Journal of Chromatography, 386 (1987) 273–282
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 961

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF BIOLOGICAL MACROMOLECULES ON NEW SILICA-BASED ION EXCHANGERS

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SUMMARY

The fundamental characteristics of a new series of ion-exchange columns, specifically designed for high-speed analysis of biological macromolecules, have been examined. Each of these columns is packed with a chemically modified silica gel, that has a controlled pore-size distribution and a uniform particle diameter. Surface silanol groups of these materials are chemically modified with hydrophilic polymers to produce a neutral silica surface, and ionic functional groups are chemically bonded to this neutral support, producing a stable ion exchanger. The separation of biological macromolecules on these columns has been investigated, and the selection of a mobile phase is discussed.

INTRODUCTION

High-performance ion-exchange chromatography (HPIEC) is one of the most useful separation techniques for the biochemist. Biological macromolecules are separated according to their surface charge in solution by means of gradient elution, and separated solutes can be recovered in good yield with little loss of bioactivity.

A recent advance in HPIEC is the development of different types of packing materials for the separation of proteins and oligonucleotides[1–6]. Since Regnier and co-workers developed suitable inorganic matrices for biomolecules[3–5], some packing materials have become commercially available[6–9]. However, the method of preparing the packing materials affects the separation of biopolymers and the stability of these materials. Pearson *et al.*[10] have demonstrated the contribution of various large-pore-diameter silica matrices to the resolution of proteins in reversed-phase chromatography, and Kopaciewicz *et al.*[11] have examined the influence of the coatings of silica gel in an attempt to correlate physicochemical characteristics of the stationary phase with protein retention. Therefore, it may be possible to produce even more suitable packing materials for practical separations of macromolecules.

This paper describes the fundamental characteristics of a new series of silica-based ion-exchange columns and the use of these columns for the separation of a complex mixture of biopolymers. The effect of mobile phase composition on protein retention is also examined.

0021-9673/87/$03.50

EXPERIMENTAL

Equipment

Liquid chromatography was carried out with a Shimadzu Model LC-4A pumping system (Shimadzu, Kyoto, Japan), a Shimadzu Model SPD-2A spectrophotometric detector, and a Shimadzu Model C-R2A data processor. Sample injection was achieved with a Rheodyne Model 7125 injector (Rheodyne, Berkeley, CA, U.S.A.).

Columns

The columns used in this study were the Shimadzu Shim-pack WAX-1 (weak anion exchanger), WAX-2 (weak anion exchanger), and WCX-1 (weak cation exchanger), as shown in Table I. The WAX-1 is an anion-exchange column designed specifically for the rapid separation of nucleotides and oligonucleotides. This column contains a spherical silica support which has a particle diameter of 3 μm and a pore size of 100 Å. Surface silanol groups are chemically modified with a hydrophilic polymer to produce a neutral silica surface. A weakly basic stationary phase is chemically bonded to this neutral silica support, producing a stable anion exchanger.

TABLE I

COLUMN SPECIFICATION

Type	*Particle size (μm)*	*Pore size (Å)*	*Functional group*	*Column dimension*
WAX-1	3	100	*tert.*-Amino	50 × 4 mm I.D.
WAX-2	5	300	*tert.*-Amino	50 × 4 mm I.D.
WCX-1	5	300	Carboxyl	50 × 4 mm I.D.

The WAX-2 is an anion-exchange column for protein separations. This column is packed with chemically bonded spherical silica gel with a particle diameter of 5 μm and a pore size of 300 Å. The chemical modification of the WAX-2 is similar to that of the WAX-1.

The WCX-1 is a cation-exchange column for biological macromolecules. A weakly acidic stationary phase is chemically bonded to the hydrophilic polymers that forms the neutral silica surface. The silica support of WCX-1 is the same as that of the WAX-2. All columns were prepared by slurry-packing into 50 × 4 mm I.D. stainless-steel tubes.

Chemicals and reagents

Oligodeoxyribonucleotides synthesis was carried out with the phosphotriester method, using a Shimadzu Model NS-1 automatic DNA synthesizer. Control haemoglobin was obtained from Helena Laboratories (Beaumont, TX, U.S.A.). All other proteins and nucleotides were purchased from Sigma (St. Louis, MO, U.S.A.). Other reagents were of analytical-reagent grade and used without further purification. All buffers were filtered through a membrane filter of 0.22-μm pore size.

RESULTS AND DISCUSSION

Column stability

The lifetime of ion-exchange columns is determined by many factors. The choice of operating conditions (buffer components, salts, pH) is important. Stout and DeStefano[12] examined the stability of hydrophilic silica materials for gel chromatography. They found an increase in the retention time of lysozyme which was proportional to the amount of bonded phase lost due to hydrolysis.

Fig. 1 shows the result of a stability test with a WAX-2 packing material in a weakly basic solution. The pH of the mobile phase was adjusted to 9.2 in order to accelerate the degradation of the material. Glycyl-L-tyrosine was used to determine the permeation volume of the column. Under these conditions, no changes were observed in the retention times of both lysozyme and glycyl-L-tyrosine after 192 h of continuous elution.

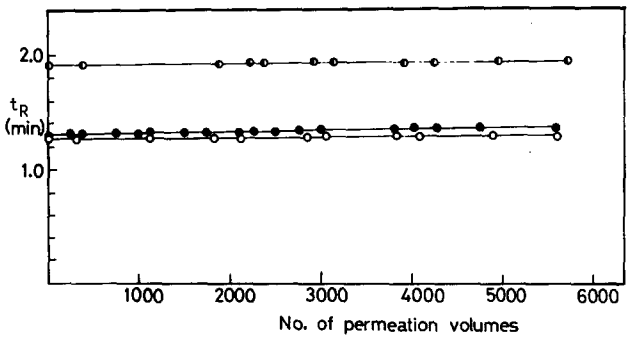


Fig. 1. Dependence of retention times on the number of permeation volumes. Column, WAX-2, 150 × 4-mm I.D.; mobile phase, 0.02 *M* borate buffer (pH 9.2), 0.1 *M* sodium sulphate; flow-rate, 1 ml/min; samples: (●) lysozyme, (○) myoglobin, (◑) glycyl-L-tyrosine; detection wavelength, 280 nm.

The lifetime of the WAX-2 column in the anion-exchange mode was investigated by making repetitive injections of protein mixtures. The results of 10 trials are listed in Table II along with the experimental conditions. Column equilibration and protein elution were carried out at intervals of 120 min. The retention data in Table II indicate that the packing material of the WAX-2 is quite stable in the mildly basic buffers, which may be chosen for the anion-exchange separation of proteins. The results of a similar study with the WAX-1 and WCX-1 revealed equivalent stability of the packing material. However, these columns would not be subjected to such basic conditions in normal usage.

Separation of oligodeoxyribonucleotides on WAX-1

Anion-exchange chromatography is a useful technique for the separation of oligonucleotides. The resolution may be increased by decreasing the particle diameter. The material of WAX-1 is as stable as that of WAX-2 in buffer solutions of high ionic strength. The separation of twelve nucleotides was achieved in 25 min as shown in Fig. 2.

TABLE II

VARIATION OF THE RETENTION TIME OF THE PROTEIN PEAKS ON THE WAX-2 COLUMN

Flow-rate, 1 ml/min; oven temperature, 35°C; detector wavelength, 280 nm; eluent A, 0.02 *M* phosphate buffer (pH 8.0); eluent B, 0.5 *M* phosphate buffer (pH 8.0); elution program, 40-min linear gradient from 0 to 100% B; sample concentration, 0.5% each; injection volume, 50 μl. Samples were injected at 120-min intervals.

Injection number	*Retention time (min)*	
	Ovalbumin	*Bovine serum albumin*
1	8.71	11.80
2	8.70	11.84
3	8.70	11.79
4	8.68	11.83
5	8.69	11.80
6	8.72	11.80
7	8.73	11.78
8	8.73	11.78
9	8.71	11.78
10	8.72	11.80
Mean	8.71	11.80
C.V. (%)	1.8	2.2

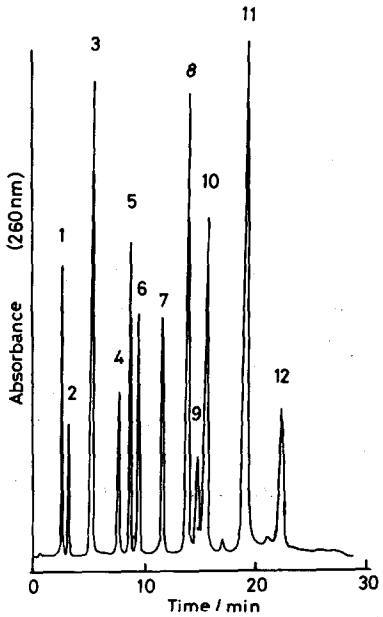


Fig. 2. Separation of a mixture of twelve nucleotides. Column, WAX-1; flow-rate, 1 ml/min; oven temperature, 35°C; eluent A, 0.02 *M* phosphate buffer (pH 7.0); eluent B, 0.48 *M* phosphate buffer (pH 6.85); gradient, 10 min 0–50% B, 5 min 60–100% B, 10 min 100% B; peaks: 1 = 5'-UMP, 2 = 5'-CMP, 3 = 5'-AMP, 4 = 5'-GMP, 5 = 5'-UDP, 6 = 5'-CDP, 7 = 5'-ADP, 8 = 5'-UTP, 9 = 5'-GDP, 10 = 5'-CTP, 11 = 5'-ATP, 12 = 5'-GTP.

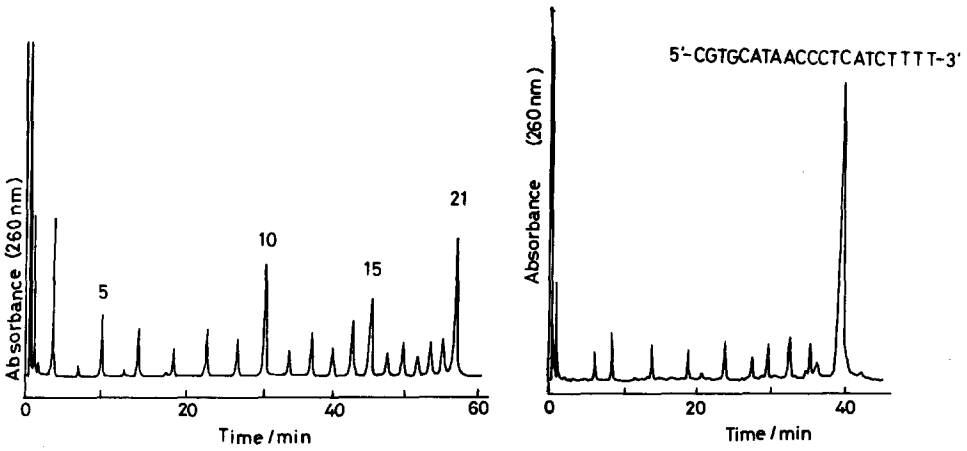


Fig. 3. Chromatogram of a mixture of synthetic oligothymidylates. Column, WAX-1; flow-rate, 1 ml/min; oven temperature, 35°C; eluent A, 20% acetonitrile in 0.01 *M* phosphate buffer (pH 6.8); eluent B, 20% acetonitrile in 0.3 *M* phosphate buffer (pH 6.8); elution program, 120-min linear gradient from 0 to 100% B. Chain lengths are indicated in the figure.

Fig. 4. Chromatogram of synthetic oligodeoxyribonucleotides. Elution, 60-min linear gradient from 0 to 100% B. Other conditions as in Fig. 3.

Mixtures of oligonucleotide were completely separated. Fig. 3 shows the chromatogram of a mixture of synthetic oligothymidylates with chain length of up to 21 bases. The fractionation of synthetic oligodeoxyribonucleotides is shown in Fig. 4. Small peaks indicate by-products in the condensation reaction. A separation with quantitative recovery was obtained for a mixture of oligonucleotides of up to 21 bases in as little as 60 min. We conclude that the WAX-1 can be applied for fast separation of nucleotides and oligonucleotides.

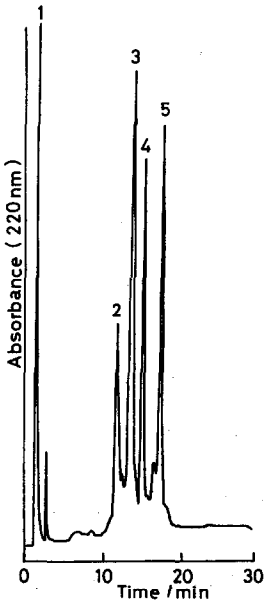
Anion-exchange chromatography of proteins on WAX-2

On a WAX-2 column, packed with a wide-pore, silica-based anion exchanger for protein separations, it was possible to fractionate proteins in 20 min or less. Fig. 5 shows the separation of a standard protein solution on the WAX-2. The ion-exchange process is largely governed by electrostatic interactions, but protein retention is the result of the interaction between the surface charge (not the net charge) of the proteins and that of the packing[13]. Consequently, the elution order of the proteins shown in Fig. 5 did not follow the order of p*I* values.

In an attempt to evaluate the capacity of the WAX-2, mixtures of up to 3 mg were injected. As shown in Fig. 6, similar chromatograms were obtained. These results indicate that the WAX-2 is suitable for the purification of trace proteins in complex mixtures.

The selectivity of an ion-exchange column is greatly dependent upon the mobile phase composition[14]. Chromatograms of a mixture of four proteins at different pH values are shown in Fig. 7. The effect of mobile phase pH on the elution order of α-lactalbumin and α_1-acid glycoprotein is readily apparent in Fig. 7.

The choice of salts also affects the retention of proteins on the WAX-2. Fig. 8 shows chromatograms of proteins with different salts in the eluent. For the sepa-



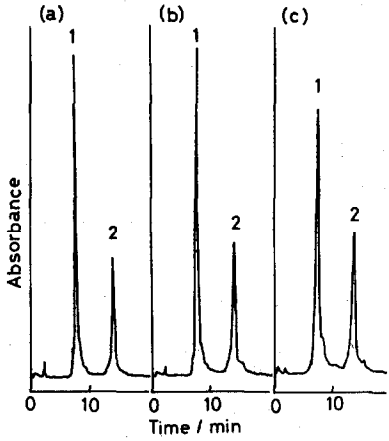


Fig. 5. Chromatogram of a mixture of five proteins. Column, WAX-2; flow-rate, 0.5 ml/min; detection wavelength, 220 nm; eluent A, 0.02 *M* phosphate buffer (pH 8.0); eluent B, 0.5 *M* phosphate buffer (pH 8.0); elution program, 20-min linear gradient from 0 to 100% B; peaks: 1 = myoglobin, 2 = transferrin, 3 = α_1-acid glycoprotein, 4 = α-lactalbumin, 5 = soybean trypsin inhibitor.

Fig. 6. Load capacity effect on the separation of proteins. Column, WAX-2; flow-rate, 1 ml/min; eluent A, 0.02 *M* Tris–H_2SO_4 (pH 7.5); eluent B, 0.5 *M* sodium sulphate in A; elution program, 20-min linear gradient from 0 to 100% B; peaks: 1 = transferrin, 2 = soybean trypsin inhibitor; sample loading: (a) 100 μg each, (b) 1 mg each, (c) 3 mg each; detection wavelength, 300 nm; sensitivity, (a) 0.04 a.u.f.s., (b) 0.16 a.u.f.s., (c) 0.32 a.u.f.s.

ration of α_1-acid glycoprotein and α-lactalbumin, sulphate was more effective than chloride. These data show that the composition of the mobile phase is important with respect to protein separation.

The separation of the A and B genetic variants of β-lactoglobulins, which differ by only 0.1 pH unit in their isoelectric points, is shown in Fig. 9. β-Lactoglobulins are made up of two different kinds of polypeptide chains: α-chains and β-chains. β-Lactoglobulin A (made up of two α-chains) differs from β-lactoglobulin B (made up of two β-chains) by two amino acid residues in the β-chain: Asp, found in the α-chain at position 64, is replaced by Gly in the β-chain. Val, found in the α-chain at position 118, is replaced by Ala in the β-chain. Except for these two differences, the amino acid sequence is identical in the α- and β-chains.

Cation-exchange chromatography of proteins on WCX-1

A mixture of five proteins was well resolved in 2 min on the WCX-1 column (Fig. 10). Analytical conditions were similar to those employed with carboxymethyl-type materials. Most proteins which cannot be retained on the WAX-2 can be separated on the WCX-1.

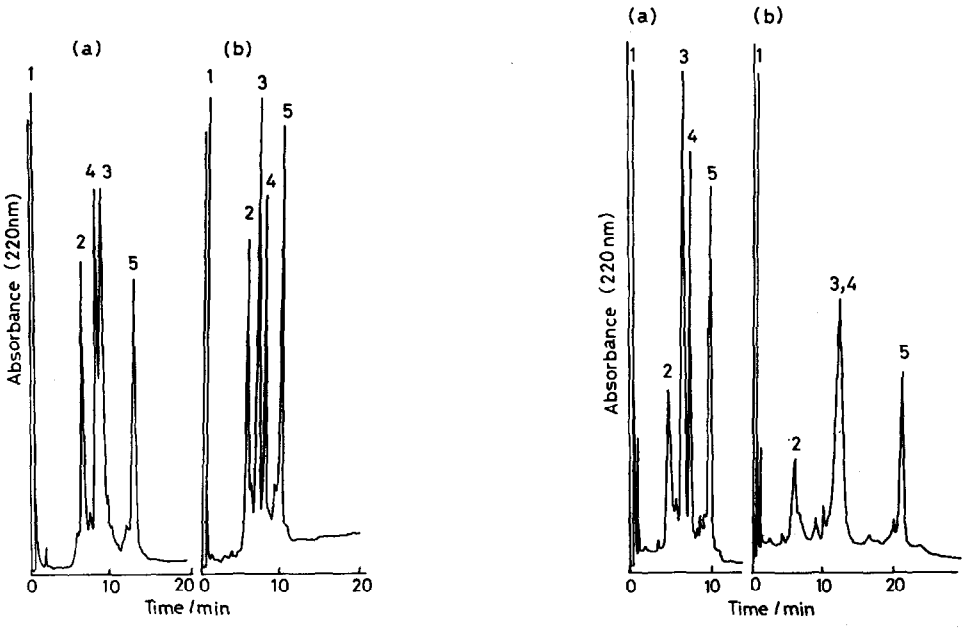


Fig. 7. Chromatographic profiles of a mixture of five proteins at different pH values. Column, WAX-2; flow-rate, 1 ml/min; eluent A, 0.02 *M* phosphate buffer, (a) pH 6.0, (b) pH 8.0; eluent B, 0.5 *M* phosphate buffer, (a) pH 6.0, (b) pH 8.0; elution, 20-min linear gradient from 0 to 100% B. Peaks are labelled using the same numbers as in Fig. 5.

Fig. 8. Chromatographic profiles of a mixture of five proteins with two anionic species. Column, WAX-2; flow-rate, 1 ml/min; eluent A, 0.02 *M* phosphate buffer (pH 8.0); eluent B, (a) 0.5 *M* sodium sulphate in A, (b) 0.5 *M* sodium chloride in A; elution program, 20-min linear gradient from 0 to 100% B. Peaks are labelled using the same numbers as in Fig. 5.

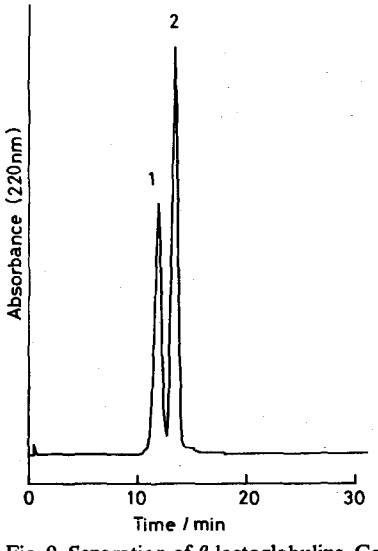


Fig. 9. Separation of β-lactoglobulins. Column, WAX-2; flow-rate, 1 ml/min; eluent A, 0.02 *M* phosphate buffer (pH 8.0); eluent B, 0.5 *M* sodium sulphate in A; elution program, 20-min linear gradient from 0 to 100% B; peaks: 1 = β-lactoglobulin B, 2 = β-lactoglobulin A.

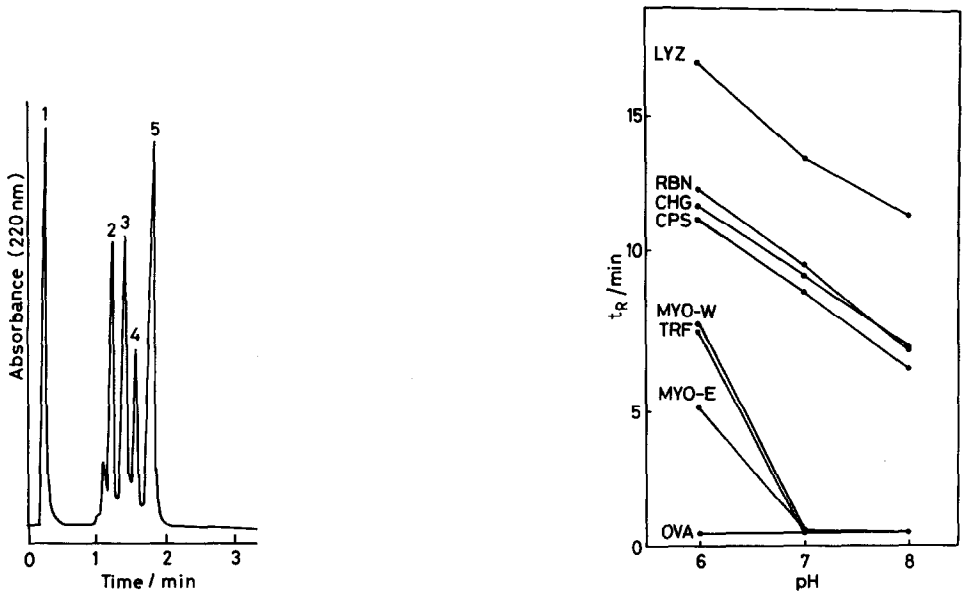


Fig. 10. Fast separation of a mixture of five proteins. Column, WCX-1; flow-rate, 2 ml/min; eluent A, 0.02 *M* phosphate buffer (pH 6.0); eluent B, 0.5 *M* sodium sulphate in A; elution program, 1-min linear gradient from 0 to 50% B; peaks: 1 = ovalbumin, 2 = myoglobin (whale muscle), 3 = α-chymotrypsinogen A, 4 = ribonuclease A, 5 = lysozyme.

Fig. 11. Dependence of the retention time of proteins on the pH of the mobile phase. Column, WCX-1; flow-rate, 1 ml/min; eluent A, 0.02 *M* phosphate buffer; eluent B, 0.5 *M* phosphate buffer; elution program, 20-min linear gradient from 0 to 100% B; abbreviations, MYO-E = myoglobin (equine), MYO-W = myoglobin (whale), TRF = transferrin, CPS = α-chymotrypsin, CHG = α-chymotrypsinogen A, RBN = ribonuclease A, LYZ = lysozyme, OVA = ovalbumin.

The selectivity of the WCX-1 also depends on the mobile phase composition. Fig. 11 shows the dependence of the retention time of proteins on the pH of the mobile phase. The fact that the retention times increase with decreasing pH suggests a change in protein surface charge. Kopaciewicz *et al.*[12] have shown that many proteins are retained at their respective isoelectric points. As shown in Fig. 11, transferrin was retained at its p*I* (6.0) on the WCX-1. This result is consistent with previous observations in this respect.

The effect of different anions on the cation-exchange chromatography of proteins is shown in Fig. 12. The selectivity relative to ribonuclease A varied considerably with the nature of the anionic species. Gooding and Schmuck[9] have shown the effects of anions on the retention characteristics of proteins in the cation-exchange mode. Protein retention on the WCX-1 was also affected by the cationic species.

Cation-exchange chromatography is a useful technique for the analysis of haemoglobin samples in a clinical environment. Fig. 13 shows the separation of a control mixture of haemoglobin variants. These haemoglobin molecules are made up of different kinds of polypeptide chains. It is well-known that the sickle-shaped haemoglobin (in sickle-cell anemia) differs from normal haemoglobin only by one amino acid in the polypeptide chain. In spite of the similarity of the haemoglobins in amino

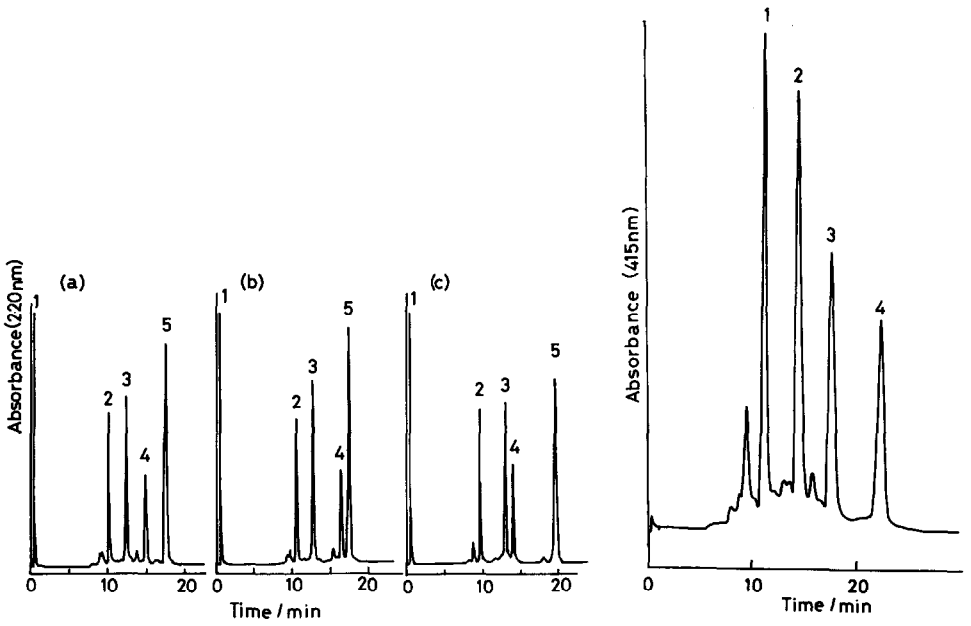


Fig. 12. Chromatographic profiles of a mixture of proteins with various anionic species. Column, WCX-1; flow-rate, 1 ml/min; eluent A, 0.02 *M* sodium phosphate (pH 6.0); eluent B, 0.5 *M* sodium salt in A; gradient rate, 2.5 m*M*/min for Na^+;counter anions, (a) sulphate, (b) chloride, (c) phosphate. Peaks are labelled using the same numbers as in Fig. 10.

Fig. 13. Chromatogram of a control mixture of haemoglobins. Column, WCX-1; flow-rate, 1 ml/min; eluent A, 0.02 *M* phosphate buffer (pH 6.5) containing 0.004 *M* potassium cyanide; eluent B, 0.1 *M* sodium sulphate in A; elution program, 20-min linear gradient from 0 to 100% B; peaks: 1 = Hb F, 2 = Hb A, 3 = Hb S, 4 = Hb C.

acid sequence, haemoglobin variants were well separated on the WCX-1 column using gradient elution.

CONCLUSIONS

A new series of ion-exchange column can be used for the high-speed analysis of biological macromolecules. These columns are quite stable, even in weakly basic solutions, and should prove reliable in practical use for the separations of nucleotides, oligonucleotides, and proteins. It is important to select the appropriate mobile-phase conditions for the optimization of practical separations. Applications of these columns to the preparative purifications of biomolecules will be the subject of future research.

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