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High-performance liquid chromatographic separation of biomolecules using calcium phosphate supported on macroporous silica microparticles

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SUMMARY

The preparation of a chromatographic support showing similar selectivity and chemical inertness to hydroxyapatite (HA) and mechanical resistance to the pressures generally used in high-performance liquid chromatography is described. A mixed matrix was formed by covering macroporous silica microparticles with a thin layer of calcium phosphate (CaP–HA). The porous (500, 1000 and 4000 Å) silica microparticles (15 and 10 μm) had been previously inactivated with glycidoxypropyltrimethoxysilane in acidic medium, to convert the silanol groups into hydrophilic groups, producing a biocompatible support with excellent properties in terms of mechanical resistance to high pressure (5000 p.s.i.), selectivity, chemical inertness and efficiency. Columns packed with small particles (5 μm) were run at high pressure and high flow-rates for prolonged periods. Mixed silica WP-DIOL/CaP–HA matrices were used to separate a standard protein mixture antimelanoma monoclonal antibody and some small molecules such as carnitine derivatives and sugars.

INTRODUCTION

Hydroxyapatite [HA; $Ca_{10}(PO_4)_6(OH)_2$] has long been used for the separation and purification of biomolecules. It is chemically inert, stable over a wide pH range (5.5–10.0), heat resistant and allows for a high recovery of biomolecules with unaltered physico-chemical properties.

Tiselius *et al.*[1,2] first used columns packed with HA for the liquid chromatography of proteins; the 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 HA by Bernardi and

co-workers[3–5] and others[6–9] have made this adsorbent especially popular. However, it is well known that HA has some serious drawbacks; in particular, its fragility often precludes the use of high flow-rates and high pressures such as are realized in high-performance liquid chromatography (HPLC) systems. Better results in terms of mechanical resistance and permeability have been achieved only recently by several workers[10–17] and new HPLC supports based on irregular microparticles or spherical ceramic aggregates of microcrystals of HA are now commercially available.

In order to rectify the above problems, we studied the possibility of preparing a support showing the chromatographic selectivity and the chemical inertness of HA and, at the same time, satisfactory mechanical resistance to the high pressures generally used in HPLC. For this purpose, we prepared a mixed matrix by covering spherical macroporous (500, 1000 and 4000 Å) silica microparticles (5 and 10 μm) with a thin calcium phosphate (CaP)–HA layer. In order to reduce the chemical reactivity of the support, the siliceous matrix was previously treated with glycidoxypropyltrimethoxysilane to convert the reactive silanol groups into diol groups to produce a biocompatible support[18] (Fig. 1, first step).

The mixed supports we synthesized showed excellent mechanical resistance, selectivity, chemical inertness and efficiency.

In this paper the preparation of these supports is reported. Their physico-chemical properties are described in addition to their chromatographic performance with low-molecular-weight organic molecules and with proteins.

EXPERIMENTAL

Equipment

Fourier transform infrared (FT-IR) spectra were recorded as KBr pellets on a Nicolet 20SX FT-IR spectrometer. Analytical liquid chromatography was performed on a Waters Assoc. (Milford, MA, U.S.A.) chromatograph equipped with a U6K universal injector, two M510 solvent-delivery systems, a temperature control module (TMC) and an M490 programmable multi-wavelength detector. Chromatographic data were collected and processed on a Waters 840 data and chromatography control station. Analytical data (% C, H, Ca, P) were obtained from Mikroanalytisches Laboratorium, Dr. H. Pascher (Bonn, F.R.G.). Thermogravimetric data were obtained by means of a Mettler TA3000 system consisting of a Mettler TG50 thermogravimeter, TC10A microprocessor and GRAPHWARE TA70.1 data station. Micrographs were obtained with ISI-Akashi Model S × 40A electron microscope system.

Reagents

Nucleosil silica gels were obtained from Macherey, Nagel & Co. (Düren, F.R.G.). All chemicals were purchased from Carlo Erba (Monterotondo, Italy), except 3-glycidoxypropyltrimethoxysilane (GOPTMS), from Janssen (Beerse, Belgium). Transferrin (bovine), myoglobin (from horse skeleton muscle), lysozyme (from chicken egg white) and cytochrome *c* (from beef heart) were obtained from Sigma (St. Louis, MO, U.S.A.). Anti-melanoma monoclonal antibody (IgG class) was kindly supplied by Prof. Soldano Ferrone (Department of Microbiology and Immunology, New York Medical College, Valhalla, NY, U.S.A).

Preparation of wide-pore glycidoxypropylsilica gel (WP-GPSG)

Nucleosil 1000-5 silica gel (3 g) was suspended in toluene (65 ml) and the suspension boiled under argon at 110°C for about 2 h to remove traces of water by azeotropic distillation. Glycidoxypropyltrimethoxysilane (1.5 ml, 6.8 mmol) was then added; the mixture was boiled for 3 h under the same conditions until about 10 ml of distillate had been collected. The suspension was cooled, filtered and washed successively with 20 ml each of toluene, methanol, methanol–water (50:50), methanol and acetone. The wide-pore glycidoxypropylsilica gel (WP-GPSG-1000-5) obtained was dried under vacuum (C, 1.10%; H, 0.18%).

Preparation of wide-pore silica gel DIOL (WP-DIOL)

WP-GPSG 1000-5 (1 g) was suspended in water (20 ml) and the pH was adjusted to 3.5 with 1 *M* sulphuric acid. The suspension was heated at 90°C for 2 h, then filtered and washed successively with 20 ml each of water, methanol and dichloromethane. The wide-pore silica gel DIOL (WP-DIOL 1000-5) obtained was dried under vacuum (C, 1.03%; H, 0.17%).

Preparation of supported calcium phosphate–HA (CaP–HA) on WP spherical microparticles of silica gel DIOL (WP-DIOL)

Procedure A (matrices I and II). An amount of $CaCl_2 \cdot 2H_2O$ [87.8 mg (0.5976 mmol) for CaP–HA 10%; 43.9 mg (0.2988 mmol) for CaP–HA 5%; 21.9 mg (0.1494 mmol) for CaP–HA 2.5%] was dissolved in water (30 ml). WP-DIOL (1 g) was suspended in this solution. Water was removed by distillation *in vacuo* (80°C, 14 mmHg). The product was washed with methanol and dried under vacuum (0.1 mmHg). The dried powder was slowly added to a volume of 0.5 *M* sodium phosphate[a] of pH 6.8 (3.42 ml for CaP–HA 10%; 1.71 ml for CaP–HA 5%; 0.85 ml for CaP–HA 2.5%) under sonication at room temperature. The suspension (brushite-WP-DIOL) was filtered, washed with water, resuspended in a saturated solution of $Ca(OH)_2$[b] (100 ml) and boiled for 30 min [always checking the $Ca(OH)_2$ consumption with methanolic phenolphthalein) to convert brushite into CaP–HA[19]. The suspension was cooled, filtered and washed with water until the pH paper was neutral; it was then washed successively with 20 ml each of methanol and dichloromethane and dried (80°C, 0.1 mmHg).

Procedure B_1 (matrices III–VII). WP-DIOL (1 g) was suspended in water (30 ml). Equal volumes of 0.5 *M* sodium phosphate (pH 6.8) and 0.5 *M* $CaCl_2 \cdot 2H_2O$ (1.71 ml for CaP–HA 5%; 0.85 ml for CaP–HA 2.5%) were added dropwise to the suspension, always with stirring at 60°C. The precipitate (brushite-WP-DIOL) was filtered, washed with water and suspended in water (50 ml) together with a few drops of methanolic phenolphthalein; saturated $Ca(OH)_2$ was added dropwise until the suspension turned pink (pH $\geqslant$8.5). The suspension was boiled for 30 min, with

[a] 0.5 *M* sodium phosphate buffer (pH 6.8) was prepared by mixing 0.5 *M* $NaH_2PO_4 \cdot H_2O$ and 0.5 *M* $Na_2HPO_4 \cdot 2H_2O$ until the desired pH was achieved.

[b] Saturated solutions of $Ca(OH)_2$ were prepared by boiling $Ca(OH)_2$ in deaerated water (helium) and filtering under argon to prevent precipitation of calcium carbonate. Similarly, treatment of brushite with boiling saturated $Ca(OH)_2$ was performed under an argon atmosphere.

TABLE I

KINETIC PERFORMANCES OBTAINED USING DIFFERENT SILICEOUS MATRICES, DIFFERENT Ca PERCENTAGES AND DIFFERENT PREPARATIVE PROCEDURES FOR CaP–HA

Matrix		*Ca(%, w/w) (Ca/P)*[a]	*Preparative procedure*	*Efficiency (plates/m)*[b]	*φ*	*Column dimensions, length × I.D. (mm)*
No.	*Type, particle size, shape*					
–	HPHA (10 μm, irregular)	–	Commercial	8000	3000	30 × 4.6
I	Nucleosil 1000-DIOL (5 μm spherical)	4.50 (1.3)	A	20 000	4000	50 × 4.0
II	Nucleosil 1000-DIOL (5 μm spherical)	2.30 (1.3)	A	26 000	2400	50 × 4.0
III	Nucleosil 500-DIOL (5 μm spherical)	2.33 (1.55)	B_1	25 000	1300	50 × 4.0
IV	Nucleosil 1000-DIOL (5 μm spherical)	2.0 (1.65)	B_1	30 000	2500	50 × 4.0
V	Nucleosil 1000-DIOL (5 μm spherical)	0.98 (1.58)	B_1	50 000	1500	100 × 6.0
		0.98 (1.58)	B_1	55 000	1300	50 × 4.0
VI	Nucleosil 1000-DIOL (10 μm spherical)	0.99 (1.54)	B_1	30 000	2000	100 × 6.0
VII	Nucleosil 4000-DIOL (5 μm spherical)	2.10 (1.55)	B_1	19 700	620	50 × 4.0
VIII	Nucleosil 1000-DIOL (5 μm spherical)	0.99 (1.3)	B_2	24 000	1200	50 × 4.0
IX	Nucleosil 1000-DIOL (5 μm spherical)	2.0 (1.3)	B_2	22 000	2600	50 × 4.0

[a] Calcium/phosphate molar ratio.

[b] See *Chromatographic performance* for the definition of the reported symbols.

dropwise addition of $Ca(OH)_2$ as necessary to maintain the pink coloration. The suspension was then cooled, filtered and washed as described in procedure A.

Procedure B_2 (matrices VIII and IX). WP-DIOL (1 g) was suspended in 0.5 *M* NaCl (15 ml). Equal volumes of 0.5 *M* $Na_2HPO_4 \cdot 2H_2O$ (pH 9.2) and 0.5 *M* $CaCl_2 \cdot 2H_2O$ (0.69 ml for CaP–HA 5%; 0.35 ml for CaP–HA 2.5%) were added dropwise to the suspension, stirring at room temperature. The precipitate was filtered, washed with water and resuspended in water (50 ml) containing a few drops of methanolic phenolphthalein. Saturated $Ca(OH)_2$ was added dropwise until the suspension turned pink (pH 8.5). The suspension was boiled for 30 min, always adding $Ca(OH)_2$ as required to keep the pink coloration; it was then cooled, filtered and washed as described in procedure A.

Chemical and physical characterization of mixed silica WP-DIOL/CaP–HA matrix

Definitions of the components of "mixed silica WP-DIOL/CaP–HA matrix" are functionalized macroporous silica = WP-DIOL and CaP–HA = calcium phosphate as calcium-deficient hydroxyapatite. The Ca/P molar ratio is always less than the theoretical value of pure HA (1.67) (see Table I).

Microanalytical data (Ca, P) of mixed silica WP-DIOL/CaP–HA matrices are reported in Table I; FI-IR spectra (KBr pellets) show bands at 604 and 562 cm^{-1} identical with those found in an authentic sample of HA.

Column packing

Columns (50 × 4.0 mm or 100 × 10 mm I.D.) made of stainless steel with titanium frits were packed using the slurry-packing procedure. Mixed silica WP-DIOL/CaP–HA matrices (0.7 or 4.0 g) were dispersed in 0.35 *M* sodium phosphate buffer (pH 6.8) (30 ml for the small column, 50 ml for the large column) and then sonicated for 5 min. The resulting slurry was packed with a Haskel Model DSTV-122 pump using water as pressurizing agent.

Chromatographic performance

Efficiency test. Mixed silica WP-DIOL/CaP–HA supports were tested using a mixture of benzene, methyl benzoate, nitrobenzene and 1,3-dinitrobenzene [eluent, *n*-hexane–chloroform (90:10, v/v); flow-rate, 1 ml min]. Only the last peak (1,3-dinitrobenzene) of the chromatogram was considered for the calculation of the number of theoretical plates.

The column dead volume (V_0) was determined from the elution time of an unretained marker (benzene; eluent, dichloromethane). Dimensionless parameters such as reduced plate height (h), flow resistance parameter (Φ) and separation impedance (E) were calculated according to Bristow and Knox[20]. Diffusion coefficients of solutes in the mobile phase were determined using the empirical Wilke–Chang equation[20]. The results are reported in Table I.

Applications. Protein solutions (10–50 μl; 1 μg μl/protein) were loaded onto columns and eluted with a linear gradient of sodium phosphate (pH 6.8) (1–350 m*M*). Different flow-rates and different run times were tried in order to optimize the resolution of a standard protein mixture; the effluent was monitored at 280 nm. Separation of some small polar molecules was also attempted (see Figs. 9 and 10) using acetonitrile–water (80:20, v/v) as the eluent; the effluents were monitored at 220 nm.

RESULTS AND DISCUSSION

The utility of HA for the chromatographic separation of biomolecules is dependent on the bioactivities of the biomolecules not being affected by adsorption to HA[3,9,21] Nevertheless, HA has some serious disadvantages: the fragile crystals break easily under mechanical load, especially if high-pressure micropumps are used. In order to improve its mechanical resistance without compromising its chromatographic performance, we produced a mixed matrix by covering functionalized silica microparticles with a thin layer of CaP–HA. The preparative procedure is presented in Fig. 1[22].

The siliceous matrix was inactivated by treatment with 3-glycidoxypropyltrimethoxysilane of the reactive silanolic groups followed by acid hydrolysis of the epoxide rings; the same result was also obtained following a single-step procedure[18] involving derivatization of the siliceous matrix with 3-glycidoxypropyltrimethoxylsilane in acidic medium. The principal difference between these two methods is that in the two-step method, described in this paper, polymerization reactions occurring during derivatization are avoided.

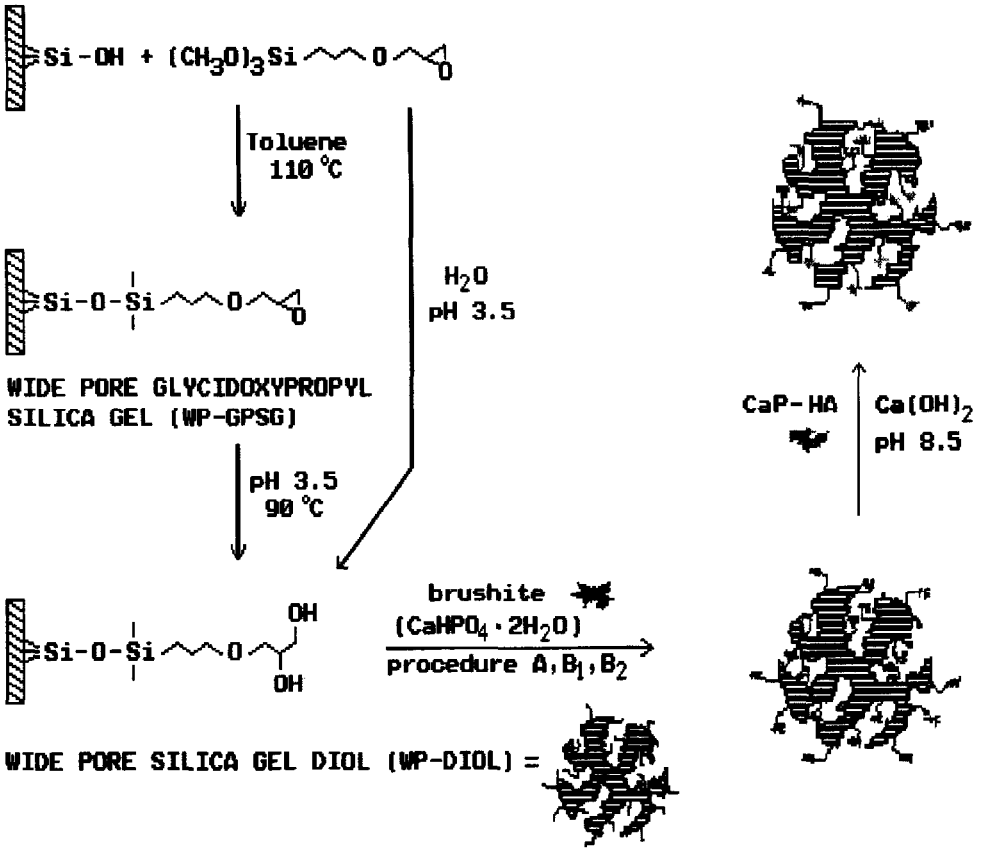

Fig. 1. Preparative procedure for calcium phosphate supported on functionalized macroporous silica microparticles (WP-DIOL/CaP–HA mixed matrices).

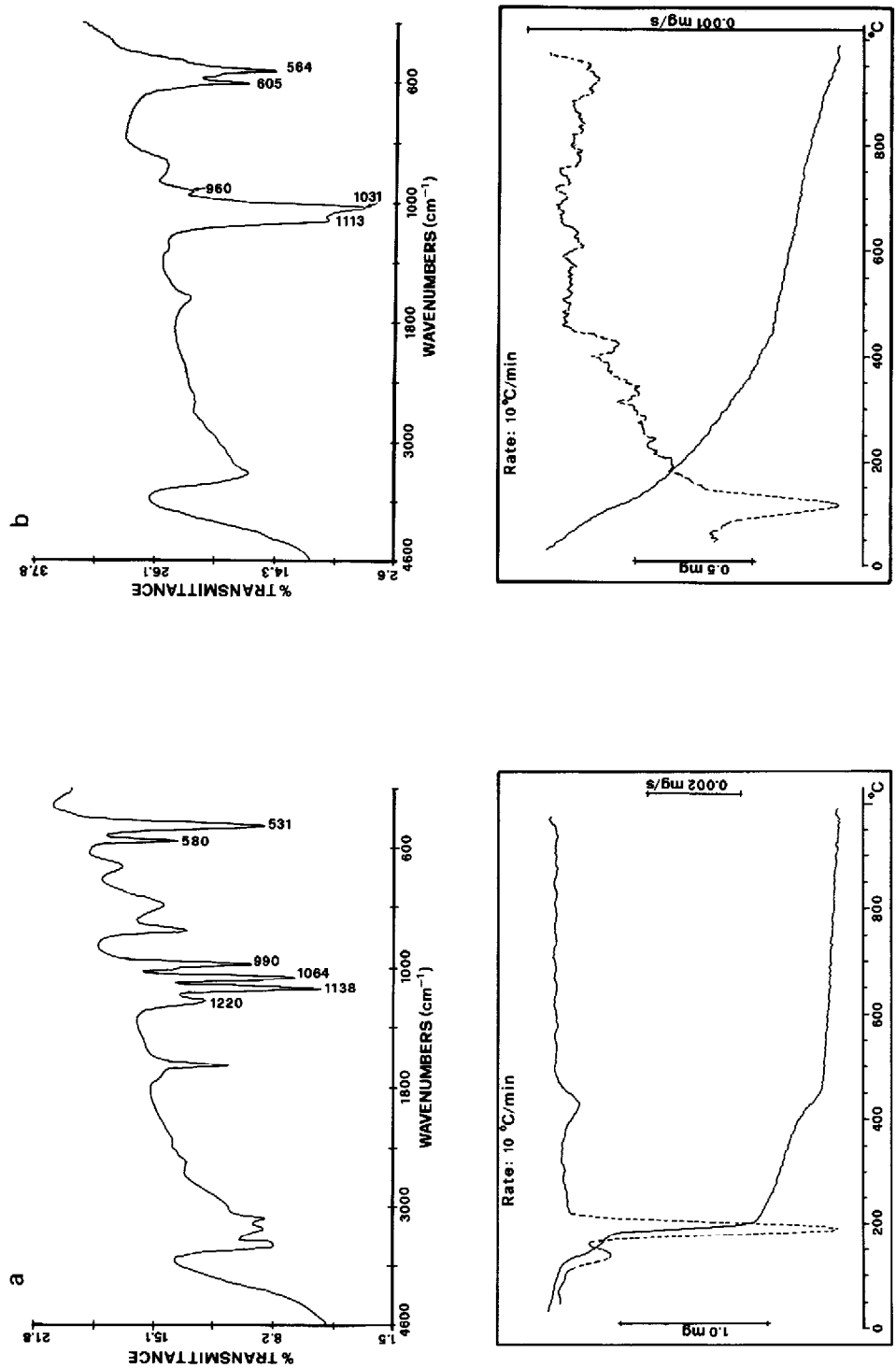

Fig. 2. FT-IR spectra and thermogravimetric analysis of (a) brushite obtained according to procedure B_1 up to 60°C and (b) octacalcium phosphate obtained according to procedure B_2 at 95°C.

For the deposition of CaP–HA two different procedures were adopted; in the first step of procedure A Ca^{2+} ions were adsorbed on the silica surface by evaporation *in vacuo* of a suspension of silica gel and a $CaCl_2$ solution of known concentration; a convenient volume of sodium phosphate solution was then added to convert Ca^{2+} ions into calcium phosphate crystals. In the first step of procedures B_1 and B_2, $CaHPO_4 \cdot 2H_2O$ (brushite) crystals were deposited on silica microparticles by slow addition of $CaCl_2$ and sodium phosphate solutions. Sodium phosphate buffers of different pH were tested (B_1, pH = 6.8; B_2, pH = 9.2).

Preliminary precipitation tests, performed at different temperatures in acidic and basic media (without silica microparticles), showed that in procedure B_1 brushite ($CaHPO_4 \cdot 2H_2O$) is obtained up to 60°C[6,17], whereas in procedure B_2, at temperatures higher than 60°C octacalcium phosphate [$Ca_8H_2(PO_4)_6 \cdot 5H_2O$] is formed[6,17]. These results are supported by FT-IR spectra[23–25] and by the thermogravimetric analyses reported in Fig. 2. These matrices were subsequently treated with $Ca(OH)_2$ to convert the brushite to hydroxyapatite[19].

Fig. 3 compares the FT-IR spectra of a mixed matrix (1000-5 DIOL + CaP–HA, matrix V) obtained according to procedure B_1, the glycolated siliceous matrix (Nucleosil 1000-5 DIOL) and a sample of commercial HA. Spectrum (b) and more clearly (c) (the latter obtained by subtracting (a) from (b) show typical absorption peaks at 604 and 562 cm^{-1} of commercial HA crystals. Hence the total conversion of brushite into CaP-HA follows from lack of IR absorption at 580 and 531 cm^{-1} and it is in agreement with the reported results[19] for a similar conversion of brushite into Ca-deficient HA by $Ca(OH)_2$.

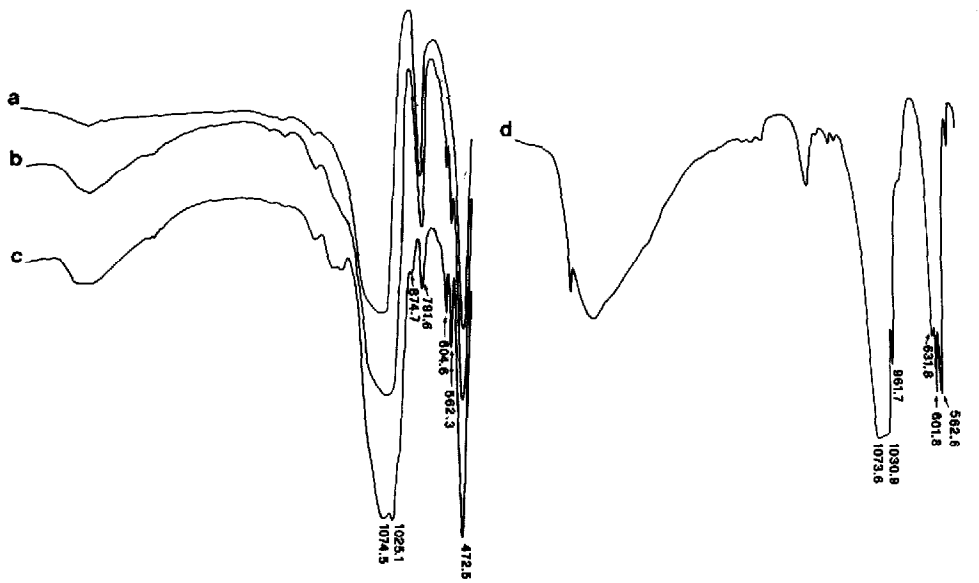

Fig. 3. FT-IR spectra: (a) control siliceous matrix Nucleosil 1000-5 DIOL; (b) Nucleosil 1000-5 DIOL CaP–HA (matrix V) mixed matrix obtained according to procedure B_1 (see Table I); (c) difference spectrum [(b)–(a)]; (d) irregular commercial HA.

Fig. 4 shows some micrographs (2000–5000 × enlargements) of a mixed matrix of 5 μm and 1000 Å porosity, obtained according to procedure B_1 (matrix V; see Table I). The macroporous structure of the support and the uniform distribution of CaP–HA are evident.

Spherical siliceous microparticles of different diameters and porosities were examined. Column kinetic performances were evaluated according to Bristow and Knox[20], and the results are reported in Table I.

It can be seen that CaP–HA supports prepared by procedure B_1 allowed us to adjust and optimize the chromatographic performance of the support in terms of efficiency, permeability and resolution of protein mixtures. The less satisfactory performance of supports prepared by procedure A might be due to the formation of unadsorbed crystals which are subsequently lost, causing a lower column efficiency and permeability.

In order to evaluate kinetic and thermodynamic performances for the separation of biomolecules, we prepared supports of different porosities (500, 1000 and 4000 Å) and particle sizes (5–10 μm); data obtained using different standard protein samples (see examples) showed that the 1000 Å support was the best, because it had a suitable porosity and high mechanical resistance (5000 p.s.i.), thereby ensuring a long column lifetime; moreover, a particle size of 5 μm in conjunction with a porosity of 1000 Å results in a high chromatographic efficiency. However, good efficiency and low column pressure can also be obtained with a particle size of 10 μm.

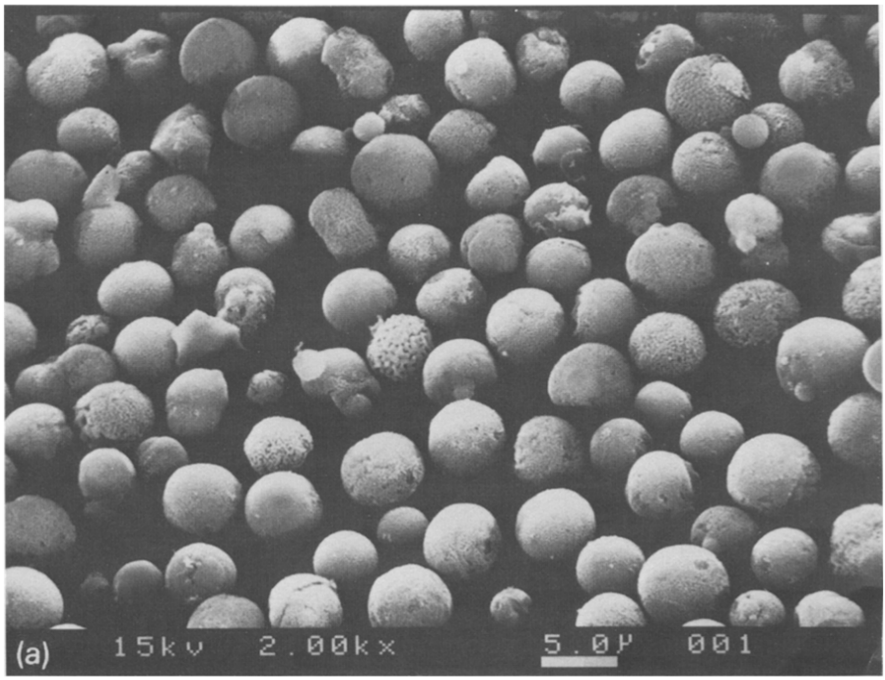

Fig. 4.

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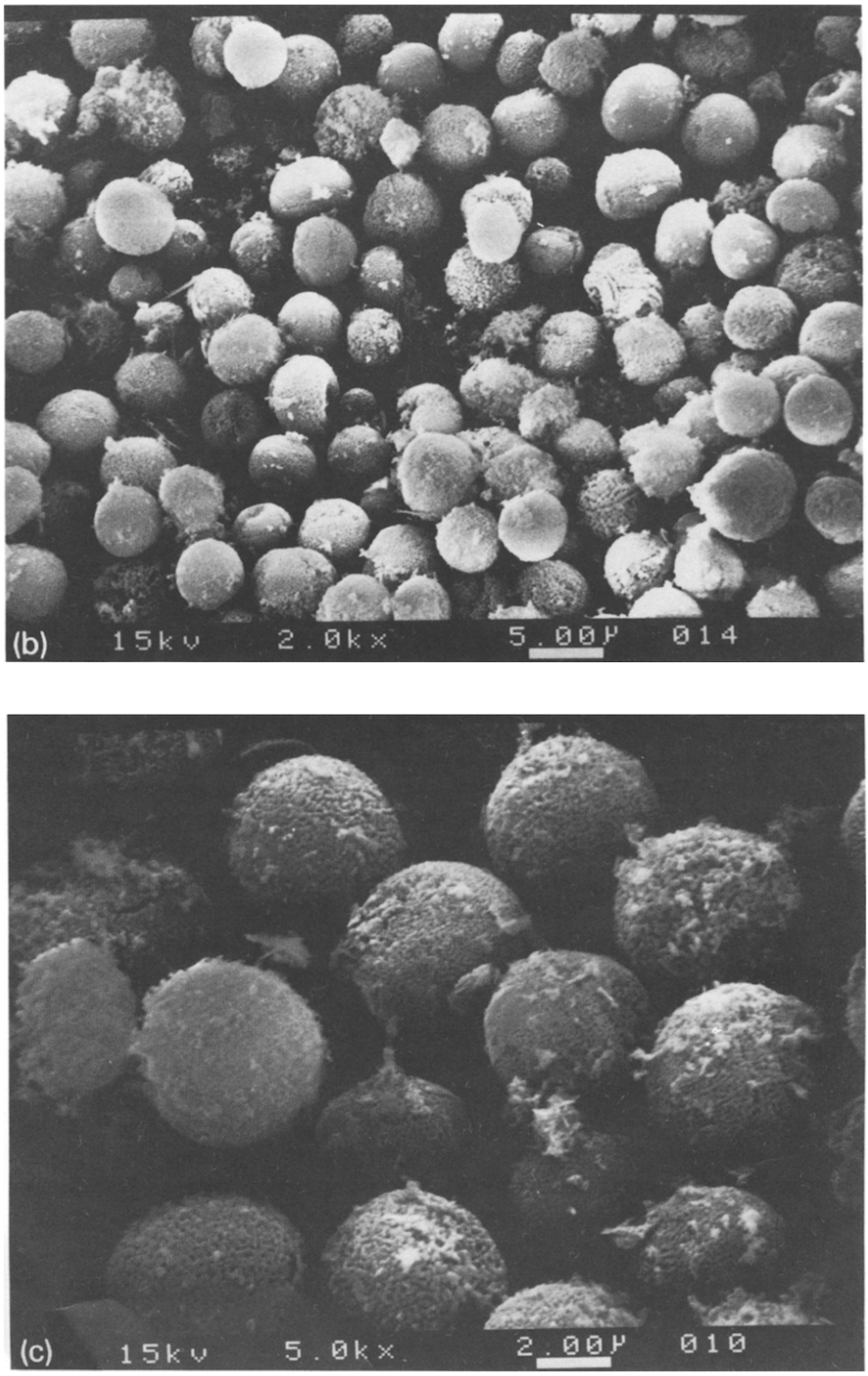

Fig. 4. Micrographs of some supports: (a) Nucleosil 1000-5 DIOL (1:2000); (b) and (c) Nucleosil 1000-5 DIOL CaP–HA (matrix V, see Table I) (1:2000 and 1:5000, respectively).

Applications

In order to investigate the separation properties of the mixed CaP–HA matrix columns, protein samples (single proteins and protein mixtures) were loaded onto the column and eluted with a linear phosphate gradient. The identity of each peak in a protein mixture was established by means of an internal standard, and/or by comparison of the capacity factors (k') of the peaks with those of the individual protein components.

We achieved separations of complex mixtures of proteins using CaP–HA mixed matrices; the small particles gave sharp peaks with minimum tailing at high flow-rates, allowing a fast analysis and more sensitive UV detection. The resolving power of the columns was demonstrated by separating a standard protein mixture containing transferrin, myoglobin, lysozyme and cytochrome *c* (Fig. 5). These chromatograms also illustrate the chromatographic behaviour of acidic and basic proteins: acidic proteins (transferrin, p*I* 5.5–5.9; myoglobin, p*I* = 7.2) are eluted at lower sodium phosphate concentrations than basic proteins (lysozyme, p*I* = 10.7; cytochrome *c*, p*I* 9.8–10.3). The interaction of acidic and basic moieties of proteins with hydroxyapatite crystals has been extensively studied by several groups[8,27–30].

A blank experiment with the above mixture of model proteins on a column packed only with WP-DIOL 1000-5 shows a very low retention of the basic proteins (lysozyme and cytochrome *c*), and no retention of the acidic proteins.

The support macroporosity and the reduced particle size allow very fast kinetics of the chromatographic process. Fig. 6 shows the results obtained in the separation of the same protein mixture using a flow-rate twice that employed for the chromatogram in Fig. 5; the elution is faster with no decrease in resolution.

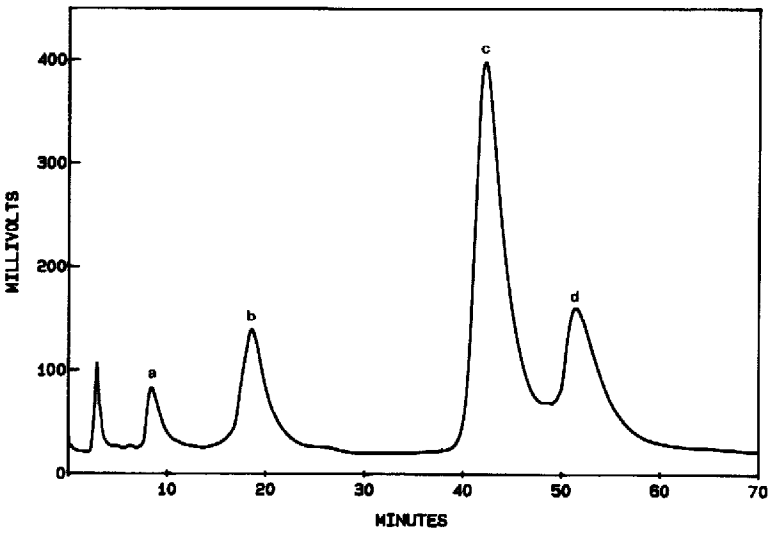

Fig. 5. Separation of a protein mixture containing (a) transferrin, (b) myoglobin, (c) lysozyme and (d) cytochrome *c* (oxidized form). Packing: Nucleosil 1000-5 DIOL CaP–HA 2.5% (matrix V) (100 mm × 6.0 mm I.D.). Linear gradient of sodium phosphate (pH 6.8), 1–350 mM (60 min); flow-rate, 1.0 ml/min; temperature, 25°C; detection, UV (280 nm).

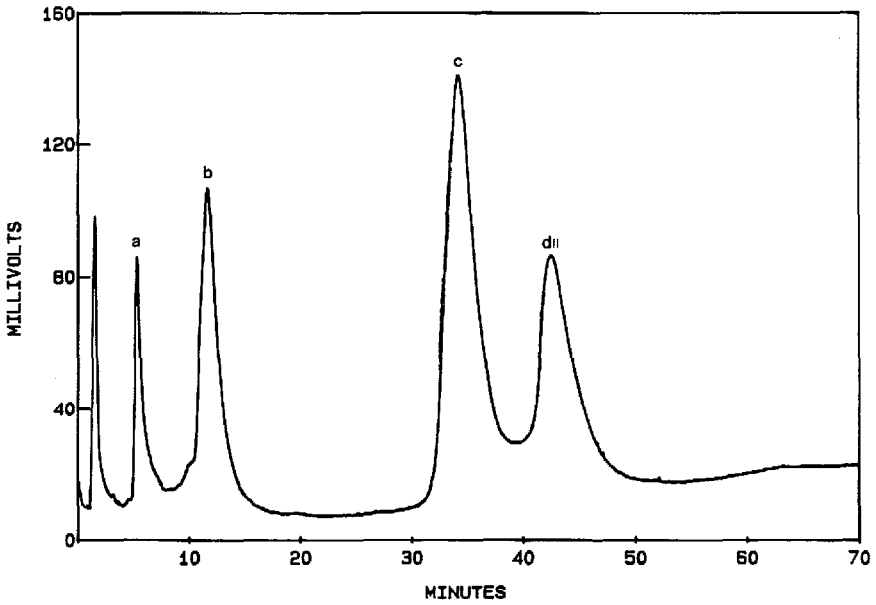

Fig. 6. Separation of a protein mixture containing (a) transferrin, (b) myoglobin, (c) lysozyme and (d) cytochrome *c* (oxidized form). Flow-rate, 2.0 ml/min; other conditions as in Fig. 5.

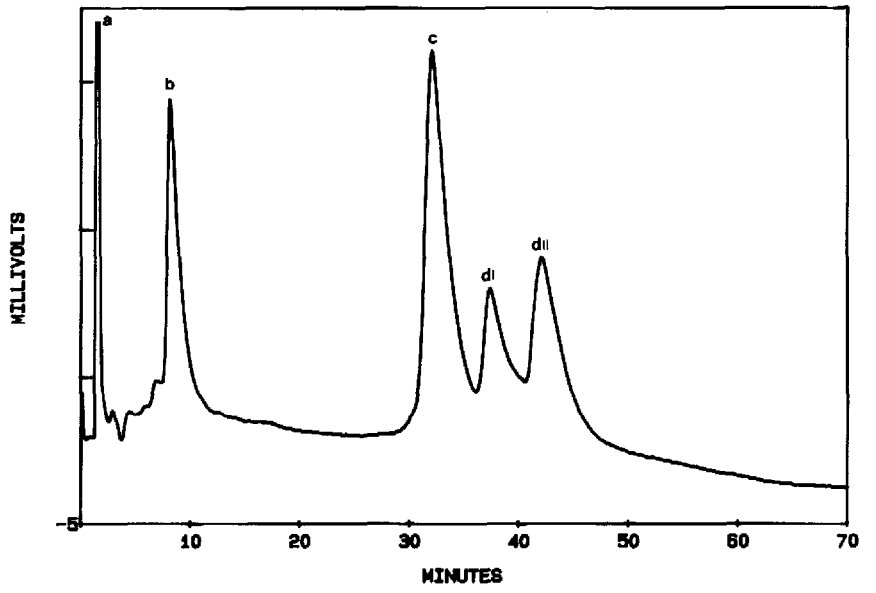

Fig. 7. Separation of a protein mixture containing (a) transferrin, (b) myoglobin, (c) lysozome and (d) cytochrome *c* (I = reduced; II oxidized). Temperature, 40°C; other conditions as in Fig. 5.

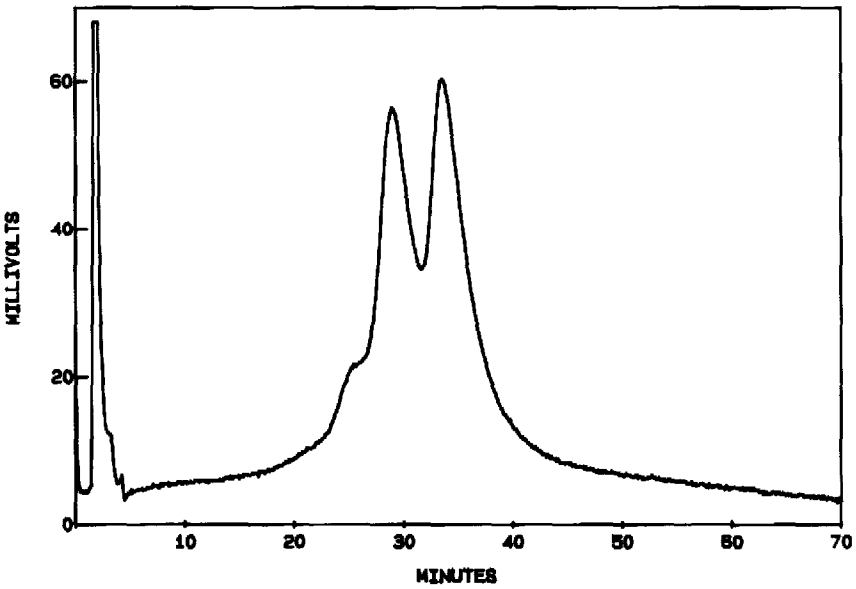

Fig. 8. Anti-melanoma monoclonal antibody (IgG class). Conditions as in Fig. 5.

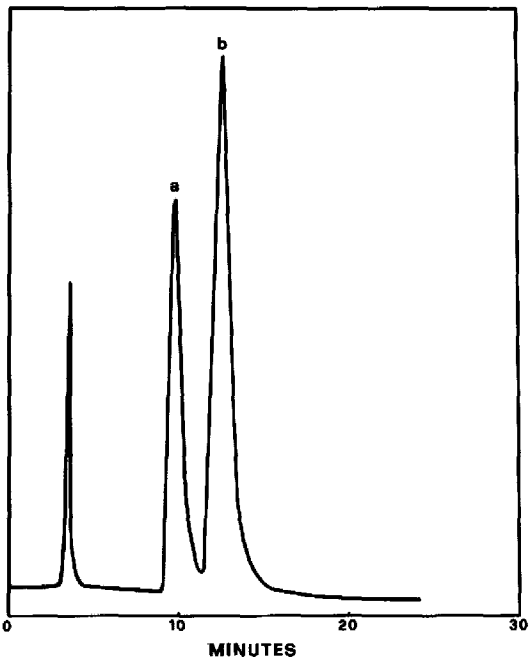

Fig. 9. Separation of (a) D,L-carnitine and (b) acetyl-D,L-carnitine. Packing: Nucleosil 1000-10 DIOL CaP–HA (matrix VI) (100 mm × 6.0 mm I.D.). Eluent, acetonitrile–water (80:20, v/v); flow-rate, 2.0 ml/min; temperature, 25°C; detection, UV (220 nm).

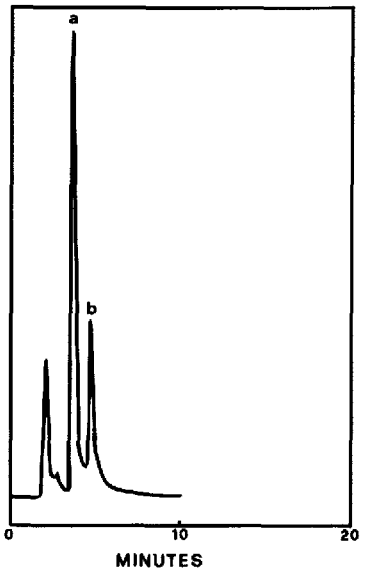

Fig. 10. Separation of (a) fructose and (b) maltose. Packing: Nucleosil 1000-10 DIOL CaP–HA (matrix VI) (100 × 6.0 mm I.D.). Conditions as in Fig. 9.

The column can also be utilized at higher temperatures; Fig. 7 shows the separation of the standard protein mixture performed at 40°C; the transferrin is very little retained under these conditions, whereas a better resolution is obtained for lysozyme and cytochrome *c*, the latter being completely separated in both its oxidized and reduced forms.

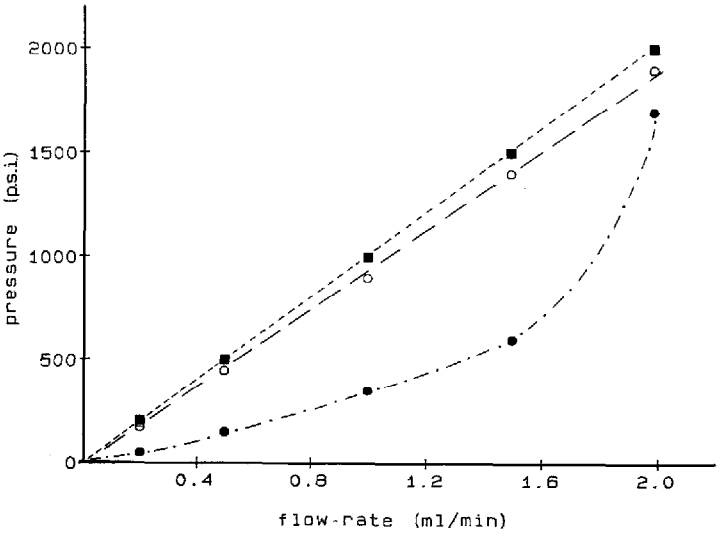

Fig. 11. Flow-rate *versus* pressure. ● = Irregular HA (10 μm, 30 × 4.6 mm I.D.; ○ = matrix III, Nucleosil 500-5 DIOL (5 μm, 50 × 4.0 mm I.D.); ■ = matrix V, Nucleosil 1000-5 DIOL (5 μm, 50 × 4. 0 mm) I.D. Mobile phase, 1 m*M* sodium phosphate (pH 6.8); temperature, 25°C.

These supports have proved to be particularly selective in the separation of monoclonal antibodies, as shown in Fig. 8 for an IgG-type monoclonal antibody.

Apart from analytical and semi-preparative separations of proteins, the column can also be used for the separation of small molecules containing quaternary ammonium groups. Fig. 9 shows a separation of some carnitine derivatives; good selectivity, peak symmetry and high efficiency are evident. A good separation of small polar molecules, such as sugars, can also be achieved (Fig. 9)[31].

The columns have been repeatedly used at high flow-rates over a period of 6–12 months with no reduction in column performance (kinetic or thermodynamic). Fig. 10 indicates the relationship between pressure and flow-rate; its linearity confirms the great mechanical resistance of the support. The use of a 0.45-μm prefilter before the analytical column is an advisable precaution. The usable pH range is 4.5–8.5.

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