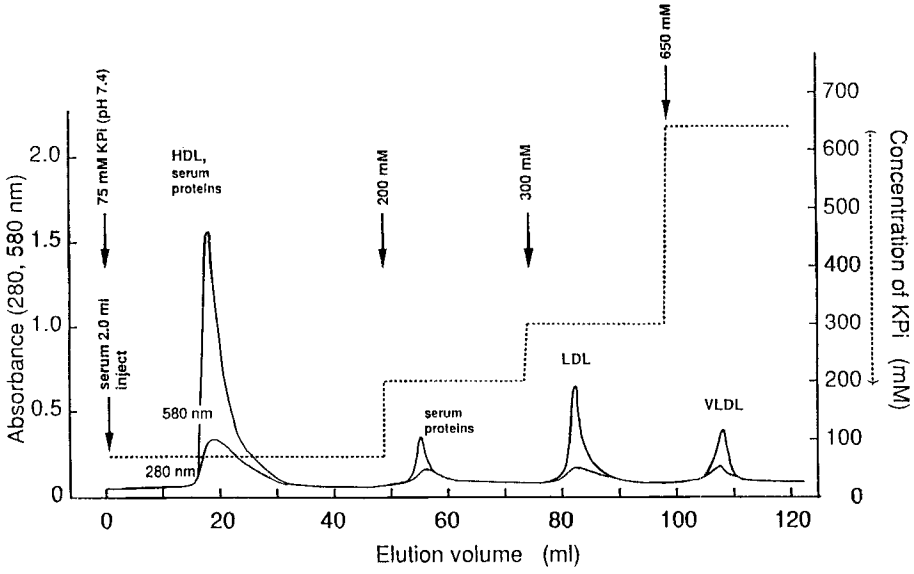

Fig. 3 Stepwise elution profile of human serum lipoproteins. Column: Bio-Gel HTP DNA grade (25 x 1.0 cm I.D.); eluents: 25, 200, 300 and 650 mM KPi (pH 7.4); flow-rate: 12.0 cm/h; sample: 2.0 ml human serum.

of human serum was loaded on the column and eluted with 75, 200, 300 and 650 mM KPi at pH 7.4. Fig. 3 shows the elution profile of human serum and four peaks were detected. The first one contained HDL and serum proteins (fr. 1). Further, the serum proteins were eluted from the column by a KPi concentration of 200 mM (fr. 2). The fractions eluted at 300 mM KPi were mainly LDL (fr. 3) and VLDL was eluted at 650 mM KPi (fr. 4). The frs. 3 and 4 were not contaminated with serum proteins. The amount of the lipoproteins in the both fractions were 4.49 mg (2.25 mg/ml serum) for LDL and 0.68 mg (0.34 mg/ml serum) for VLDL, respectively. The

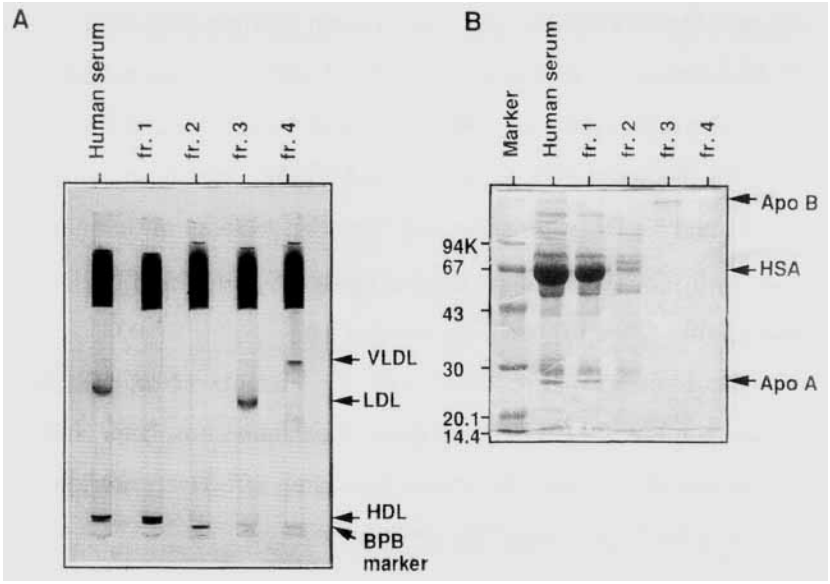

Fig. 4 3% polyacrylamide disk (A) and 12% SDS polyacrylamide (B) slub gel electrophoretic profiles of the fractions collected from hydroxyapatite chromatography of human serum.

lipoproteins and the serum proteins in the fractions were identified by the electrophoresis. Disk PAGE patterns of the fractions are shown in Fig. 4A. The lipid moiety of the lipoproteins was stained by Sudan black B. The fractions eluted by 75, 300 and 650 mM KPi (frs. 1, 3 and 4), corresponding to center cuts of the first, third and forth peaks in the chromatogram contained HDL, LDL and VLDL, respectively. The second peak may represent serum protein, because the fraction showed no lipid staining (Fig. 4A). The serum proteins contained in the fraction were identified by the slub SDS PAGE

patterns with Coomasie briliant blue protein staining (Fig. 4B). The SDS PAGE analysis revealed that the frs. 1 and 2 were contained the most of the serum proteins. The frs. 3 and 4 show no protein band except for the apoprotein B in LDL and VLDL. It was shown that the LDL and VLDL were separated from the proteins in the human serum by hydroxyapatite chromatography of Bio-Gel HTP DNA grade column (25 x 1.0 cm I.D.) within 10 h.

The hydroxyapatite developed by Tiselius *et al.* [12] for chromatographiy is a crystallized form of calcium phosphate. It has been suggested [13] that the crystal size is an effective criterion of the binding strength of hydroxyapatite packing.

It is considered that the separation of the HDL from the serum proteins is difficult on several types of hydroxyapatites. It will be necessary to use materials that provide another separation mode of chromatography to separate HDL from serum proteins.

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