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Short communication

Lead phosphate hydroxyapatite high-performance liquid chromatography

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Abstract

Spherical aggregates of lead phosphate hydroxyapatite (PbHA) have been developed as an adsorbent in high-performance liquid chromatography. There are effectively two types of crystal surface, a (or b) and c which appear on the crystal, analogous to the case of calcium phosphate hydroxyapatite. Basic proteins adsorb to the c surface, and can be eluted by chloride or phosphate salts. Acidic proteins adsorb to the a (or b) surface, and are eluted only by phosphate. All proteins were adsorbed at pH 6 and eluted at pH 8–10. The binding capacity of PbHA for bovine serum albumin was determined by frontal analysis; the shape of the adsorption isotherm is of the Langmuir type.

1. Introduction

The calcium phosphate hydroxyapatite (CaHA), crystal chemical composition $Ca_{10}(PO4)_6(OH)_2$, belongs to the space group $P6_{3/m}$; the crystal unit cell is characterized by the primitive vectors $\vec{a}$, $\vec{b}$ and $\vec{c}$ with $\vec{b}\Lambda\vec{b} = 120°$, $\vec{b}\Lambda\vec{c} = 90°$, $a = b = 9.420$ Å and $c = 6.882$ Å [1]. Column chromatography using CaHA as adsorbent was originally introduced by Tiselius et al. [2] for the separation and purification of biomolecules. It is stable over a wide pH range (5.5–10.0), heat-resistant, and allows for a high recovery of biomolecules with unaltered physicochemical properties. A method has also been successfully utilized to separate nucleic acids such as native and denatured DNA, linear and circular DNA and RNA [3]. Studies on the structure, mode of action and applicability of CaHA by Bernardi and Giro [3–5] and others [6–9] have made this adsorbent especially popular. High-performance liquid chromatography (HPLC) using spherical aggregates (particle size 5–10 μm) of CaHA [10,11] and strontium phosphate hydroxyapatite (SrHA) micro-crystals [12] was recently developed.

CaHA is a particular type of a group of crystals, called apatite, that have a general chemical composition represented as $M_{10}(XO_4)_6Y_2$. The M (metal) and the XO_4 sites of the structure can, in general, be filled respec-

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tively with mono- to trivalent cations and di- to tetravalent groups, and the Y site can be filled with substances with mono- to trivalent anions; the Y site and M can often be vacant [13]. Thus, the M site can be occupied by Ca, Pb, Cd, Sr, Ni, Na or vacancy; the XO_4 site by PO_4, ASO_4, VO_4, CrO_4, SiO_4, CO_3; and the Y site by OH, F, Cl, Br, I, O, N, CO_3, . . . or vacancy [13].

Lead phosphate hydroxyapatite (PbHA) is obtained by substituting Pb^{2+} for Ca^{2+}, thus yielding $Pb_{10}(PO_4)_6(OH)_2$ with a = b = 9.920 Å and c = 7.480 Å; in the PbHA structure, the M site is occupied by lead ions with a radius of 1.19 Å [14] as compared with the CaHA structure in which the M site is occupied by calcium ions with a smaller radius, 1.00 Å [14].

We have developed spherical aggregates (5–10 μm diameter) of microcrystalline PbHA to be packed into HPLC columns. This paper reports their preparation and their physico-chemical properties. Performances of HPLC columns packed with this material were evaluated with protein standards.

2. Materials and methods

PbHA micro-crystals (average particle size 0.1 μm) as starting materials were prepared by slowly dropping ammonium phosphate solution (0.186 *M*, 3 l) into a boiling lead nitrate solution (0.31 *M*, 3 l) in basic media for 3 h at 100°C; the precipitate was matured for 30 min. A small amount of the solution was picked up and characterized. After dilution at a convenient viscosity with deionised water, the moist precipitate was sprayed in a centrifugal spray-dry apparatus model (RAMM) with a flow-rate of 1.2–1.4 l/h at 200°C. The resulting sprayed drops were dried at 95°C into spherical aggregates with diameters of 2–30 μm. The 5–10 μm fraction (Fig. 1) was isolated by an elutriation method.

On the basis of both the X-ray diffraction analysis (Fig. 2) and elemental analysis, it was confirmed that the PbHA aggregates are pure apatite (a = b = 9.920 Å and c = 7.480 Å) with a chemical composition represented as $Pb_{10}(PO_4)_6(OH)_2$ and a Pb/P molar ratio equal to one of a stoichiometric apatite, 1.67. The total surface area (15 m^2/g) was determined by the Brunauer–Emmet–Teller (BET) method with a mixture of nitrogen–helium (30:70) on a Quantasorb II apparatus (Quantachrome).

The proteins used in the present work were albumin (from bovine serum), lysozyme (from chicken egg) and cytochrome *c* (from horse heart) purchased from Sigma (Saint-Quentin, France).

The spherical PbHA aggregates (5–10 μm) were packed in a stainless-steel column (75 mm × 4.6 mm I.D.) using the slurry packing procedure. PbHA microparticles (2 g) were dispersed in CCl4 (20 ml) and then sonicated for 5 min. The resulting slurry was packed with a Haskel Model MCP-110 pump using ethanol as

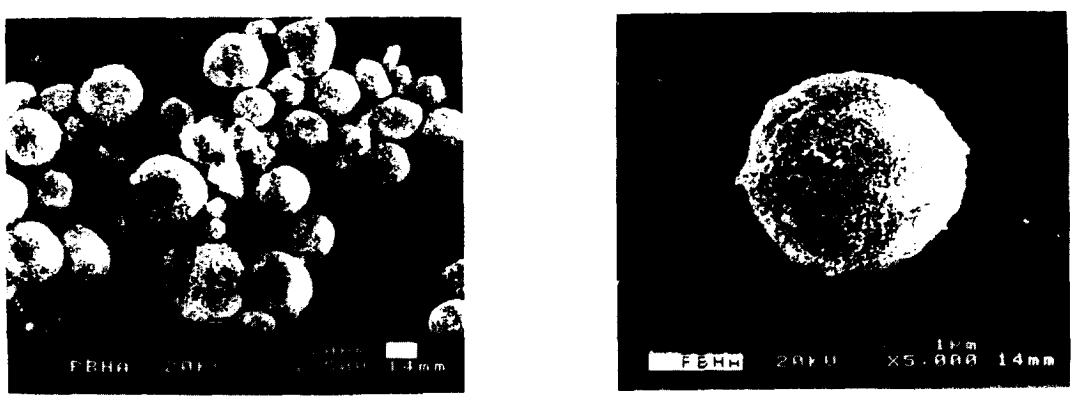

Fig. 1. Scanning electron micrograph of spherical PbHA with an average diameter of ca. 7 μm.

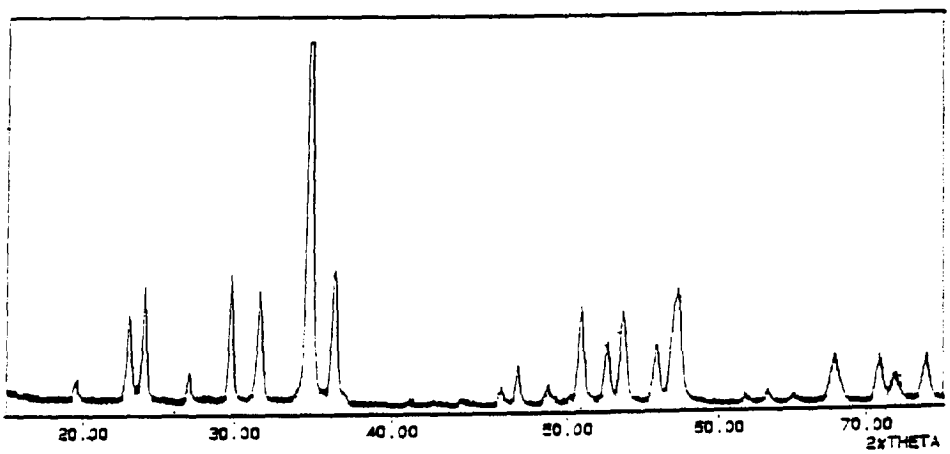

Fig. 2. X-Ray diffraction patterns of PbHA.

pressurizing agent under pressure (100 bar). All chromatographic measurements were carried out at room temperature with a system consisting of a Model 112 (Beckman) HPLC pump equipped with a Model 165 variable-wavelength UV detector (Beckman). Samples were eluted using a molarity gradient of potassium phosphate buffer at pH 6.8 (i.e., an equimolar mixture of K_2HPO_4 and KH_2PO_4); buffer A was 5 m*M* while buffer B was 600 m*M*; alternatively, a molarity gradient was used of 5–600 m*M* KCl in buffer A. The elution was monitored by measuring the UV absorption at 230 nm.

The binding capacities of PbHA and CaHA for bovine serum albumin (BSA) were measured by frontal analysis, as follows: various amounts of BSA, dissolved in buffer A, were pumped through the column as a mobile phase, at a flow-rate of 1 ml/min and at 25°C. Since the 5 m*M* phosphate concentration does not suffice to elute adsorbed BSA, the appearance of protein in the eluate permits the determination of the capacity of the column at saturation.

3. Results and discussion

Fig. 3 is a typical chromatogram of a standard mixture of BSA, lysozyme and cytochrome *c* obtained by applying a phosphate buffer molarity gradient to the PbHA packed column. The first eluted peak, BSA, is followed by a peak of

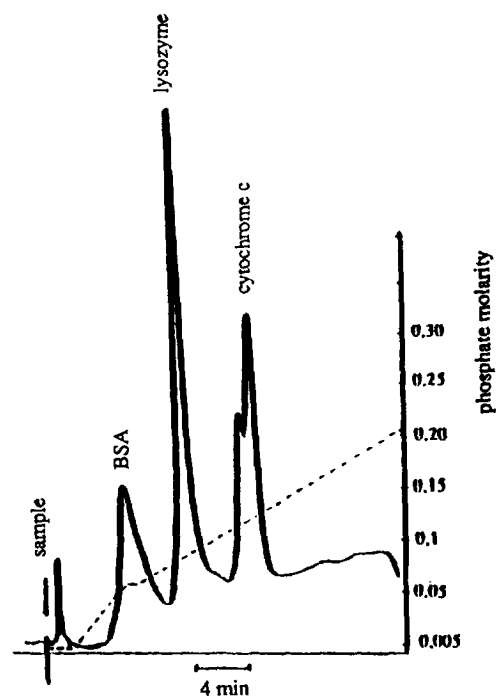

Fig. 3. Chromatogram of a standard mixture of BSA, lysozyme and cytochrome *c* as obtained on the PbHA packed column (75 × 4.6 mm). Conditions: sample load 13.2 μg for albumin, 6.6 μg for lysozyme and 6.6 μg for cytochrome *c*; flow-rate 1 ml/min; inlet pressure 30–32 bar; detection UV at 230 nm. Sample was eluted using a gradient of buffer A to buffer B (0–35% B in 21 min).

lysozyme and a double peak of cytochrome *c* which probably correspond to the oxidized and the reduced forms of cytochrome *c*.

By using the double-gradient method (Fig. 4), it can be seen that BSA, with an acidic p*I*, is eluted with the second potassium phosphate gradient whereas both cytochrome *c* and lysozyme, with basic p*I*, are eluted with the first KCl gradient. Two types of effective surfaces appear on the crystal. From the work of Kawasaki et al. [15] on CaHA, acidic protein molecules are adsorbed mainly onto the a (or b) surface, whereas basic protein molecules are adsorbed mainly onto the c surface. Knowledge of this mechanism permitted selective elution of acidic components in the protein mixture to be developed on the PbHA column. The basic proteins that had been adsorbed on c crystal surfaces were first desorbed through competition with potassium ions from the KCl gradient while the acidic molecules adsorbed on a (or b) crystal surfaces remained adsorbed. When elution of the basic molecules was completed, the phosphate buffer gradient was applied; the acidic molecules were desorbed from a (or b) crystal surfaces through competition with phosphate ions.

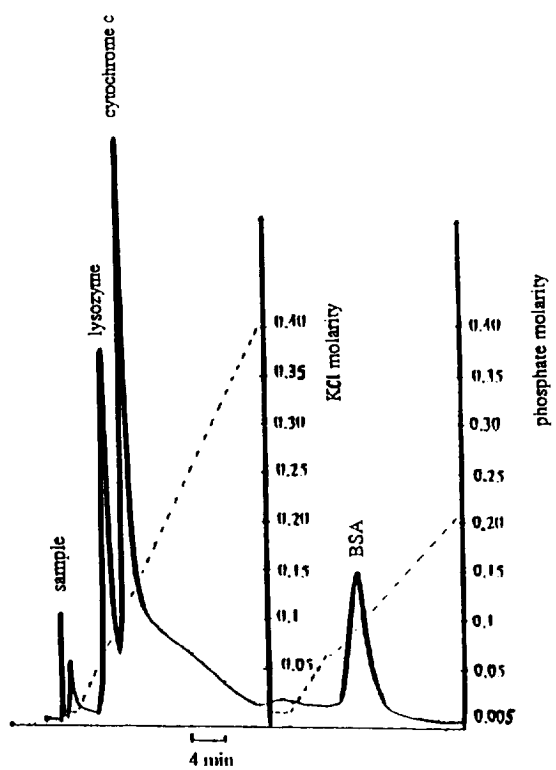

Fig. 4. Double gradient chromatogram for a mixture of BSA (20 μg), lysozyme (10 μg) and cytochrome *c* (10 μg) obtained on the 75 × 4.6 mm PbHA column. First gradient: 5–600 m*M* KCl in buffer A (5–356 m*M* KCl in 20 min). Second gradient: buffer A to buffer B (0–35% in 21 min). Flow-rate = 1 ml/min.

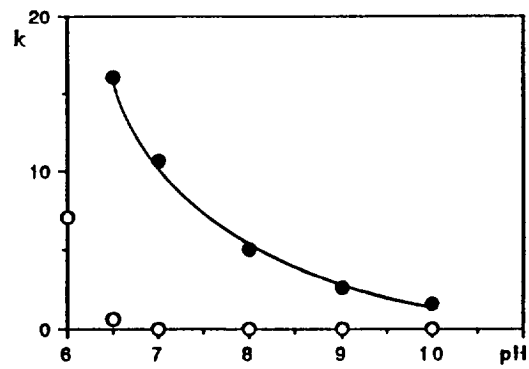

Fig. 5. Effect of pH of eluent on the retention factor (k) of proteins. Proteins were eluted isocratically (0.1 *M* potassium phosphate solution) at various pH values. $k = (t_R - t_0)/t_0$, where t_R is the elution time and t_0 is the elution time for a non-retained solute. ○ = BSA; ● = lysozyme.

Fig. 5 illustrates the effect of the pH of the eluent on the retention factor (k) for BSA and lysozyme. The proteins were injected onto the

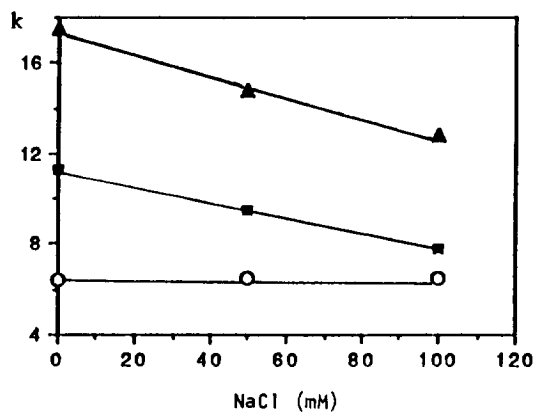

Fig. 6. Effect on NaCl on the retention of proteins on PbHA. A mixture of BSA (○), lysozyme (■) and cytochrome *c* (▲) was eluted from the column under the same conditions as in Fig. 3.

Table 1
Parameters A and Q_m determined from the linearized form of a Langmuir equation

Protein	Adsorbent	Q_m		A
		mg BSA/g adsorbent	mg BSA/mol adsorbent	
BSA	PbHA	4.97	$13.32 \cdot 10^3$	0.308
	CaHA	12.45	$12.54 \cdot 10^3$	0.226

column, and were eluted isocratically at various pH values of the 0.1 *M* phosphate buffer solution. Both proteins were adsorbed at pH 6. BSA, an acidic protein, was eluted at pH 6.5. Lysozyme, a basic protein, was eluted at pH 10. Generally proteins are adsorbed strongly in low-pH eluent and weakly in high-pH eluent, because adsorption depends on the charges existing on the local molecular surface.

We analysed the effect of sodium chloride on the PbHA chromatography of proteins at pH 6.8. As demonstrated in Fig. 6, addition of sodium chloride to the phosphate buffers had a significant effect on retention; elution of basic proteins (lysozyme and cytochrome *c*) was facilitated by the addition of sodium chloride whereas no effect was observed on the elution of an acidic protein (BSA). The adsorption of sodium ions to the c crystal surface is analogous to that of potassium ions, and the effects of NaCl on the chromatography are similar to those of KCl.

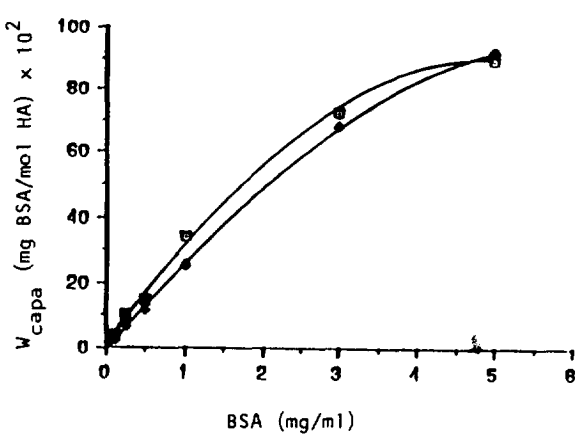

Fig. 7. Adsorption isotherm of bovine serum albumin (BSA) to PbHA (□) and CaHA (◆). The ordinate indicates the binding ratio of BSA to PbHA and CaHA; the abscissa indicates the concentration of BSA (mg/ml) at pH 6.8.

The adsorption isotherms of PbHA and CaHA packings were measured and the amounts of BSA adsorbed per unit mass of the adsorbent (W_{capa}, mg/g) was determined together with C_M, the concentration of BSA in the supernatant.

The adsorption isotherm can be represented by a Langmuir equation:

$$W_{capa} = AQ_mC_M/(1 + AC_M)$$

where A is related to the affinity of the adsorbent and Q_m indicates the maximum capacity of the adsorbent.

To determine the relevant parameters, a linearized form of the above equation can be employed:

$$1/W_{capa} = (1/A)(1/Q_m)(1/C_M) + 1/Q_m$$

Fig. 7 displays the adsorption isotherms of BSA on both CaHA and PbHA. The A and Q_m parameters were determined (Table 1). Little difference is evident when comparing CaHA and PbHA. The affinity A and Q_m are slightly higher for PbHA than CaHA.

The Pb released from PbHA during the adsorption of BSA and lysozyme was determined by inductively coupled plasma mass spectrometry (ICP-MS). The amount of Pb is, in the range 0.2–0.4 ppm, quite low and we can consider that the contamination is minimum. It shall be kept in mind however that this work was more aimed at analytical than preparative separations.

This study shows that it is possible to prepare lead apatites for use as adsorbents in HPLC. Initial experiments with proteins show that the chromatographic behavior of this adsorbent is

similar to CaHA. This may be due to the similar apatitic structure between PbHA and CaHA; the large variation between the a (or b) and c parameters of these two apatites does not lead to a significant difference in protein adsorption. Nevertheless the differences in these parameters, and the difference in electronegative potential between Pb^{2+} and Ca^{2+}, could result in differences in selectivity for closely related proteins. Further study is required to determine the utility of PbHA in chromatography of proteins as well as other small molecules, such as sugars.

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