Journal of Chromatography, 566 (1991) 67–76
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5799

Separation of human serum lipoproteins into three major classes by hydroxyapatite chromatography

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(First received May 15th, 1990; revised manuscript received December 26th, 1990)

ABSTRACT

The separation of normolipidemic male serum lipoprotein fraction, prepared by ultracentrifugal flotation, was studied on hydroxyapatite columns. Potassium phosphate buffers in the pH range 5.6–7.4 were evaluated as eluents. The three main classes of the lipoproteins (high density, low density and very low density) can be separated on the Tiselius-type hydroxyapatite (Bio-Gel HTP DNA grade) column by elution with 75, 250 and 300 m*M* potassium phosphate buffer (pH 7.4), respectively.

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 lipoprotein are known as high-density, low-density and very low-density lipoproteins (HDL, LDL and VLDL, respectively). The density gradient ultracentrifugal procedure [1] requires *ca.* two days for the separation of HDL, LDL and VLDL. 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 is restricted both because of their large molecular size and because of their remarkable property, that means they form their micelle-like spheres only in aqueous media. Therefore, size-exclusion chromatography has been performed on Bio-Gel A 50 m [2] and/or Bio-Gel A 5 m [3], however, elution on these soft gel columns took *ca.* 20 h. Human lipoprotein fraction, which was prepared by ultracentrifugal flotation, has been separated into VLDL, LDL and HDL on a column of Superose 6B (6HR) [4] *ca.* 4 h for a single run.

0378-4347/91/$03.50

Recently, the use of high-performance (HP) size-exclusion chromatography has been reported [5–7] for the separation of serum lipoproteins into the three main classes. The separation is performed by combining columns packed with a larger pore-sized silica gel matrix (*e.g.* TSK GEL 3000–4000 SW) and/or a porous polymer matrix (*e.g.* TSK GEL 5000–6000 PW). In this technique, monitoring of the absorbance at 280 nm is not sufficient for lipoprotein evaluation, as the amounts of the lipoproteins of lower molecular mass are low compared with those of albumin and immunoglobulins, which elute with similar retention times. The HP exclusion chromatographic technique [8,9] has involved separation of the eluate and additional post-chromatographic reactions, such as on-line determination of cholesterol and other lipids. However, relatively costly columns and complex equipment are required. This method is also expensive because it uses enzyme reagents for the colorimetric determination.

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 HDL fraction is divided into five (HDL_2) to six (HDL_3) subclasses [11], and LDL has been separated from other proteins [12] by using hydroxyapatite chromatography. However, no published report can be found concerning simultaneous separation of the three main classes of serum lipoproteins by means of this chromatographic technique.

We therefore tried to fractionate, without quantitative determination, the human serum lipoprotein fraction prepared by ultracentrifugal flotation into their three major classes by means of stepwise elution on hydroxyapatite columns. We also examined the retention behaviour of serum lipoproteins on several types of commercially available hydroxyapatite. Their crystalline morphology and the crystal size are different, since they were produced by different procedures.

EXPERIMENTAL

Materials

Bio-Gel HTP DNA-grade hydroxyapatite (crystal size 10–170 μm) (Bio-Rad Labs., Richmond, CA, U.S.A.), 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 cm × 0.8 cm I.D.) pre-packed with microporous spherical hydroxyapatite beads, bead size 4–10 μm, average size 6 μm (Kanto Chemical, Tokyo, Japan) were purchased from commercial sources. Lyphogel, the polyacrylamide gel granules (Gelman Sciences, Ann Arbor, MI, U.S.A.) and Spectrapor 3 membrane (Spectrum Medical Industries, Los Angeles, CA, U.S.A.) were used for concentration and dialysis. Other reagents were of analytical-reagent grade.

Instruments

A Hitachi Model 70P-72 equipped with a swing rotor RPS 65T or a vertical rotor RPV 50T ultracentrifuge (Hitachi, Tokyo, Japan) was used for collection of lipoprotein fractions floated from human serum. 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 light scattering of eluate at right angles at 580 nm, respectively.

Eluents were pumped with either a 2120 Varioperpex II peristaltic pump (LKB, Bromma, Sweden), or an Instrument model controlled-volume mini pump (Milton Roy, Philadelphia, PA, U.S.A.). A disk electrophoresis apparatus (Atto, Tokyo, Japan) was used for characterization and identification of the lipoprotein classes chromatographed.

Preparation of lipoprotein fraction

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 proteins, except for a small amount of albumin. The lipoprotein fraction in the KBr solution was dialysed against 0.154 *M* NaCl solution.

Preparation of standard HDL, LDL and VLDL fractions

Each main class of lipoproteins was collected by ultracentrifugation using a multiple discontinuous density gradient, as proposed by Sclavons *et al.* [13]. Human blood was collected from fasting normolipidemic healthy males in tubes containing 0.15% EDTA. The plasma was separated by centrifugation (7°C, 700 *g*, 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 1.24, 1.063, 1.019 and 1.006 g/ml, and 0.5 ml of doubly 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.

Hydroxyapatite chromatography

After swelling in starting phosphate buffers, two hydroxyapatite packings were packed into columns (10 cm × 1.0 cm I.D.). The pre-packed hydroxyapatite

column (10 cm × 0.8 cm I.D.) was equilibrated with starting buffers before use. Lipoprotein fractions (50–100 ml) were loaded onto these columns, and then eluted stepwise with several concentrations (50–650 m*M*) of potassium phosphate (KPi) buffers (pH 5.6, 6.2, 6.8 and 7.4). A flow-rate of 7–32 ml/h was maintained by the pump. The column recovery was determined by isocratic elution of the lipoprotein fraction with 500 m*M* KPi (pH 7.4). The recovery was evaluated as the ratio of the peak area (absorbance at 280 nm) from a column to the area obtained from an empty accumulating tube (0.1 mm I.D.).

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.* [14]. 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) poly(ethylene glycol) 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% 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.

RESULTS AND DISCUSSION

Chromatography on the Tiselius-type hydroxyapatite at pH 6.8

In general, proteins and nucleic acids have been eluted from conventional hydroxyapatite columns by a stepwise increase of the concentration of phosphate buffer: potassium phosphate (KPi) buffer (pH 6.8) is the most commonly used eluent.

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* KPi buffers from the Tiselius-type hydroxyapatite (Bio-Gel HTP DNA grade) column (10 cm × 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 particles was monitored with a fluorescence detector at 580 nm. Then

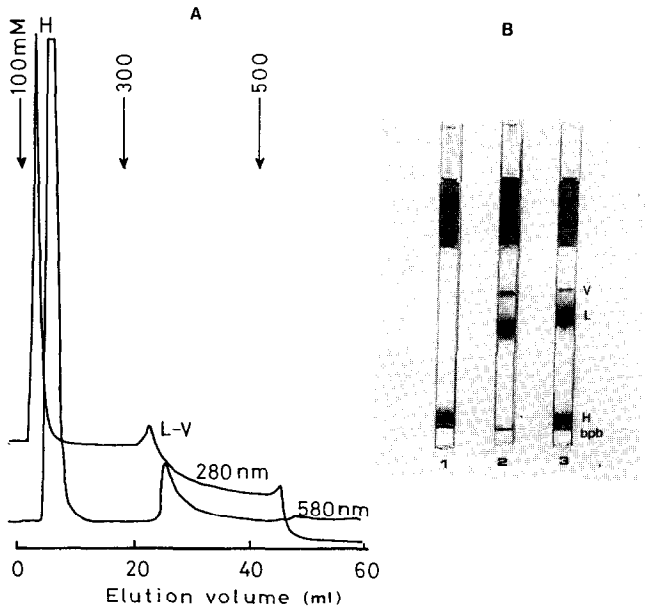

Fig. 1. (A) Stepwise elution profile of human serum high (H), low (L) and very low (V) density lipoproteins. Column, Bio-Gel HTP DNA grade (10 cm × 1.0 cm I.D.); eluents, 100, 300 and 500 m*M* potassium phosphate buffer (pH 6.8); flow-rate, 7.4 ml/h. Lipoprotein fractions of 100 μl were prepared as described in Experimental and loaded on the column. (B) Polyacrylamide gel disk electropherogram of the chromatographic fractions of lipoproteins. The anode is placed at the bottom. The proteins were applied at the upper sample gel. The sample was prestained with Sudan black B in the sample gel. The separation gel was 3.1% (w/v). Lanes: 1 = H in 100 m*M* KPi fraction; 2 = L and V in 300 m*M* KPi fraction; 3 = loaded lipoprotein fraction prepared as described in Experimental.

the eluate was fractionated. HDL were eluted with 100 m*M* KPi, and LDL and VLDL were eluted with 300 m*M* KPi buffer. Characterization and identification were by polyacrylamide gel disk electrophoresis. 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 LDL and VLDL is higher than that for HDL on hydroxyapatite columns [11,12]. The same elution order was observed on the chromatogram shown in Fig. 1.

However, it was desirable to increase the retention of LDL and VLDL, and so we used ammonium phosphate (NH_4Pi) buffer (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. HDL were completely eluted with 100 and 300 m*M* NH_4Pi buffer. LDL were eluted with 400 m*M* buffer mainly, and the remainder was eluted with VLDL at 500 m*M* concentration. Thus LDL and VLDL are retained more strongly in the NH_4Pi buffer than in the KPi buffer. It seemed

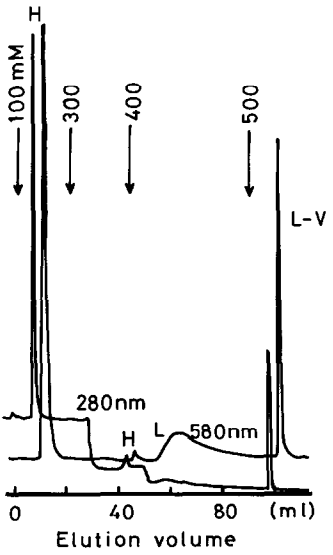

Fig. 2. Stepwise elution profile of human serum high (H), low (L) and very low (V) density lipoproteins. Column, Bio-Gel HTP DNA grade (10 cm × 1.0 cm I.D.); eluents, 100, 300, 400 and 500 m*M* ammonium phosphate buffer (pH 6.8); flow-rate, 15.2 ml/h; lipoprotein fraction volume, 50 μl.

advantageous to use the NH_4Pi eluent for separation of the LDL fraction from the VLDL–LDL fractions. However, this was not favourable for HDL elution, because of long elution times.

Effects of the pH of the potassium phosphate buffer and of the hydroxyapatite morphology on elution behaviour

The pH value of the KPi buffer affects the retention of proteins in hydroxyapatite chromatography. The effect on the peak elution molarity for standard lipoprotein fractions isolated by 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 potassium mono- and dihydrogenphosphates for the three main classes at each pH value. The retention of these standard lipoproteins was improved at pH 5.6 and 6.2 compared with pH 6.8. The elution molarity of VLDL was the same, however, as that of LDL. At pH 7.4, LDL were retained less strongly than at pH 6.8 and VLDL was eluted with the 300 m*M* buffer. This indicates that it is preferable to use KPi buffer at pH 7.4 for the separation of VLDL from LDL. It is not yet clear why the VLDL adsorption on the Tiselius-type hydroxyapatite suddenly increases at pH 7.4.

The human serum lipoprotein fraction was separated into three main classes by three-step elution of KPi molarity at pH 7.4 (Fig. 4), as expected from the elution behaviour of standard lipoproteins (Fig. 3). HDL, LDL and VLDL were

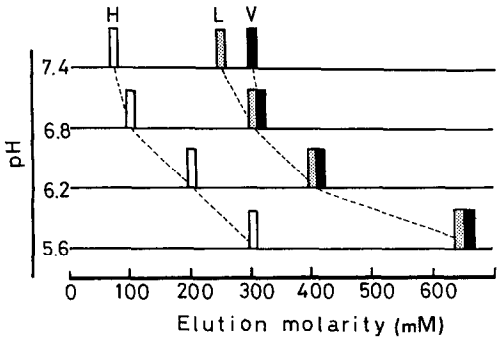

Fig. 3. Effect of pH of potassium phosphate buffer on elution molarity of high (H), low (L) and very low (V) density lipoproteins. Column, Bio-Gel HTP DNA grade (10 cm × 1.0 cm I.D.); eluents, potassium phosphate buffer, pH 5.6–7.4; flow-rate, 15.2–16.8 ml/h; 50 µl of a mixture of standard lipoproteins were separated.

eluted from the column with 75, 250 and 300 m*M* KPi, respectively. The eluates were identified by polyacrylamide disk electrophoresis on 3.1% (w/v) 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* KPi

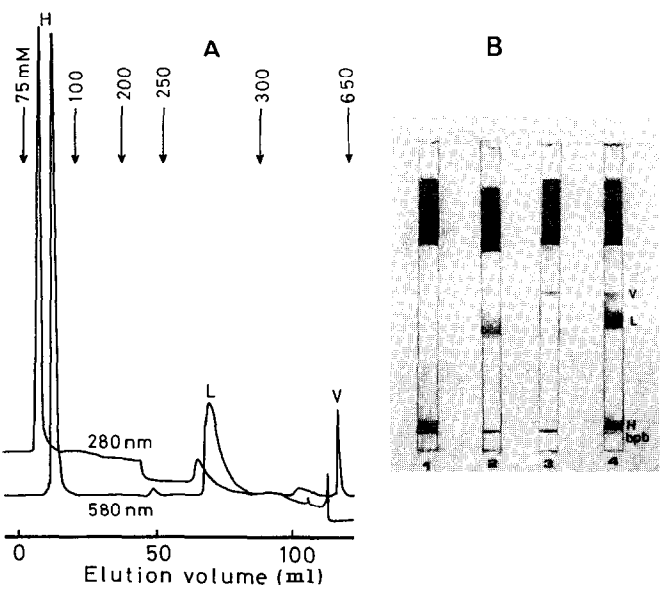

Fig. 4. (A) Stepwise elution profile of human serum high (H), low (L) and very low (V) density lipoproteins. Column, Bio-Gel HTP DNA grade (10 cm × 1.0 cm I.D.); eluents, 75, 100, 200, 250, 300 and 650 m*M* potassium phosphate buffer (pH 7.4); flow-rate, 15.6 ml/h; lipoprotein fraction volume, 50 µl. (B) Polyacrylamide gel disk electropherogram of the chromatographic fractions of lipoproteins, as in Fig. 1B). Lanes: 1 = H in 75 m*M* KPi fraction; 2 = L in 250 m*M* KPi fraction; 3 = V in 300 m*M* KPi fraction; 4 = loaded lipoprotein fraction prepared as described in Experimental.

(pH 7.4). These recoveries were evaluated as the ratio of the peak area of one elution band, which was detected by absorbance at 280 nm, to the area obtained from an empty accumulating tube (0.1 mm I.D.).

The hydroxyapatite preparation named Bio-Gel HTP DNA grade is one of the commercial products made by the Tiselius procedure. According to the manufacturers' specification, this preparation is composed of hexagonal prisms with a wide range of crystalline sizes (10–170 μm). The elution behaviour of the serum lipoprotein fraction isolated by ultracentrifugal flotation was evaluated at pH 7.4 with columns packed with different types of hydroxyapatite packing purchased commercially. Two types of hydroxyapatite were evaluated: a type of high flow-rate preparation and a microporous spherical aggregate developed for HP liquid chromatographic pre-packed columns.

The manufacturers' specification describes the fast flow-type hydroxyapatite preparation as thin plate-like lamellae (100–200 mesh) whose longest dimension is *ca.* 150 μm, with a high flow-rate of *ca.* 0.5 ml/min/cm^2 suitable for large open columns. When the serum lipoprotein fraction was eluted with 100, 200 and 500 m*M* KPi buffer from this column (10 cm × 1.0 cm I.D.), two HDL peaks were resolved at 100 m*M* and combined LDL and VLDL were eluted at 200 m*M* (elution profile not shown). LDL and VLDL were weakly retained on the large lammellar crystals.

The spherical aggregate (Hibar Hydroxyapatite-MP) is composed of microcrystal beads (4–10 mm). This microporous hydroxyapatite is produced by the spray-pyrolysis technique from methanol–water–nitric acid solution of calcium nitrate and phosphoric acid above 1200°C [15,16]. The lipoprotein fraction was eluted from the pre-packed column (10 cm × 0.8 cm I.D.) with 75, 100, 150, 200, 250 and 650 m*M* KPi buffer. After the elution of HDL at 75 m*M*, even the discernible shoulder peak was observed at 150 m*M*, and VLDL could not be separated from LDL (elution profile not shown).

The relatively slow acceptance of chromatography on hydroxyapatite columns seems to be largely due to the rather laborious preparation procedure and the uncertain mechanism of interaction of proteins with hydroxyapatite. The former difficulty seems to have been overcome with the advent of commercially available preparations. The hydroxyapatite developed by Tiselius *et al.* [10] for chromatographic packings is a crystallized form of calcium phosphate, $Ca_{10}(PO_4)_6(OH)_2$. A number of different methods of preparation have been published [17,18]. The binding strength that is shown by the elution molarity of a macromolecule can vary from one preparation to another. The resolution between peaks can also vary considerably. It has been suggested [18] that the crystal size is an effective criterion of the binding strength of hydroxyapatite packings.

The three main lipoprotein classes were separated on the Tiselius-type hydroxyapatite (Bio-Gel HTP DNA grade) column in our experiments by pH 7.4 buffer (Figs. 3 and 4). This preparation requires the use of relatively low flow-rates, but the resolution between the double- and single-stranded DNA molecules

is greater. The molar ratio of Ca/P of this preparation has been reported [18] as 1.51, and this is lower than the stoichiometric value of 1.67. This is believed to be due to lattice defects, and the low Ca/P ratio of hydroxyapatite induces stronger interactions with acidic proteins. Spencer and Grynpas [18] have noted that the Ca/P ratio appears to be indirectly correlated with the binding properties. The values of the Ca/P ratio of the fast flow-type hydroxyapatite with large plate-like crystals and the microporous spherical aggregate of microcrystal hydroxyapatite (Hibar Hydroxyapatite-MP) are much closer to the stoichiometric value. Therefore, one of reasons for the separation of VLDL from LDL at pH 7.4 (Figs. 3 and 4) may be the lower Ca/P ratio caused by lattice defects in the Tiselius-type hydroxyapatite.

Several recent investigations have led to an understanding of the interactions between proteins and hydroxyapatite [19] and the parameters that determine the resolving power of the columns [20,21]. The main conclusions concerning the mechanism of adsorption of proteins [19,22,23] are that the calcium sites of hydroxyapatite crystal surfaces (a or b crystal surface) appear to bind acidic groups, phosphates and carboxyls, and that the phosphate sites (c surface) bind basic groups. Elution is achieved with phosphates, which compete with the phosphate or carboxyl groups of proteins for the calcium sites of hydroxyapatite, or by cations such as K^+, Na^+ and NH_4^+ ions, which compete with the basic groups of proteins for the phosphate sites.

Several different values have been reported for the isoelectric point of human serum HDL and LDL: 4.6–5.1 [11], 3.8–7.4 [24], 4.7–5.4 [25] and 4.9–5.5 [26] for HDL; and 5.3–5.5 [27] or 5.28 [28] for LDL. We were unable to find a value for the isoelectric point of VLDL in the literature. It seems reasonable to suggest that serum lipoproteins are negatively charged in the eluents of pH 6.8 and 7.4. Therefore, the lipoproteins interact principally with the calcium sites of hydroxyapatite surfaces and are eluted with phosphates, which compete with the calcium sites. In addition to this, there are certain differences in elution molarities between KPi and NH_4Pi buffers at pH 6.8 (Figs. 1 and 2); therefore it is suggested that some basic moieties of lipoproteins also interact with the phosphate sites. Potassium ions may compete more strongly than ammonium ions with the positively charged moieties of the proteins because of the higher affinity of K^+ ions than that of NH_4^+ ions for the phosphate sites on hydroxyapatite surfaces.

It has been pointed out by Spencer *et al.* [29] that the order of elution of proteins with NaPi or KPi, at two pH values, depended on both the buffer cation and the pH. The molarity at which HDL and LDL started to elute was the lowest at high pH (Fig. 3), suggesting that HPO_4^{2-} ions are more effective than $H_2PO_4^-$ in displacing these lipoproteins from hydroxyapatite. Moreover, the net negative charge of the proteins would be changed by the pH values of the eluent. HPO_4^{2-} ions may compete more effective than $H_2PO_4^-$ with the negatively charged lipoproteins for the adsorption sites of hydroxyapatite. Thus the elution of the lipoproteins from the column was facilitated when the molar ratio of HPO_4^{2-} ions was increased at higher pH.

In conclusion, serum HDL, LDL and VLDL 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.

ACKNOWLEDGEMENT

The authors thank Nacalai Tesque Inc. for kindly supplying fast flow-type hydroxyapatite produced by Taihei Chemical Industry Co., Ltd.

REFERENCES

1 M. J. Chapman, S. Goldstein, D. Lagrange and P. M. Lapland, *J. Lipid Res.*, 22 (1981) 339.
2 T. Sata, D. L. Estrich, P. P. S. Wood and L. W. Kinsell, *J. Lipid Res.*, 11 (1970) 331.
3 L. L. Rudel, J. A. Lee, M. D. Morris and J. M. Felts, *Biochem. J.*, 139 (1974) 89.
4 P. M. Clifton, A. M. Mackinnon and P. J. Barter, *J. Chromatogr.*, 414 (1987) 25.
5 I. Hara, M. Okazaki and Y. Ohno, *J. Biochem.*, 87 (1980) 1863.
6 M. Okazaki, Y. Ohno and I. Hara, *J. Biochem.*, 89 (1981) 879.
7 M. Okazaki, K. Shiraishi, Y. Ohno and I. Hara, *J. Chromatogr.*, 223 (1981) 285.
8 K. Makino, I. Sasaki, T. Uenishi, T. Takeuchi, I. Hara and M. Umino, *Nippon Kagaku Kaishi*, (1984) 524.
9 I. Hara and M. Okazaki, *Methods Enzymol.*, 129 (1986) 57.
10 A. Tiselius, S. Hjertén and O. Levin, *Arch. Biochem. Biophys.*, 65 (1956) 132.
11 G. M. Kostner and A. Holasek, *Biochim. Biophys. Acta*, 488 (1977) 417.
12 M. Gnezda, M. Jacoby and J. Kerkay, *Anal. Lett.*, 21 (1988) 29.
13 M. M. Sclavons, C. M. Cordonnier, P. M. Mailleux, F. R. Heller, J.-P. Desager and C. M. Harvengt, *Clin. Chim. Acta*, 153 (1985) 125.
14 C. S. Frings, L. B. Foster and P. S. Cohen, *Clin. Chem.*, 17 (1971) 111.
15 S. Inoue and A. Ono, *Yogyo Kyokai-shi*, 95 (1987) 759.
16 S. Inoue, M. Kobayashi and A. Ono, *J. Ceram. Soc. Jpn. Inter. Ed.*, 96 (1988) 183.
17 G. Bernardi, *Methods Enzymol.*, 22 (1971) 325.
18 M. Spencer and M. Grynpas, *J. Chromatogr.*, 166 (1978) 423.
19 G. Bernardi and T. Kawasaki, *Biochim. Biophys. Acta*, 160 (1968) 301.
20 T. Kawasaki and G. Bernardi, *Biopolymers*, 9 (1970) 257.
21 T. Kawasaki, *Biopolymers*, 9 (1970) 291.
22 G. Bernardi, M. G. Giro and C. Gaillard, *Biochim. Biophys. Acta*, 278 (1972) 409.
23 T. Kawasaki, S. Takahashi and K. Ikeda, *Eur. J. Biochem.*, 152 (1985) 361.
24 G. S. Sundaram, S. L. MacKenzie and H. S. Sodhi, *Biochim. Biophys. Acta*, 337 (1974) 196.
25 P. Eggena, W. Tivol and F. Aladjem, *Biochem. Med.*, 6 (1972) 184.
26 C. Luley, H. Watanabe and H. U. Kloer, *J. Chromatogr.*, 278 (1983) 412.
27 J. L. Oncley, J. R. N. Gurd and M. Melin, *J. Am. Chem. Soc.*, 72 (1950) 458.
28 S. Ghosh, M. K. Basu and J. S. Schweppe, *Proc. Soc. Exp. Biol. Med.*, 142 (1973) 1322.
29 M. Spencer, E. J. Neave and N. L. Webb, *J. Chromatogr.*, 166 (1978) 447.