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RAPID PROTEIN PROFILING WITH A NOVEL ANION-EXCHANGE MATERIAL

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SUMMARY

A new anion-exchange material has been developed which allows very rapid resolution of protein mixtures. The Microanalyzer™ MA7P matrix consists of small (7 μm), spherical, non-porous, polymethacrylate beads with polyethyleneimine (PEI) covalently coupled to the surface. When packed 30 × 4.6 mm I.D. columns, this matrix is particularly well suited for applications in which 1–300 μg of a protein mixture must be resolved in a minimum of time. Recoveries of injected proteins are usually quantitative, even when the amounts of individual proteins are in the submicrogram range.

Chromatography on Microanalyzer MA7P columns is characterized by very narrow bandwidths, even at relatively high flow-rates. This is due to the combined effects of short column length, high selectivity, and the lack of velocity-dependent bandbroadening attributable to diffusion into and out of pores. These columns have no discernable gel filtration effects in the molecular weight range from 10^3 to 10^6 daltons. Columns are very rapidly equilibrated with new solvents, further reducing cycle-to-cycle times.

INTRODUCTION

Two major types of chromatographic column materials are currently being used for analytical anion-exchange high-performance liquid chromatography (HPLC) of proteins. These are porous silica materials, like AX-300 (Synchrom, Linden, IN, U.S.A.) and Bakerbond (J. T. Baker, Phillipsburg, NJ, U.S.A.)[1–6], and organic polymer-based materials, such as DEAE-5PW (Toyo Soda, Tokyo, Japan) and Mono-Q (Pharmacia, Uppsala, Sweden)[6–9]. Also, two classes of ion-exchange functional groups are used on these supports. The first type consists of isolated point charges (tertiary or quaternary amines) distributed across the surface of the bead[6–8]. The second type consists of short polycationic chains, *e.g.* polyethylenimine (PEI), crosslinked onto the surface of the support[1,2,5]. In many applications, PEI-containing matrices perform better in protein separations due to their higher capacities and improved selectivities[1–6].

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The materials described above were designed for packing columns which allow relatively large masses of protein (up to 100 mg) to be loaded onto a single column. However, these columns have some deficiencies when very small samples (0.2 mg or less) are applied. In these cases, intraparticulate diffusion resistance results in unnecessary bandspreading, and entrapment of protein in the microporous space results in poor recovery. The ion-exchange capacities, the available surface areas, and the column volumes are all in great excess over what is required for the separation. The result is that there is more bandspreading than is necessary for these small sample applications with a concommitant loss of sensitivity, recovery, and speed of separation. The goal of this project was to design an anion-exchange column specifically tailored for very rapid separations in the numerous biochemical research applications in which small amounts (under 0.2 mg) of proteins must be resolved.

Previous work[10] has shown that very rapid separations could be accomplished using non-porous (or pellicular) materials, 40–70 μm in diameter. Those materials suffered from very low capacities and poor chemical stability[2,10,11]. In the current work, a new material (Microanalyzer MA7P), with PEI covalently coupled to 7 μm, non-porous, polymethacrylate spheres, is shown to be an excellent material for the rapid resolution of protein mixtures. The PEI coating results in improved capacity and selectivity compared to other non-porous suports. This support is chemically stable in the pH range from 2 to 12. Protein bands eluted from columns packed with this non-porous material were extremely sharp when compared to bands eluted from identical columns packed with porous silica materials containing similar ion exchange functionality. This effect is especially pronounced under high speed chromatographic conditions. This reduction in peak width is attributable to the fact that there is no diffusion of solutes into and out of pores. A higher capacity form of this material (Microanalyzer MA7P+) is useful in the analysis of mixtures of small peptides.

MATERIALS AND METHODS

Columns

Microanalyzer MA7P (Cl^- binding capacity = 100 μequiv./ml) and Microanalyzer MA7P+ (capactity = 300 μequiv./ml) cartridge columns were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. Electron micrographs of the non-porous beads used to synthesize these matrices are shown in Fig. 1 (electron microscopy was performed with an ISI SX-40 scanning electron microscope). This material was shown to be non-porous in a chromatographic sense, since columns packed with these materials were unable to separate thyroglobulin (molecular weight = 670000) from vitamin B_{12} (molecular weight = 1350) by gel filtration chromatography in high salt buffers[13]. Columns were 30 × 4.6 mm cartridges packed with appropriate material. Cartridges were housed in Bio-Rad cartridge holders.

HPLC system

The HPLC system used in these studies was a Bio-Rad Protein Microanalyzer™ System, consisting of two Model 1330 pumps, a gradient mixer (1.8 ml volume), and either a Model 7125 manual injector or a Model AS-48 autosampler.

Connections between the mixer and the injector, between the injector and the column, and between the column and the detector were kept to a minimum by using

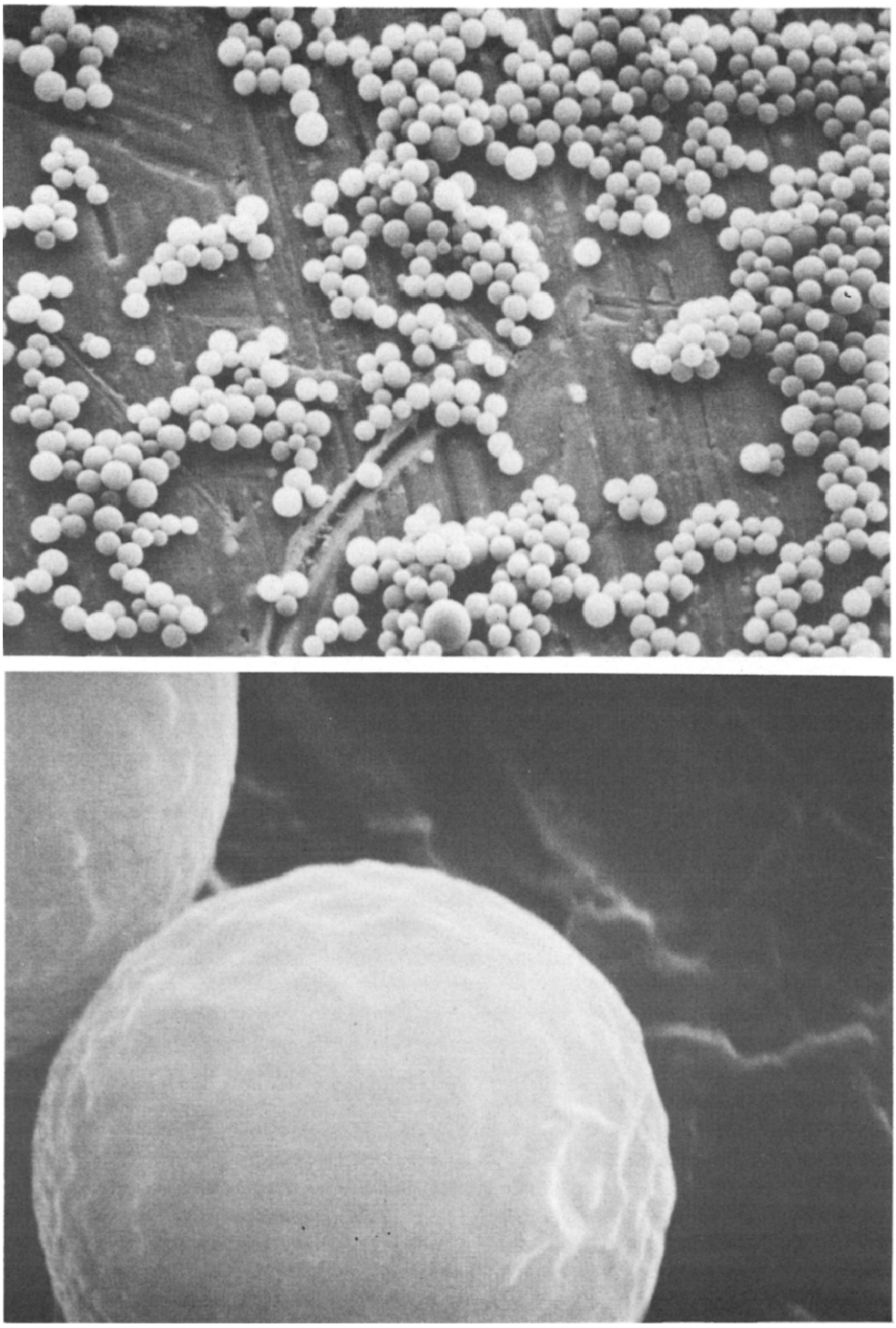

Fig. 1. Scanning electron micrographs of non-porous beads, used to make Microanalyzer MA7P and Microanalyzer MA7P+. Top micrograph: 527×, 1.0 cm = 20.1 μm. Bottom micrograph: 15 820×, 1.0 cm = 0.67 μm.

short lengths of 0.01 in. I.D. tubing. The tubing length from the injector to the detector was 10 cm. Since the detector cell had a volume of 8 μl, the extra-column volume (injector through detector) was about 20 μl. Cartridges had a bed volume of 500 μl, and a void volume of approximately 175 μl.

The system was operated by an Apple IIe computer with dual disk drive and Bio-Rad Gradient Processor System (Version 3.7) software. Data from the Bio-Rad Model 1305A detector was integrated with a Model 3392A integrator, interfaced with the computer.

Materials

All buffer solutions were made with distilled, deionized water and reagent grade solutes. Human hemoglobin A was donated by Bio-Rad Clinical Division, Richmond, CA, U.S.A. Horse myoglobin, conalbumin, ovalbumin, insulin, and bovine serum albumin were obtained from Sigma, St. Louis, MO, U.S.A. Hemoglobin AFCS and Hemoglobin A/C standard mixtures were obtained from IsoLab (Akron, OH, U.S.A.). Carbonic anhydrase and glucagon were obtained from Calbiochem (San Diego, CA, U.S.A.). Soybean trypsin inhibitor was from United States Biochemicals (Cleveland, OH, U.S.A.). The Protein-A MAPS kit with buffers was obtained from Bio-Rad and was used according to the manufacturer's recommended protocol[10]. Ascites fluid P3X63 was obtained from Dr. Lesley Walker, Scripps Clinic, San Diego, CA, U.S.A., and ascites fluid L1 was obtained from Dr. Roger Walker, Bio-Rad Clinical Division. AX-300 cartridges (30 × 4.6 mm) were obtained from Brownlee Labs., Santa Clara, CA, U.S.A. Bakerbond MAb was from J. T. Baker, Phillipsburg, NJ, U.S.A.

RESULTS

Column design optimization

For most small-scale protein separations on a non-porous matrix, a material with a Cl^- binding capacity of approximately 100 μequiv./ml (Microanalyzer MA7P) gave the best overall performance. A material with a capacity of 250–325 μequiv./ml (Microanalyzer MA7P+) performed better in separating peptides. This higher-capacity material may also be better for protein applications that require more retentiveness.

For analytical protein separations, it is important to balance resolution and sensitivity with speed of separation. For the separation of up to 200 μg of proteins using the packings described above, the optimum column dimensions were 30 × 4.6 mm. Longer columns gave slightly better resolution, but resulted in significantly longer analysis time and broader bands than a 30-mm column. Typical 30-mm columns have greater than 300 plates (corresponding to about 10000 plates/meter).

Speed of analysis

The principal goal of this project was the very rapid analysis of protein mixtures. An example showing speed of separation is presented in Fig. 2. A mixture of five proteins (some of them heterogeneous) is separated in a 42-s gradient at 4.5 ml/min. The entire analysis is complete in less than 90 s, and the cycle-to-cycle time is 180 s. It should be noted that a large portion of that cycle time is due to the volume

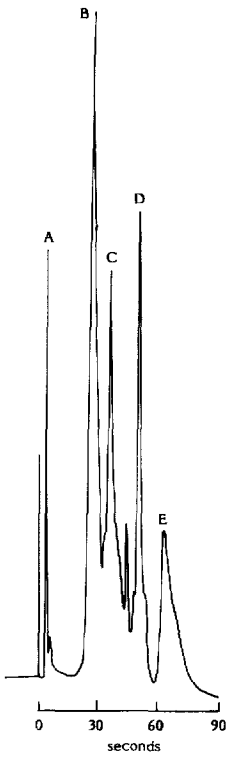

Fig. 2. High-speed separation of a mixture of horse myoglobin (A), human hemoglobin A (B), conalbumin (C), ovalbumin (D), and bovine serum albumin (E). Buffer A, 20 m*M* Tris, (pH 8.5); buffer B, 20 m*M* Tris–0.5 *M* NaCl, (pH 8.5); gradient: 0–100% B in 42 s; flow-rate, 4.5 ml/min.

of the mixer. When a low-volume mixer is used, the time necessary to recycle the column from 0.5 *M* sodium chloride to 0.02 *M* buffer is *ca.* 20 s at 2.0 ml/min (data not shown).

The utility of Microanalyzer MA7P columns in such separations is demonstrated in Fig. 3. Separations are shown at three different flow-rates for both a Microanalyzer MA7P column and a commercially available AX-300 column with the same dimensions (AX-300 is an anion exchanger in which PEI is coupled to a porous silica support). While the AX-300 column does partially resolve the bands of interest, the Microanalyzer MA7P column is superior in resolution, bandwidth, and sensitivity, especially at the faster flow-rates. It should be noted that the AX-300 column is able to resolve this mixture adequately at slower flow-rates. However, when speed of separation is essential, the Microanalyzer MA7P column is superior.

Another example demonstrating the speed and resolution of the Microanalyzer MA7P column is the analysis of mouse ascites fluid. Fig. 4 shows a 6-min analysis of an ascites fluid (P3X63). The IgG in this sample is partially resolved into two peaks and a shoulder. IgG purified from this sample by affinity chromatography on Affi-Gel Protein-A shows the same two peaks and the trailing shoulder (not shown). Fig. 5 shows a separation of the same ascites fluid on a Bakerbond MAb column

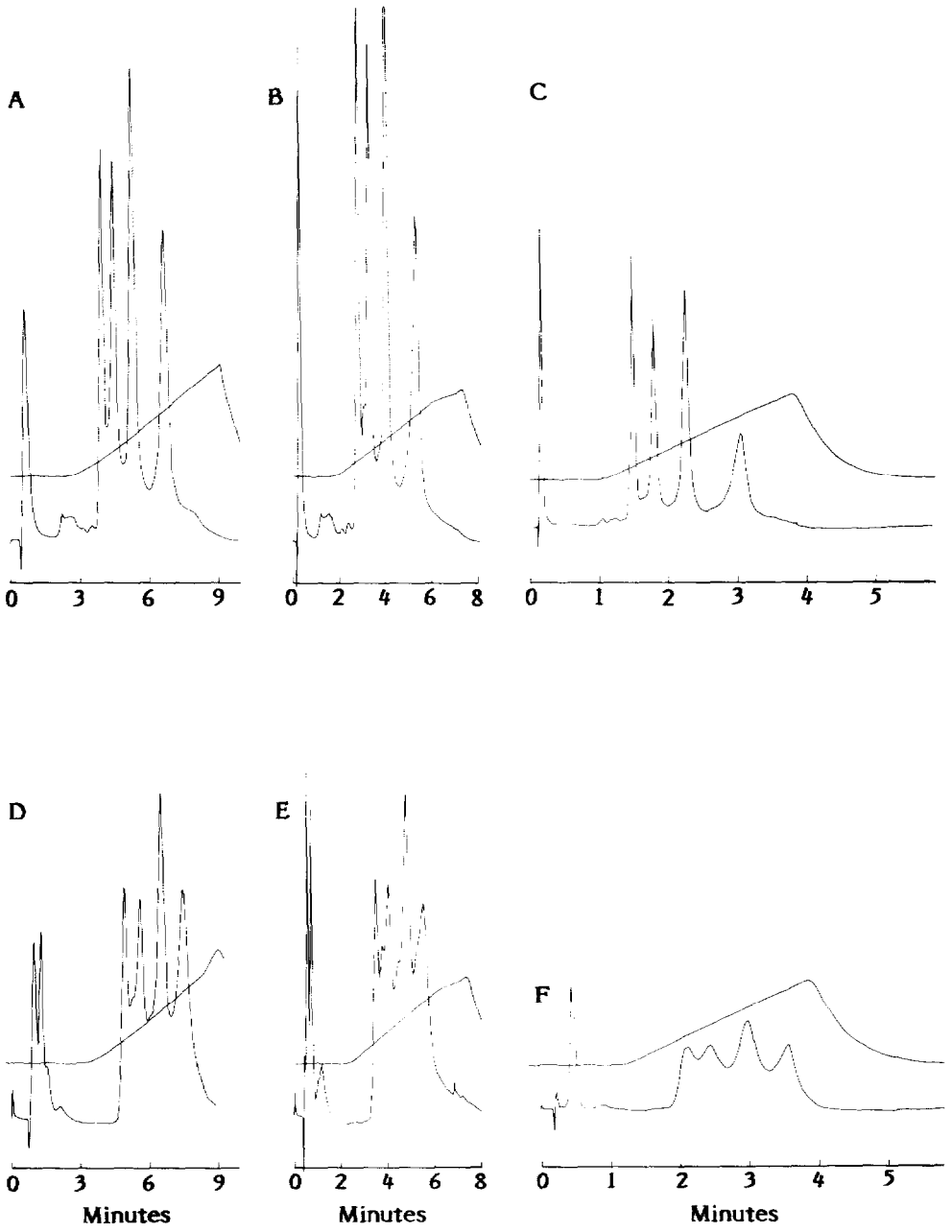

Fig. 3. Comparison of chromatography on Microanalyzer MA7P and AX-300 columns. The Hb AFSC mixture was chromatographed on 30 × 4.6 mm columns of the two materials at three different flow-rates. Panels A, B, and C are chromatograms on Microanalyzer MA7P at 0.5, 1.0, and 2.0 ml/min, respectively. Panels D, E, and F are chromatograms on AX-300 under the same conditions. Buffer A, 16 m*M* Tris chloride (pH 8.5); buffer B, 20 m*M* Tris chloride–0.2 *M* NaCl (pH 8.5). Chromatograms A and D, 0–70% B from 0 to 7 min; chromatograms B and E, 0–50% B from 0 to 5 min; chromatograms C and F, 0–40% B from 0 to 3 min.

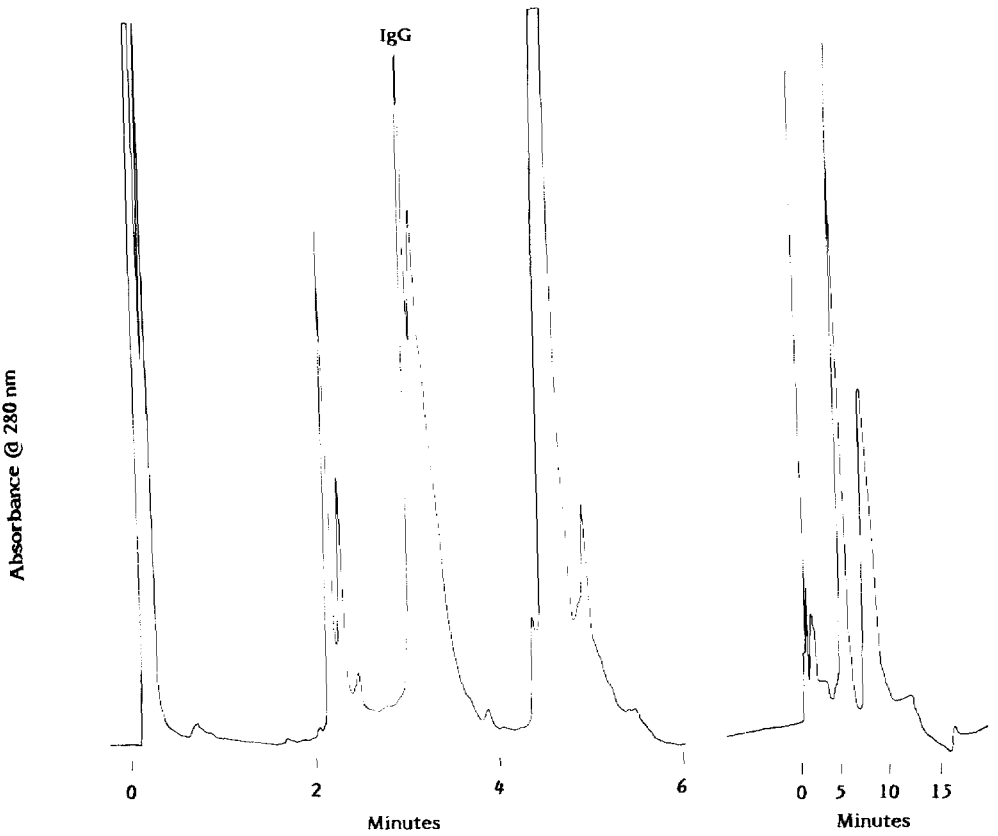

Fig. 4. Analysis of P3X63 ascites fluid on a Microanalyzer MA7P column (30 × 4.6 mm). Buffer A, 16 m*M* Tris (pH 8.5); buffer B, 20 m*M* Tris–500 m*M* NaCl (pH 8.5); gradient, linear from 0 to 30% B in 4 min, step to 100% B; flow-rate, 2.0 ml/min.

Fig. 5. Analysis of P3X63 ascites fluid on a Bakerbond column (30 × 4.6 mm). Buffer A, 16 m*M* phosphate (pH 6.8); buffer B, 500 m*M* phosphate (pH 6.4); gradient, linear from 0 to 25% B in 15 min; flow-rate, 2.0 ml/min.

(packed into the same 30 × 4.6 mm cartridge format). Bakerbond MAb is a porous column material which was designed explicitly for this separation. Note that there is no sign of IgG heterogeneity, and that the analysis time is significantly longer.

Van Deemter plot

Fig. 6 shows a plot of HETP (height equivalent of a theoretical plate) as a function of flow-rate. Over the range from 0.1 to 1.5 ml/min, this particular column has about 6000 plates/meter. Recent improvements in packing procedures allow columns with 10000 plates/meter to be produced routinely. The most interesting feature of this curve is that there is a broad minimum, indicating that high efficiency is retained over a broad range of flow-rates (see also Fig. 3). This is in contrast to the porous AX-300, which has an optimum flow-rate of 0.25 ml/min[4] and which loses protein resolution at higher flow-rates (Fig. 3).

The relatively small number of plates (hence short column length) required for

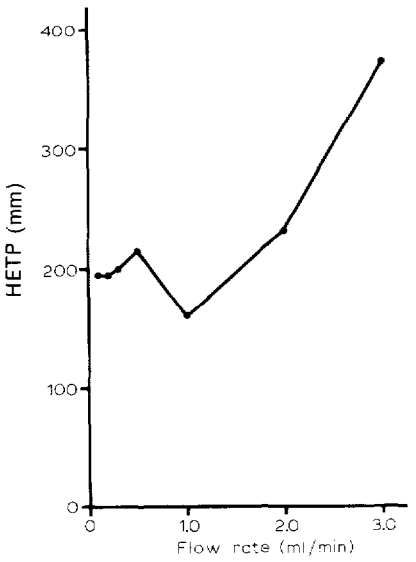

Fig. 6. Plot of HETP as a function of flow-rate for a 30 × 4.6 mm Microanalyzer MA7P column with glycyltyrosine as the non-retained solute. The eluent was 20 m*M* Tris–0.5 *M* NaCl (pH 8.0). Duplicate determinations were obtained at each flow-rate and the average value of HETP was plotted.

protein separations on Microanalyzer MA7P columns show that the PEI coating results in a very high selectivity for protein mixtures[3].

Protein recovery and column capacity

Data on the recovery of various proteins from Microanalyzer MA7P columns are shown in Table I. Recoveries were determined by loading 100 μg of protein onto the column in 20 m*M* Tris buffer at pH 8.5, eluting with buffer containing 0.5 *M* sodium chloride, collecting the protein peak, and determining the amount of protein in the effluent.

An important property of ion exchangers being used in analytical applications

TABLE I

PERCENT RECOVERY OF VARIOUS PROTEINS FOR MICROANALYZER MA7P COLUMNS

Protein	*Recovery (%)*
Hemoglobin A	93
IgG (affinity purified)	91
Bovine serum albumin	100
Ovalbumin	95
Conalbumin	93
Carbonic anhydrase	97
Soybean trypsin inhibitor	96
Insulin	91
Glucagon	89
Average	94

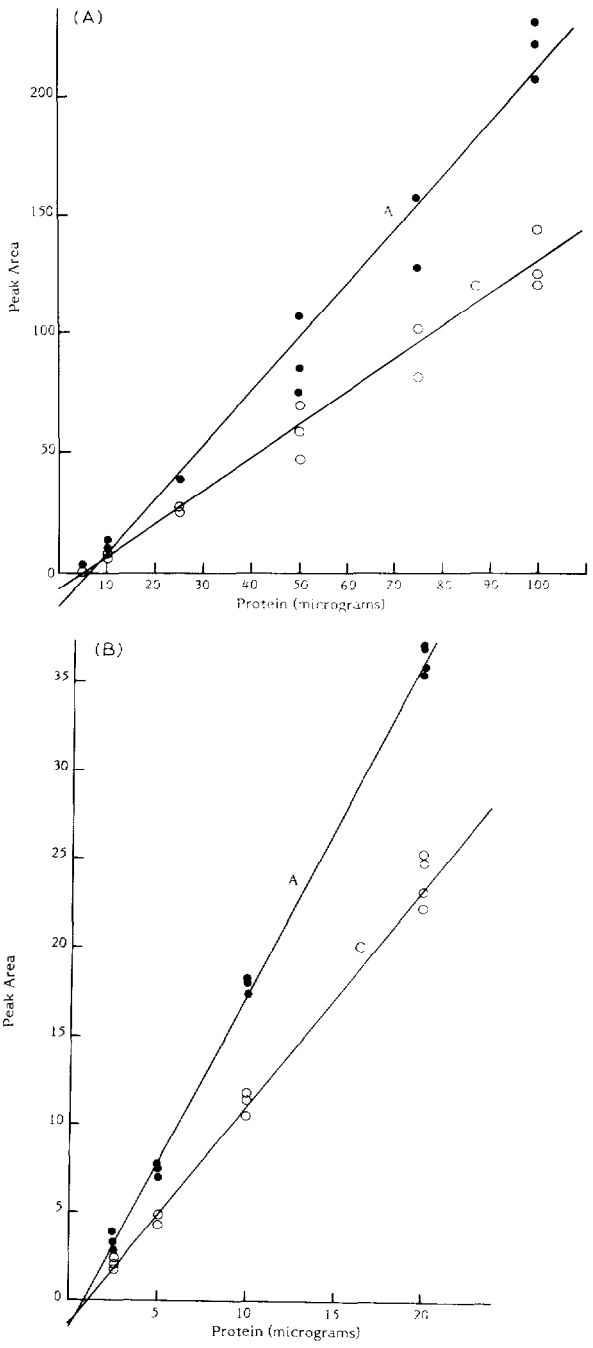

Fig. 7. Linearity of the curve of peak area *versus* amount of protein injected for hemoglobin A and hemoglobin C. A 1.0 mg/ml solution of HbA plus HbC was prepared (62% HbA, 38% HbC). Serial dilutions of this solution were also prepared. Duplicate or triplicate injections of 100 μl of these solutions were analyzed by rapid chromatography on Microanalyzer MA7P columns, as described in the legend to Fig. 3B. Panel A shows peak area *versus* amount injected over the range from 0 to 100 μg of protein. Panel B shows data from a second series of injections over the range from 2.5 to 20 μg of total protein.

in recovery of proteins injected in very small amounts. As shown in Table I, the recovery of hemoglobins is excellent when 100 μg of protein is injected. In order to assess the recovery at much lower loads, we prepared serial dilutions of a mixture of hemoglobins A and C, injected a constant volume (20 μl) of each dilution, eluted with a linear salt gradient, and quantitated the area of each peak in each chromatogram. A plot of peak area *versus* amount of protein injected (Fig. 7) was linear over a wide range of sample loads. High recoveries of proteins have also been claimed for other supports[1,5,7]. However, in those experiments, protein recoveries were not analyzed at such low protein inputs. The experiment shown in Fig. 7 was repeated with the AX-300 column. Recovery of protein was nearly quantitative at 100 μg of input protein, but dropped off sharply below that value (data not shown).

The protein-binding capacities of 30 × 4.6 mm Microanalyzer MA7P columns were determined by two tests. In the first test, protein solutions (in 20 m*M* Tris, pH 8.5) were injected in sequential 0.10-mg shots until protein was detected in the breakthrough volume. In this test, capacities ranged from 3.2 to 4.6 mg protein per ml packing material. The second test consisted of injecting different amounts of a 1:1 mixture of bovine serum albumin (BSA) and ovalbumin (OVA). The BSA and OVA were then resolved by gradient elution with a linear gradient from 0 to 0.5 *M* sodium chloride in 20 m*M* Tris buffer (pH 7.5). The upper limit for separating BSA and OVA was 300 μg of protein. Above this load, band broadening led to a loss of resolution.

As in the case of hemoglobin, the recovery of affinity-purified IgG is excellent (Table I). A plot of peak area *vs.* amount of IgG injected shows that there is a linear response down to 300 ng or less[14]. This shows that recovery of these proteins is excellent, even at very low protein inputs.

Reproducibility and column lifetime studies

The data in Table II show that chromatography on Microanalyzer MA7P columns is highly reproducible, either when one is injecting into the same column repeatedly or injecting the same sample into different columns, packed with the same material. More than 200 injections can be made into a single column (Fig. 8). Columns that are beginning to lose resolution can be regenerated with washing with

TABLE II

COLUMN-TO-COLUMN REPRODUCIBILITY

A mixture of four hemoglobins (A, F, S and C) was injected onto eight different Microanalyzer MA7P columns, and the four peaks were resolved by using the same salt gradient. Values of the mean and standard deviation were computed for the retention times (t), peak-widths at half-height (w), and peak areas (A) of the four bands, as well as the resolution of adjacent pairs of bands (R). Standard deviations are in parenthesis.

Parameter	*HbC*	*HbS*	*HbA*	*HbF*
t	2.22 (0.03)	2.73 (0.03)	3.43 (0.03)	4.71 (0.04)
w	0.157 (0.009)	0.168 (0.013)	0.213 (0.012)	0.312 (0.022)
A	7.00 (0.26)	8.48 (0.46)	14.26 (0.55)	10.54 (0.52)
R	0.78 (0.05)	0.91 (0.03)	1.26 (0.08)	

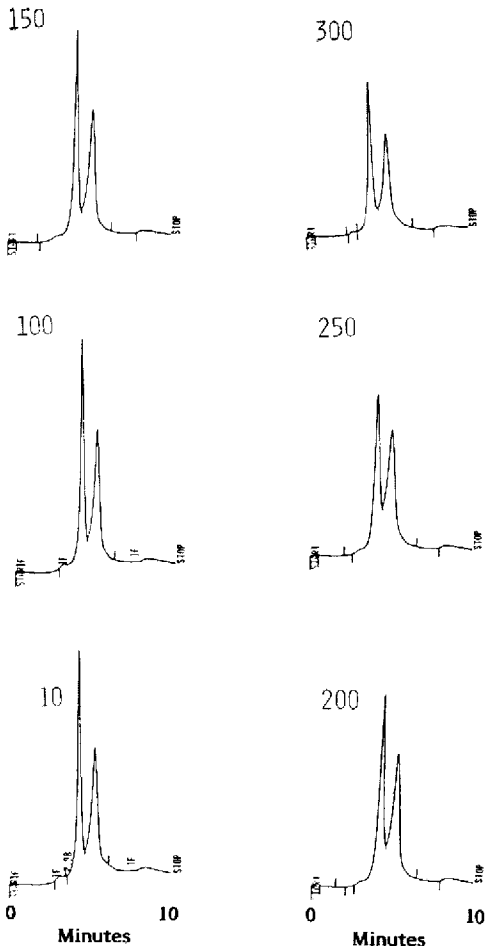

Fig. 8. Column lifetime study. Resolution of the hemoglobin A/F pair is shown as a function of the number of injections. The number of the injection appears in each of the panels. Buffer A, 16 m*M* Tris chloride (pH 8.5); buffer B, 20 m*M* Tris chloride–0.2 *M* NaCl (pH 8.5); gradient, 0–40% B from 0 to 5 min; flow-rate, 2.0 ml/min.

0.05–0.1 *N* sodium hydroxide. The column in Fig. 8 was washed with 0.1 *N* sodium hydroxide prior to the 300th injection.

Applications

As shown in Fig. 3, Microanalyzer MA7P columns are useful for the rapid analysis of clinical samples. In applications where a large number of similar samples must be analyzed quickly, the very short cycle times of these columns are particularly advantageous.

Another potential application of Microanalyzer MA7P columns is on-line monitoring of protein purifications. An example of this type of application is shown in Fig. 9. IgG from Ascites L1 was purified by affinity chromatography on Affi-Gel Protein-A. The purified IgG was then analyzed as shown. The IgG peak correspond-

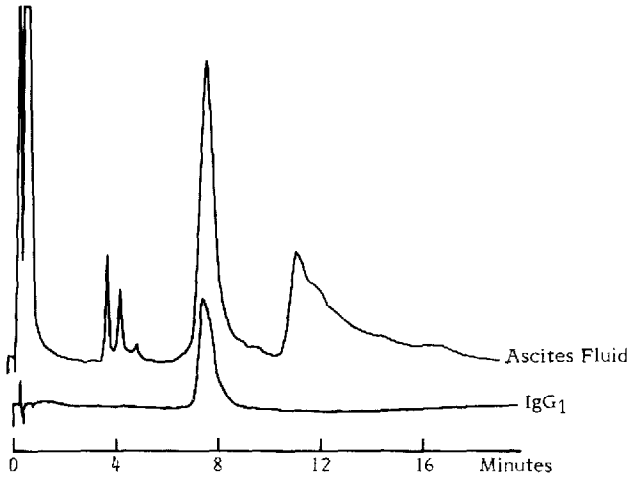

Fig. 9. Analysis of mouse Ascites Fluid L1 (upper tracing) and purified IgG_1 (lower tracing) from that sample on Microanalyzer MA7P. The IgG_1 was purified by chromatography of the sample on Affi-Gel Protein A. Buffer A, 16 m*M* Tris (pH 7.5); buffer B, 20 m*M* Tris–0.5 *M* NaCl (pH 7.5); gradient, 0–100% from 0 to 20 min at 1 ml/min.

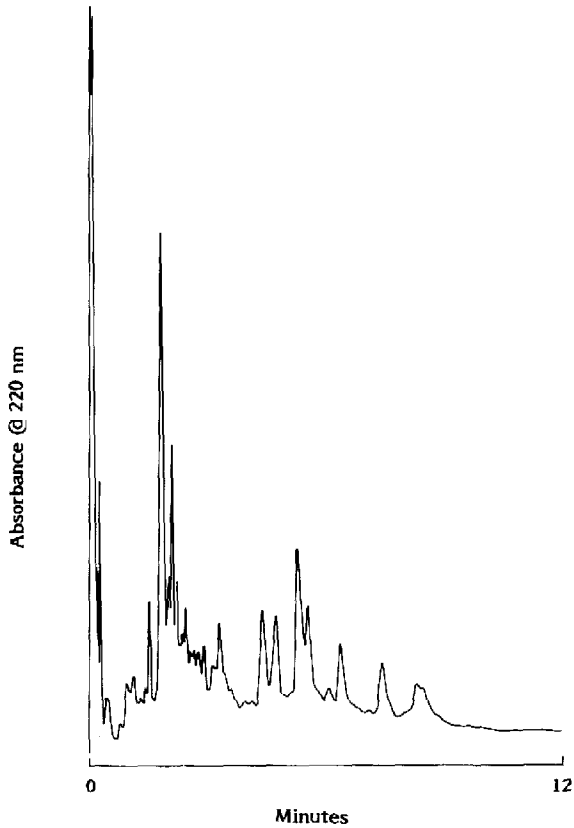

Fig. 10. Analysis of tryptic peptides of bovine serum albumin on a Microanalyzer MA7P+ high capacity anion-exchange column. Buffer A, 20 m*M* Tris (pH 8.5); buffer B, 20 m*M* Tris–1.0 *M* NaCl (pH 8.5); gradient, 0–50% B from 0 to 12 min; flow-rate, 2.0 ml/min.

ed to a well-resolved peak in the chromatogram of the crude ascites fluid. In this particular analysis, it was possible to see that the desired product was acceptably pure. The analysis time for this experiment was 20 min. However, as shown in Fig. 4, it is possible to perform this analysis in a much shorter time by adjusting the flow-rate and gradient steepness.

Fig. 10 shows a chromatogram of the tryptic peptides of bovine serum albumin on a Microanalyzer MA7P+ column. The analysis time was 12 min. This short analysis time would allow the investigator to monitor frequently and accurately the course of the enzyme digestion to determine the end-point.

CONCLUSIONS

Microanalyzer MA7P is a non-porous polymeric HPLC matrix, suitable for very rapid analysis of protein mixtures. Microanalyzer MA7P columns perform better than other available columns in applications where speed of separation is important. In the current format, Microanalyzer MA7P columns are capable of resolving complex mixtures in $\leqslant 5$ min. In addition, for many applications in which "preparative" HPLC entails injection, detection, and recovery of less than 200 μg of protein, Microanalyzer MA7P columns provide a fast, reproducible alternative to the larger but slower columns that are now available for such applications.

It seems remarkable that a 30-mm long Microanalyzer MA7P column can resolve a complex mixture in such a short period of time, in spite of the relatively small number of theoretical plates. Previous analyses suggest that this very high selectivity may result from the multiple points of interaction between the polycation (PEI) on the matrix and complex, polyanionic proteins[3,6]. By taking advantage of this high selectivity, the need for a large number of theoretical plates is reduced. Very short, low-volume columns then become ideal.

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