

Disposable Microbore High-Pressure Liquid Chromatography Columns for Protein and Peptide Separations

Christopher Southan,^{*,1} Patrick Lavery,[†] and Kenneth G. M. Fantom[†]

^{*}Department of Bioinformatics and [†]Department of Molecular Screening Technologies, SmithKline Beecham Pharmaceuticals, New Frontiers Science Park, Third Avenue, Harlow, Essex, CM19 5AW, United Kingdom

Received July 27, 1998

A range of high-performance liquid chromatography (HPLC) columns with internal diameters of 0.25 to 1.8 mm have been constructed by securing glass or plastic tubing into standard HPLC fittings. These were packed with chromatographic materials chosen for operation at moderate pressures with high flow rates. These columns were shown to be effective in a conventional HPLC instrument for peptide and protein separations in reverse-phase mode and for proteins in ion-exchange and size-exclusion modes. The simple construction and low cost of these microbore columns allow them to be considered as disposable. Using only small amounts of any type of packing material, they have the flexibility to be adapted to a wide range of analytical and micropreparative separations. © 1999

Academic Press

Key Words: HPLC; chromatography; microbore; proteins; peptides.

The advantages of reducing the internal diameter of HPLC columns for protein chromatography are well established (1). Gains in detection sensitivity for analytical work are complemented by increases in yield and concentration factors for micropreparative applications. Even where sample material is not limited, a significant saving in solvent usage is gained by reducing column size.

Chromatographic materials are now available which are specifically designed for protein and peptide separations at high horizontal flow velocities with low operating pressures (2, 3). These large-particle, wide-

pore materials are well suited for microbore columns because they can be packed at moderate pressures. This work describes the construction and evaluation of a range of small-i.d. columns that can be easily assembled from standard HPLC fittings and packed with high flow-rate packings. They can be operated in the range of 100 to 500 μ l/min using conventional HPLC instruments.

MATERIALS AND METHODS

All chemicals were analytical grade and all solvents were HPLC grade. The individual standard proteins were obtained from Sigma (Dorset, UK) and the ion-exchange protein standard mixtures were from Bio-Rad (Hertfordshire, UK). The cation-exchange standard mixture, catalogue No. 1250562, contained equine myoglobin, ribonuclease A, and cytochrome *c*. The vial was made up to 1 ml of aq. 0.08% TFA² and then a 1/10 dilution of this solution was injected onto the reverse-phase columns. The anion-exchange standard mixture, catalogue No. 1250561, contained myoglobin, conalbumin, ovalbumin, and trypsin inhibitor. The vial was made up to 1.0 ml in water and a 1/10 dilution was made up for injection onto the anion-exchange columns.

Column Components

A range of PEEK and TEFZEL tubing is available from a variety of manufacturers to fit standard 1/16-in. (1.57 mm o.d.) or 1/8-in. (3.17 mm o.d.) plastic

² Abbreviations used: TFA, trifluoroacetic acid; PEEK, polyethyl ether ketone polymer; KEL-F, tetrafluoroethylene polymer; TEFZEL, ethylene tetrafluoroethylene polymer; BSA, bovine serum albumin.

¹ To whom correspondence should be addressed. Fax: 01279-627666. E-mail: Christopher_D_Southan@sbphrd.com.

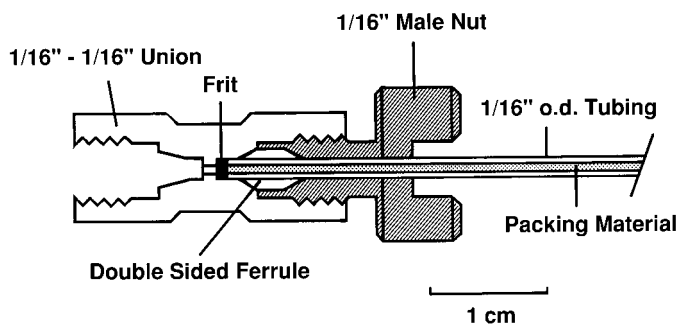


FIG. 1. A column end-fitting. This illustrates the 1/16-in. HPLC fittings constructed as a column bed support. The external view of a complete column is shown in Fig. 3. Equivalent fittings from different manufacturers can be combined. Although the double-sided ferrule is shown here, both single-sided ferrules and one-piece plastic nuts can be used. The 1/8-in. fittings are similar in design to the 1/16-in. model shown here except that only single-sided ferrules are available.

HPLC fittings. For the PEEK columns, the 1/16-in.-o.d. \times 0.75-mm-i.d. tubing (green color coded) was obtained from Upchurch (Anachem, Bedfordshire, UK). For the 1/16-in.-o.d. glass tubing 25 μ l precalibrated capillaries with an i.d. of 0.65 mm and a length of 125 mm was used (Vitrex, from Camlab, Cambridgeshire, UK). The following 1/16-in. fittings from Jour (Alltech, Lancashire, UK) were used: Kel-F low dead-volume unions, acetal male nuts (either hexagonal headed or any of the finger-tight styles), and double-sided Kel-F ferrules. The 1/16-in. frits were obtained from Upchurch (Anachem). The 1/16-in.-o.d. \times 0.25-mm-i.d. and 1/8-in.-o.d. \times 1.5-mm-i.d. TEFZEL tubing was also obtained from Upchurch. The appropriate fittings for 1/8-in. tubing

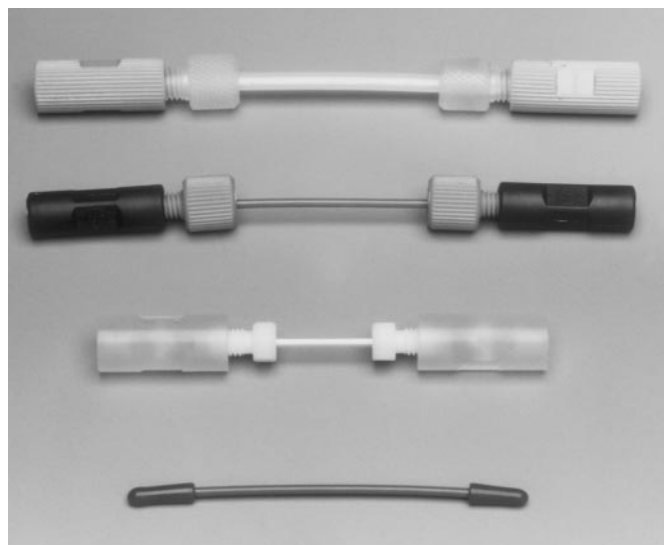


FIG. 3. Illustration of packed columns. In order from the top down these are a 1/8-in.-o.d. \times 1.5-mm-i.d. TEFZEL size-exclusion chromatography column, a shortened version of that used for the results in Fig. 6, a 1/16-in.-o.d. \times 0.75-mm-i.d. PEEK column similar to that used for the reverse-phase separations in Figs. 4a and 7, a 1/16-in.-o.d. \times 0.65-mm-i.d. glass capillary column, similar to that used for the ion-exchange separation in Fig. 5, and a PEEK column adapted for storage after end-fitting removal by the addition of 1/16-in. tubing caps.

consisted of a 1/8- to 1/16-in. adapter, 1/8-in. one-piece male nut, 1/8-in. TEFZEL ferrule, and 1/8-in. frit. These were obtained from either Upchurch (Anachem) or Optimax (JT Biosciences, Dorset, UK).

Column Packings

The Superdex 30 preparative grade 30- μ m particle size-exclusion packing material was obtained from



FIG. 2. Column packing devices. The Poros Self-Pack reservoir (top) is shown with a PEEK column used as an adapter and with a column attached ready for packing. The dry-packing assembly (bottom) is shown with the capillary tube (125 mm) connected to the pipet tip with a small section of silicone tubing and sealed with a 1/16-in. tubing cap.

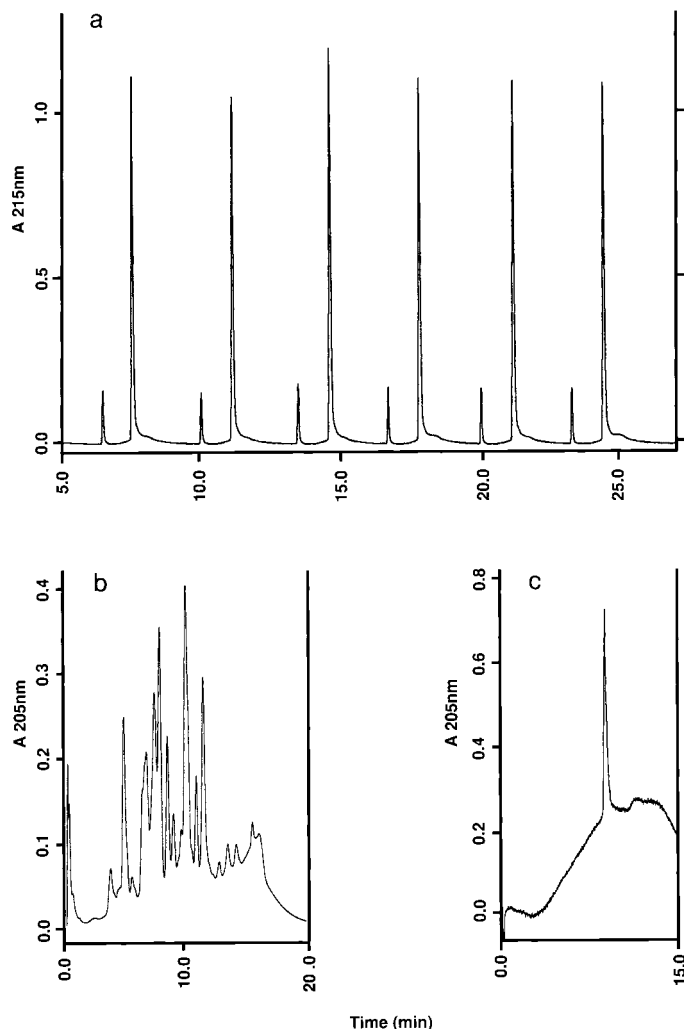


FIG. 4. Reverse-phase chromatography. For all runs solvent A was 0.08% aq. TFA, and solvent B was 0.06% TFA in 80% acetonitrile. All columns were packed with Poros 10- μ m R2. For the high-speed solvent exchange in (a) the column was 0.75-mm-i.d. \times 55-mm PEEK. The consecutive desalt series was run in manual control mode as follows. A gradient of 25 to 85% B in 1 min, at 0.5 ml/min, was started immediately after injection of 2.0 μ g of BSA in 2.0 μ l. After 1 min the solvent was returned to 25% B. As soon as the baseline returned to zero, a second sample was injected and the second gradient of 25 to 85% B was initiated. This cycle was continued for the six successive injections shown above. For the micro-preparative peptide separation shown in (b) the column was 0.65-mm-i.d. \times 110-mm glass capillary. A gradient of 0 to 50% B was run in 15 min at a flow rate of 200 μ l/min. The sample was 2 μ l of a 5 mg/ml tryptic digest of bovine lactoglobulin. For the high-sensitivity protein analysis in (c), the column was 0.25-mm-i.d. \times 50-mm TEF-ZEL clear plastic. A gradient of 10 to 90% B was run for 9 min at 100 μ l/min. The sample was 1 μ l of a BSA standard diluted to 50 μ g/ml, corresponding to 0.05 μ g of protein.

Pharmacia (Bedfordshire, UK). The Poros R2 10- μ m reverse-phase packing and the Poros Q, 10- μ m anion-exchange material, were from PE Biosystems

(Cheshire, UK). The AX-300 7- μ m anion-exchange material was extracted from a PE Biosystems cartridge column. The commercial Poros R2 and Brownlee AX-300 Aquapore columns were from PE Biosystems. For slurry packing the Poros Self-Pack reservoir from PE Biosystems was used. Alternatively a 4.6- or 10-mm-i.d. empty PEEK HPLC column from Upchurch (Anachem) was used as a packing reservoir.

Column Construction

The basic column design is shown in Fig. 1. The support for the chromatographic material is formed by

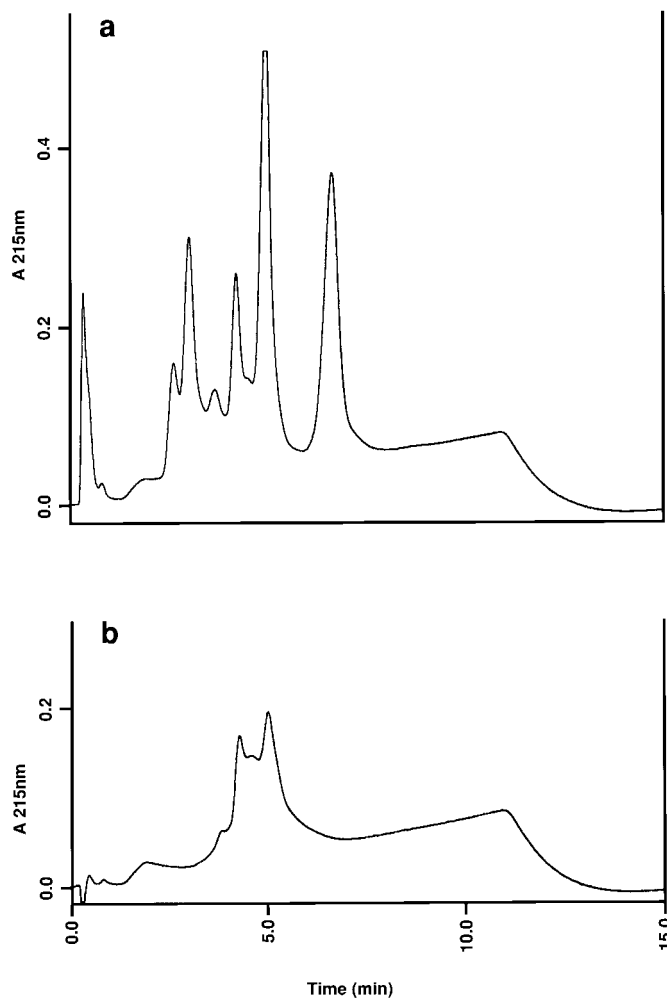


FIG. 5. Anion-exchange chromatography. The column was a 0.65-mm-i.d. \times 100-mm glass capillary packed with 20 μ m Poros Q material. Solvent A was 25 mM Tris at pH 8.0, solvent B was 25 mM Tris at pH 8.0 with 0.5 M NaCl. A gradient of 0 to 100% B was run in 10 min at 200 μ l/min. Chromatogram a was from an injection of 15 μ l of the 1:10 diluted anion-exchange standard mixture. Chromatogram b was an ovalbumin standard, 5 μ g injected in 5 μ l.

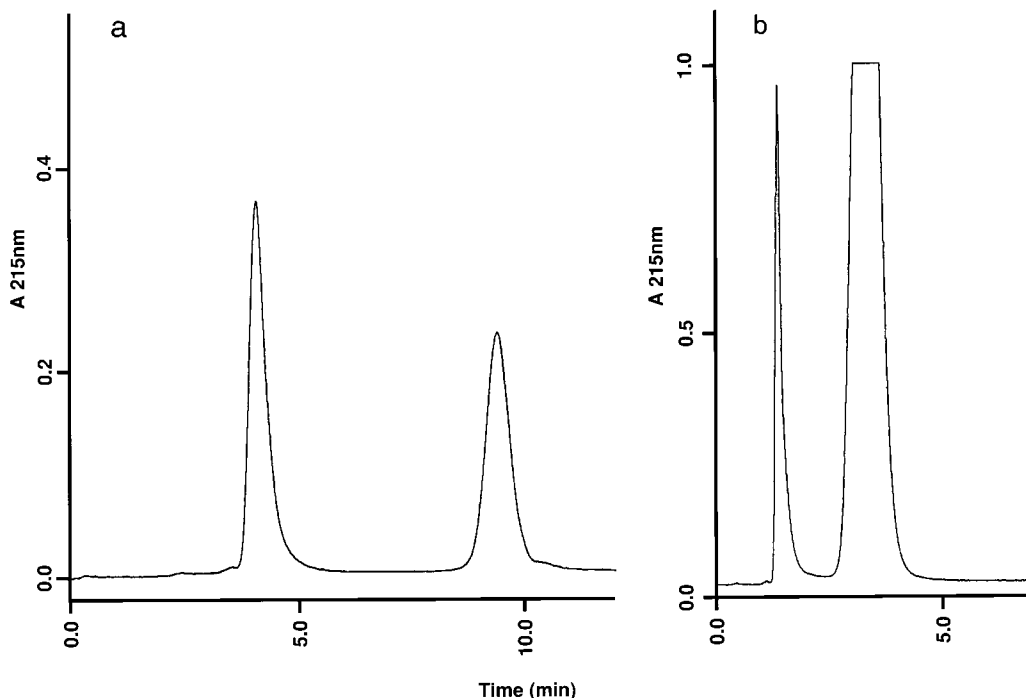


FIG. 6. Size-exclusion chromatography. The column consisted of Superdex 30 material with 30- μm particle size packed into 1.5-mm-i.d. \times 450-mm TEFZEL tubing secured into 1/8-in. fittings. The solvent was 25 mM Tris adjusted to pH 8.0. Chromatogram a shows a BSA standard, 1.5 μg , injected in 1.5 μl , with a flow rate of 100 $\mu\text{l}/\text{min}$. The later-eluting peak is the small amount of sodium azide included in the Sigma standard BSA solution as a preservative. Chromatogram b shows a fast solvent exchange, run at 300 $\mu\text{l}/\text{min}$, of 5 μl of a reduction/carboxymethylation mixture consisting of 0.6 M Tris, 8 M urea, 10 mM DTT, 10 mM iodoacetate, 0.1 M NaOH, and 0.5 μg of BSA.

seating a frit into a union. Tubing is then secured into the union with a ferrule and male nut. Packing material can be either dry-packed or slurry-packed. Dry packing is especially convenient for the transparent glass capillary tubes. A pipet tip served as a reservoir at the top and the tube was plugged with parafilm or a tube cap at the bottom (Fig. 2). Although the loose material could be simply tapped down the tube, particle settling was accelerated by using vibration from a vibrating rotary mixer, an empty sonicator bath, or an engraving tool. Because of the particle dust generated, this was performed in a fume cupboard. The packed tube was then secured into the union with a ferrule, frit, and male nut, as illustrated in Fig. 1. The same fittings, without a frit, were used to assemble the top of the column.

Packing was completed by running the appropriate starting solvent for that packing at approximately 50% higher than the expected operating flow rate. Appropriate separation flow rates are shown under Results. With reverse-phase packings 5% acetonitrile was included in the solvent to ensure complete wetting of the packing. The completion of packing was monitored by equilibration to a constant back-pressure, and, in the case of glass tubing, by observ-

ing a constant bed height. This usually occurred within 30 min and was accompanied by a 20–40% compression of the dry volume. The tubing was then cut down to the bed surface and the column was reassembled ready for use. Typically, a dry-packed 125-mm glass capillary resulted in a 100-mm column bed, or this could be cut and reassembled into two 50-mm columns.

Slurry packing was found useful for the opaque PEEK tubing where the progress of dry packing could not be observed. Reverse-phase material was suspended to approximately 10% by settled volume in 50% acetonitrile. The anion exchange and size exclusion materials were made up as 10–20% aqueous slurries. The Poros Self-Packer is shown in Fig. 2. The empty column was connected to this before the packing reservoir was filled with the appropriate amount of dilute slurry. The end of the reservoir column was then screwed on firmly with the aid of a wrench. For constant-pressure packing the Beckman Gold HPLC pump unit was used. This has the advantage of reducing flow rate when the maximal pressure is reached rather than stopping pump operation.

For the PEEK tubing, packing could be carried out with the maximal pressure set at 2000 psi. Because it was not possible to observe the packing bed, it was found convenient to pack excess tubing lengths of between two to five column lengths (e.g., 10–50 cm). These were equilibrated at 2000 psi over several hours or 1000 psi overnight. By cutting the appropriate lengths of packed tubing from the bottom, several full columns were obtained until the cut exposed empty tubing. For packing Superdex into the TEFZEL tubing the packing pressures were reduced to 500 psi. With this translucent plastic the gel surface is visible so the progress of packing can be observed. The tube could then be cut back to remove any dead volume and reassembled. Slurry packing could also be used for glass tubing provided that packing pressures were held below 1000 psi.

HPLC Instrumentation

A Beckman System Gold HPLC was used fitted with a Rheodyne 8125 injection valve fitted with a 50- μ l PEEK injection loop, a 126 solvent module, and the 167 dual-wavelength detector. The following modifications were made to the standard instrument to reduce system dead volume: (i) exchanging the standard for a microbore dynamic mixer (Beckman), (ii) bypassing the purge valve by direct connection of the mixer outlet to the injector inlet by 0.01-in. i.d. PEEK tubing, and (iii) cutting back both inlet and outlet tubes from the UV detector to 1 in. This reduced the volume delay between the electronic initiation of a gradient and the detection of B solvent in the flow cell (excluding column or injector loop volume contributions) from 1.5 to 0.5 ml. This resulted in a considerable shortening of gradient run times at flow rates of 100–300 μ l/min. The columns described in this work can be operated in HPLC systems with larger system dead volumes provided that the extended postinjection isocratic starting sections and extended run times are acceptable.

RESULTS

The range of columns used in this work is shown in Fig. 3. With practice it was possible to prepare short columns (≥ 50 mm) ready for evaluation in less than 1 h. Longer columns needed more packing and equilibration time. Testing of all column types demonstrated that the omission of a top frit, included in most commercial columns, reduced the incidence of column blockages without any noticeable effect on separation. If any sustained rise in pressure, packing bed discoloration, or column voids were observed, it was a simple procedure to dismantle the top fitting and, with either

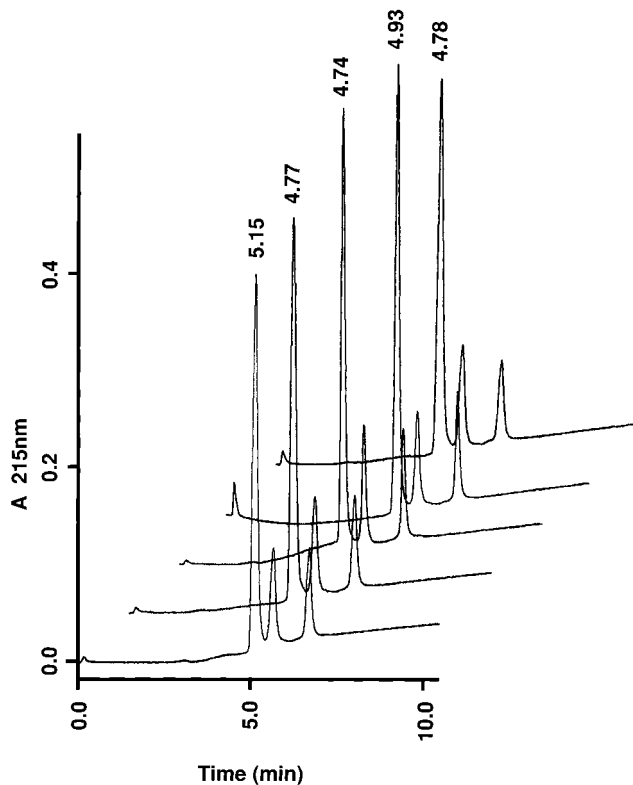


FIG. 7. Reproducibility trials. Solvents were as described for Fig. 4. The five columns were 0.75-mm-i.d. \times 50-mm PEEK packed with 10- μ m Poros R2. Flow rate was 0.3 ml/min. The gradient profiles were 0 to 100% B for 10 min. The protein sample was 5 μ l of the 1/10 cation exchange standard mixture. The variation between the five columns, expressed as the mean \pm standard deviations for the main peak, was as follows: retention time (min) 4.88 ± 0.16 , peak height (mm) 41.0 ± 4.0 , and peak area (arbitrary units) 64.9 ± 3.4 . A separate experiment to measure the variation from five injections on the same column gave the following data: retention time (min) 4.71 ± 0.02 , peak height (mm) 40 ± 0.3 , and peak area (arbitrary units) 60.8 ± 6.8 .

glass or plastic tube cutters, to clip off the top 2–5 mm of any column. This removed any voids and/or packing contamination and consequently normalized the operating pressure with negligible effects on chromatographic performance.

The results in Fig. 4 demonstrate three applications of reverse-phase columns. The rapid sequential protein separation (Fig. 4a) is facilitated by the short between-run equilibration time. With flow programming it is possible to reduce this time even further. The peptide separation (Fig. 4b) demonstrates the utility of these columns for the separation of peptide digests. The high sensitivity analysis (Fig. 4c) shows the capability of the column to analyze subpicomole amounts of protein.

The results obtained with the anion exchanger are shown in Fig. 5. The column is shown to be effective for

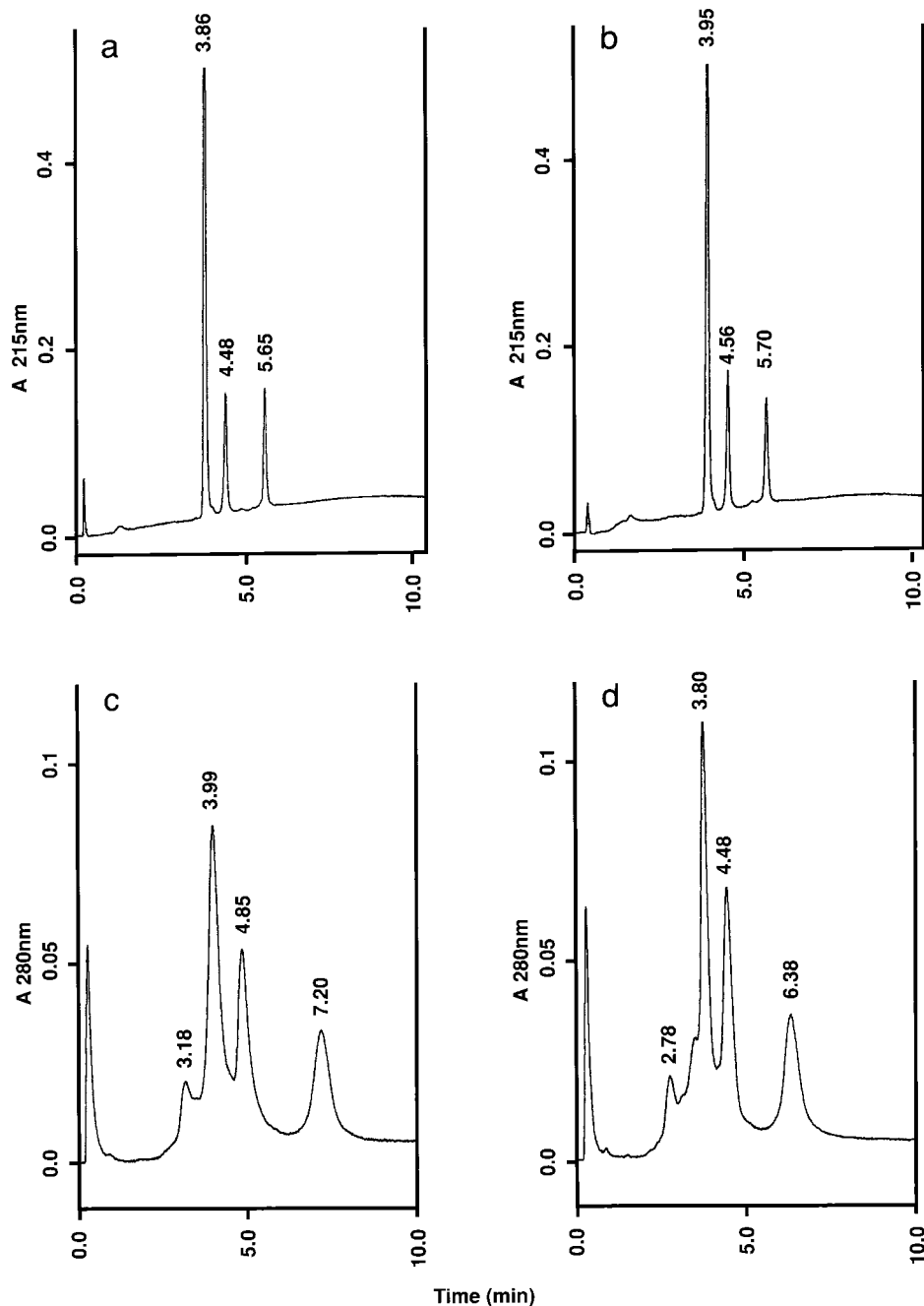


FIG. 8. Comparison with commercial columns. To facilitate accurate comparisons it was necessary to compensate for the slightly larger internal diameter of the commercial columns by adjusting both flow rate and sample volume in proportion to the cross-sectional area. For the reverse-phase comparisons the solvents and gradient profiles were as described in the legend to Fig. 7. Chromatogram a is from 10- μ m Poros R2 material packed into 1.8-mm-i.d. \times 100-mm PEEK tubing, secured into 1/8-in. end fittings as described. The flow-rate was 0.8 ml/min. and the injected sample was 10 μ l of the 1/10 cation-exchange standard. Chromatogram b is from a commercial 2.0 \times 100-mm PEEK column containing the same material, run at 1.0 ml/min, and injected with 12.5 μ l of the same standard. Chromatograms c and d show the anion exchange comparisons. These were carried out with the same solvents and gradient profiles as Fig. 5. Both columns were packed with Brownlee AX-300 7- μ m anion-exchange packing. Chromatogram c was a 1.8-mm \times 500-mm PEEK column packed in this work, run at 0.4 ml/min, and injected with 40 μ l of the 1/10 anion-exchange standard. Chromatogram d was the commercial 2.1 \times 300-mm steel cartridge run at 0.5 ml/min and injected with a 50- μ l sample of the same standard.

the rapid analysis of both mixtures (Fig. 5a) and single proteins (Fig. 5b), with a column reequilibration time of less than 5 min. The application of the Superdex column for protein solvent exchange (Fig. 6) highlights three advantages of the small-diameter columns. In terms of absolute amounts of protein injected, a high detection sensitivity can be achieved compared with conventional size-exclusion column diameters of 0.5 to 1 cm. Modern UV detectors are capable of being zeroed against high background absorbancies. This gives the flexibility to use 205–220 nm as a detection wavelength in a range of ion-exchange compatible eluents as long as Tris concentrations are kept low. This produces a 10- to 20-fold enhancement of sensitivity compared with 280 nm. Where speed is a priority over sensitivity, protein solvent exchange speeds of less than 1 min can be achieved (Fig. 6b). The third advantage, which is relevant to micropreparative work, is the small volume in which protein peaks can be collected, typically 300–500 μ l. Such volumes can be directly reinjected for orthogonal analysis without the necessity for additional concentration steps which may be associated with adsorption losses.

The reproducibility of the packing procedure was examined by preparing five columns and comparing their separation parameters. Although not up to the quality control standards expected of commercial column batches, the results in Fig. 7 show only 3% standard deviation in retention time within the series of five columns. Direct comparisons with commercially packed columns are shown in Fig. 8. For the protein standard used to compare gradient peak asymmetries, the commercially packed reverse-phase results are indistinguishable from the column packed in this work (Figs. 8a and 8b). The comparison between the anion exchange columns does show superior resolution for the commercial column but the difference would be acceptable for many applications (Figs. 8c and 8d).

DISCUSSION

The packing of conventional, silica-based, RP-HPLC materials into glass capillary tubes for protein separations has been demonstrated previously (4). This simple column design has now been extended in this work to use different types of high flow-rate chromatographic material packed into different types and diameters of tubing. The separation results demonstrate that these columns are of particular utility for small-scale separations, especially where some compromise in resolution can be accepted in favor of speed. The columns are not expected to match the batch consistency and column efficiencies of commercially packed

columns for isocratic separations. However, the comparisons carried out in this work show that the performance difference for gradient separations is small enough to be acceptable for many applications, especially when this can be balanced against the convenience, flexibility, and low cost. Because the microbore columns in this work can be used in standard HPLC equipment, they offer a useful intermediate stage before exploring the gains in sensitivity offered by home-packed fused-silica capillary columns and instrumentation manufactured or modified for gradient flow rates below 50 μ l/min (5, 6).

Additional advantages of the small columns prepared in this work are that any type of medium-pressure or low-pressure column material can be tested with minimal amounts of packing and, because of the convenient storage mode shown in Fig. 3, a suite of prepacked columns can be prepared for separation trials. Additional packings tested in the course of this work (results not shown) included the Resource R and Q series (Pharmacia), PLRPS (Polymer Laboratories), Vydac (Nest Group), HyperD (Beckman/Biosepra), Sephadex (Pharmacia), and Fractogel (Merck). Another advantage of the small packing quantities and rapid construction time is that the columns can be considered as disposable, even though both tubing and fittings can be reused. This broadens the scope of biochemical applications to the use of harsh solvents and/or crude samples that could compromise the lifetime of commercial microbore columns.

ACKNOWLEDGMENTS

We thank Paul England and Richard Smith for encouragement during this work. We are also grateful to many colleagues and manufacturers' representatives who have graciously supplied us with a wide variety of packing materials for testing.

REFERENCES

1. Nice, E. C., and Simpson, R. J. (1989) *J. Pharm. Biomed. Anal.* **7**, 1039–1053.
2. Afeyan, N. B., Gordon, N. F., Mazsaroff L., Varady, L., Fullerton, S. P., Yang, Y. B., and Regnier, F. E. (1990) *J. Chromatogr.* **519**, 1–29.
3. Joyce, J. G., Cook, J. C., Przysiecki, C. T., and Lehman, E. D. (1994) *J. Chromatogr.* **662**, 325–334.
4. Southan, C (1989) *in* Techniques in Protein Chemistry (Hugli, T., Ed.), pp. 392–398. Academic Press, San Diego.
5. Swiderek, K. M., Lee, T. D., and Shively, J. E. (1996) *in* Methods in Enzymology (Karger B. L., and Hancock, W. S., Eds.), Vol. 271, pp. 68–86. Academic Press, San Diego.
6. Tong, D., Moritz, R. L. Eddes, J. E. Reid, G. E. Rasmussen, R. K. Dorow, D. S., and Simpson, R. J. (1997) *J. Protein Chem.* **16**, 425–431.