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Application of capillary reversed-phase high-performance liquid chromatography to high-sensitivity protein sequence analysis

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ABSTRACT

A continuous gradient elution method for capillary column (<0.32 mm I.D.) liquid chromatography was developed. Gradient eluent from a microbore liquid chromatograph was split ahead of the injector so that an accurate percentage (2–3%) of the mobile phase delivered by the pump flowed through the capillary column. The outlet of the column was connected to a length of 0.075 mm I.D. fused-silica capillary tubing which, in turn, was connected to a 6-mm optical path length longitudinal capillary flow cell. Fused-silica capillary columns of 0.32 mm I.D. were slurry-packed efficiently with 7-μm spherical, 300 Å pore size, C_8 bonded-phase particles, and evaluated in terms of their ability to resolve mixtures of proteins, peptides or phenylthiohydantoin (PTH)-amino acid derivatives. The gradient elution profiles agreed with those obtained using microbore (<2.1 mm I.D.) and larger bore columns. The minimum detectable amounts for proteins and PTH-amino acids on 0.32 mm I.D. capillary columns were 50 pg and 25 fmol, respectively. At a flow-rate of 3.6 μl/min, proteins and peptides were recovered from the capillary columns in volumes of about 2–8 μl. The use of a multiple-wavelength, forward-optics detector for identifying tryptophan- and tyrosine-containing peptides is discussed.

INTRODUCTION

Microbore column liquid chromatography [1–4], combined with high-sensitivity protein sequence analysis [5], has become a routine procedure in the primary structure determination of low-abundance proteins and peptides [6–10]. Although commercially available protein sequencers are capable of yielding amino acid sequence information from 10–20 pmol of starting material [5], it is recognized that the rate-limiting step in obtaining such data resides in the ability to manipulate (*e.g.*, concentrate, buffer exchange, reduce and alkylate, remove detergents, etc.) low-microgram amounts of proteins and peptides [6,11,12].

During the past 7 years, we have been involved in the development and application of microbore column liquid chromatographic techniques that are compatible with high-sensitivity microsequencing methodologies [6–10,12]. Compared with their conventional column counterparts (4.6 mm I.D.), microbore columns (1–2.1 mm I.D.) offer enhanced mass sensitivity (5–20-fold) and decreased peak volumes (40–60 μl) without any striking diminuition of resolution [7,13–15]. Microbore column liquid chromatography is now widely used for peptide mapping with proteases [6–10,12,16], complete protein structure determinations [17–19] and the isolation of proteins form acrylamide gel electroeluates [20] and detergent mixtures [16]. More recently, microbore chromatography has been used in combination with mass spectrometry [21–25] (for reviews, see refs. 12, 26 and 27).

The potential for further miniaturization of liquid chromatography has been known for a long time [1,28]. Of the various ways of reducing column dimensions, first pioneered by Ishii *et al.* [1], slurry-packed fused-silica tubing (0.2–0.35 mm I.D.) [28–30] appears to be promising. Whereas the initial protein separation studies using capillary liquid

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chromatography were based on reversed-phase principles [1,28–32], other stationary phase materials with additional selectivities (*e.g.*, size-exclusion chromatography [32,33], chromatofocusing [34], affinity chromatography using chitosan beads [35], normal-phase chromatography [36]) have been used for capillary columns.

Other significant instrumental improvements in capillary chromatography include accurate and precise management of low flow-rates (< 5 μl/min) using microsyringe-type pumps [37] and stream-splitting devices [38], continuous [39] or stepwise gradient elutions [40] and "on-column" UV [41] and fluorescence [42], "in-column" fluorescence [43] and axial beam–longitudinal UV capillary flow cells [44].

Although the advantages of miniaturized columns are obvious [45], progress with this technology has been restricted by the availability of packed capillary columns and instrumentation designed to facilitate the operation of such columns.

In this paper we describe the design of a liquid chromatographic system allowing gradient elution from reversed-phase capillary (0.32 mm I.D.) columns, a procedure for slurry packing fused-silica capillary columns and the application of this technology to high-sensitivity protein separations and microsequencing.

EXPERIMENTAL

Chemicals

Recombinant murine interleukin-6 (mIL-6) was produced by overexpression in *Escherichia coli* and purified as described previously [46]. Chick egg lysozyme, bovine serum albumin, ribonuclease A, myoglobin, carbonic anhydrase and ovalbumin were purchased from Sigma (St. Louis, MO, USA). *Staphyloccocus aureus* V8 protease was obtained from Miles Scientific (Naperville, IL, USA).

High-performance liquid chromatographic (HPLC)-grade organic solvents were purchased from Mallinckrodt (Melbourne, Australia) and trifluoroacetic acid (Sequenal grade) from Pierce (Rockford, IL, USA). High-purity, deionized water was obtained from a tandem Milli-R015 and Milli-Q system (Millipore, Bedford, MA, USA). Ammonium hydrogencarbonate (AnalaR grade) was purchased from BDH (Poole, UK).

Conventional HPLC

The chromatographic equipment employed consisted of a Hewlett-Packard (Waldbronn, Germany) liquid chromatograph (HP 1090A), equipped with an autosampler and diode-array detector (HP 1040A). Spectral and chromatographic data were stored on electronic disk, using a Hewlett-packard HP-85 computer and a Model 9153C disk drive. Manual injections were performed with a Rheodyne (Cotati, CA, USA) Model 7125 injector, equipped with a 2-ml injection loop, installed in the column oven compartment. An Applied Biosystems (Foster City, CA, USA) liquid chromatograph (Model 120A), equipped with a Rheodyne Model 8125 injector installed in the column oven compartment, was also used.

Capillary HPLC

A schematic diagram of the two capillary HPLC systems used is shown in Fig. 1.

System 1. The solvent delivery for this system was supplied by an Applied Biosystems Model 120A liquid chromatograph. Accurate low flow-rates (2–5 μl/min) through the capillary columns and reproducible gradient formation were achieved with a preinjection solvent split that diverted most of the solvent flow through *ca.* 100 cm of 0.10 mm I.D. × 0.26 mm O.D. fused-silica tubing (SGE, Melbourne, Australia) at an Upchurch (Oak Harbor, WA, USA) 1/16-in. tee (P/N U-428). Capillary columns were direclty connected to a Rheodyne Model 8125 injector fitted with 0.5–5-μl injection loops. The flow through the column could be adjusted to 3–5 μl/min by the splitter from a pump flow-rate of 100–200 μl/min. With this split-flow approach, frequent monitoring of the actual flow through the capillary column is necessary. This is readily achieved by applying a 10-μl chromatographic syringe (Microliter 700 Series; Hamilton, Reno, NV, USA) to the effluent and accurately timing the advancing meniscus with a stop-watch. For detection, the conventional flow cell was replaced with a 6-mm optical path length U-shaped longitudinal capillary flow cell (LC Packings, Amsterdam, Netherlands).

System 2. The solvent delivery system was a Hewlett-Packard HP1090A liquid chromatograph. Accurate solvent delivery rates and gradient formation were achieved as described for system 1. Capillary columns were directly connected to a Rheodyne

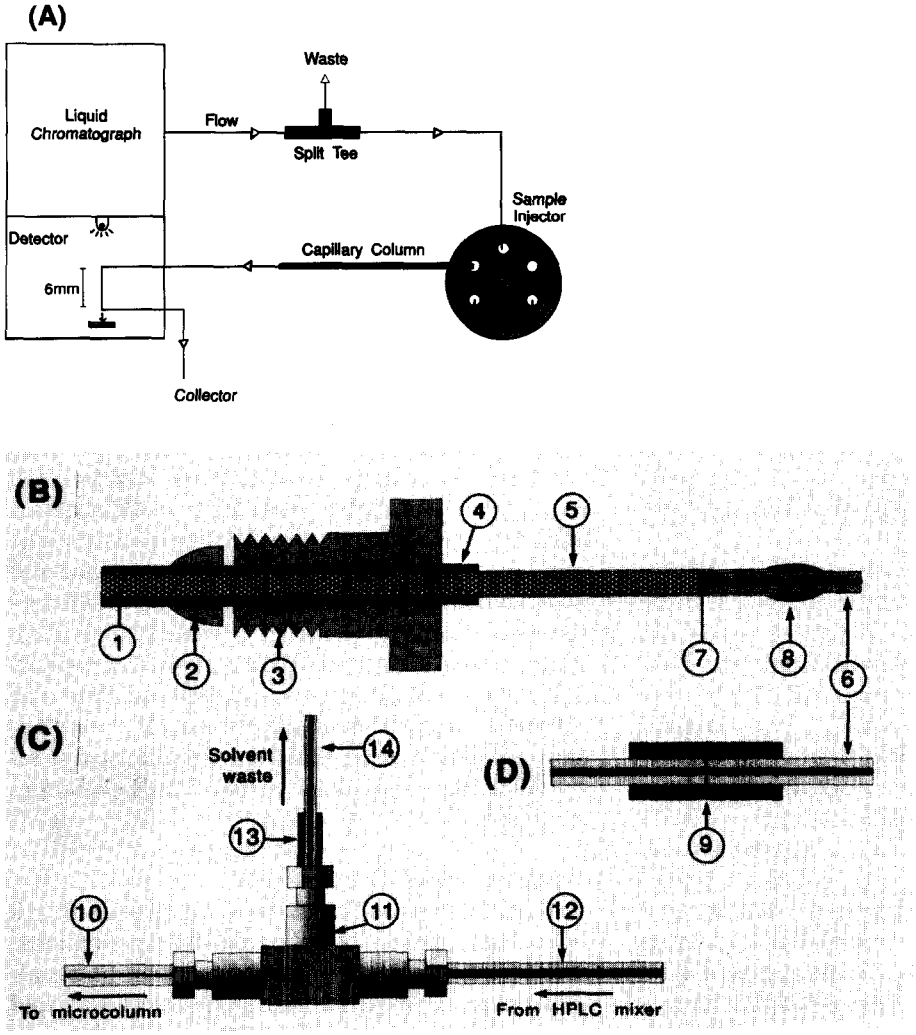

Fig. 1. Schematic diagram of the gradient capillary HPLC system. (A) System 1 consisted of an Applied Biosystems Model 120A liquid chromatograph and system 2 of a Hewlett-Packard Model 1090A liquid chromatograph equipped with a Spectra-Physics Spectra Focus multiple-wavelength detector. Further details are given under Experimental. (B) Capillary column. (C) Split-flow device. (D) Zero-dead-volume tubing union. 1 = Chromatographic stationary phase; 2 = standard 1.59-mm (1/16-in.) stainless-steel ferrule; 3 = standard 1.59-mm (1/16-in.) stainless-steel male nut; 4 = PTFE tubing, 1.59 mm (1/16-in.) O.D. × 0.5 mm I.D. (Beckman, Melbourne, Australia); 5 = polyimide-coated fused-silica 0.42 mm O.D. × 0.32 mm I.D capillary tubing; 6 = polyimide-coated fused-silica 0.275 mm O.D. × 0.075 mm I.D capillary tubing; 7 = hydrophilic poly(vinylidine difluoride) frit, 0.32 mm diameter, 0.45 μm pore size; 8 = epoxy resin; 9 = PTFE tubing, 20 mm × 1.59 mm (1/16-in.) O.D. × 0.25 mm I.D; 10 = Polysil tubing, 1.59 mm (1/16-in.) O.D. × 0.05 mm I.D (SGE); 11 = standard stainless-steel tee, 1.59 mm (1/16-in.), with 0.5-mm flow ports; 12 = PEEK tubing 1.59 mm (1/16-in.), O.D. × 0.178 mm I.D (Upchurch); 13 = PTFE tubing, 1.59 mm (1/16-in.) O.D. × 0.25 mm I.D; 14 = polyimide-coated fused-silica 0.26 mm O.D. × 0.1 mm I.D capillary tubing (SGE).

Model 7520 injector fitted with 0.2–1.0 μl internal sample rotors. A U-shaped longitudinal capillary flow cell (6 mm path length, illuminated volume *ca.* 26.5 nl) installed in a conventional Spectra-Physics forward-optics scanning detector flow cell holder (LC Packings) was used for eluate detection. Data collected were either sent to a strip-chart recorder via the single wavelength mode or collected on an

IBM PS/2 computer (Model P70 386) with Spectra Focus software installed and operated in the multiple-wavelength mode (195–340 nm, 5-nm intervals).

Column supports

The following packing materials were used: (a) Brownlee RP-300 C_8 (particle diameter 7 μm, dimethyloctylsilica, pore size 300 Å, packed into 30 × 4.6 mm I.D., 100 × 2.1 mm I.D. and 30 × 2.1 mm I.D. cartridges), obtained from Applied Biosystems; Brownlee RP-300 C_8 packed into a 50 × 0.32 mm I.D. glass-lined column was provided by LC Packings; (b) Applied Biosystems PTH-C_{18} packed into a 150 × 0.32 mm I.D. glass-lined column was supplied by LC Packings; and (c) Brownlee RP-300 C_8 packing in-house into fused-silica capillary tubing as decribed below.

Fused-silica capillary column construction. Microcolumns of 0.32 mm I.D. were constructed in the following manner. A 20-mm length of 0.420 mm O.D. × 0.320 mm I.D. polyimide-coated fused-silica tubing (Polymicro, Phoenix, AZ, USA) was used as a disc cutter to fabricate internal column frits from 0.45-μm porosity hydrophilic poly(vinylidine difluoride) (PVDF) (Millipore, Bedford, MA, USA). Using a length of 0.275 mm O.D. × 0.075 mm I.D. polyimide-coated fused silica, the frit was inserted into a 60-mm length of polyimide-coated fused silica (Polymicro) to a depth of 5 mm. A small bead of epoxy resin (E-Pox-E Glue, Loctite, Knoxfield, Victoria, Australia) (the resin was precured by stirring whilst applying heat with a heat gun until a tacky consistency was achieved) was then applied to the join and gentle heating was continued until the epoxy resin had fully cured. Proper precuring of epoxy glue is important to ensure that it does not run up the capillary and thereby block the frit [47].

Once the PVDF frit had been positioned in the fused-silica capillary column, a slurry-packing procedure was employed to pack the column. The slurry reservoir consisted of an empty 50 × 2 mm I.D. stainless-steel glass-lined tube (SGE) with standard 1/4-in. column end-fittings (Alltech, Deerfield, IL, USA) that had 0.5 mm I.D. holes as flow-through ports. The fused-silica capillary column was connected to the standard 1/4-in. column end-fitting, either by Minitight fittings (Upchurch, Part No. F-218) or by a PTFE tubing sleeve (see Fig. 1B). Brownlee RP-300 C_8 dimethyloctylsilica (600 mg) was obtained by unpacking a 30 × 4.6 mm I.D. cartridge (Applied Biosystems, P/N 0711-0055). A slurry of this material in *n*-propanol (20 mg in 500 μl) was sonicated for 15 min in a 1.5-ml polypropylene tube. Before packing, the capillary column was filled with packing solvent (*n*-propanol) using a column-packing pump (Shandon, Runcorn, UK) at a pressure of 100 bar. This step prefilled the column with packing solvent and also allowed the system to be checked for leaks. The slurry reservoir was then emptied and quickly replaced with 200 μl of the prepared packing–slurry mixture. The column was packed at a constant pressure of 100 bar for 16 h and then conditioned with 50% (v/v) aqueous methanol for a further 4 h at the same pressure. The capillary column was then carefully dismantled and plugged by inserting the bottom end into a silicone-rubber septum and placing a closed-off 1/16-in. stainless-steel zero-dead-volume union (Swagelock, Solon, OH, USA) on the top of the column. The column efficency was tested using a series of standard proteins (ribonuclease A, lysozyme, bovine serum albumin, myoglobin, carbonic anhydrase and ovalbumin).

Peptide mapping

Recombinant mIL-6 (120 μg) in 200 μl of 1% (w/v) ammonium hydrogencarbonate containing 0.01% (w/v) Tween 20 was digested with *Staphylococcus aureus* V8 protease at an enzyme-to-substrate mass ratio of 1:20 at 37°C for 18 h.

Protein determination

Lysozyme was dissolved in 1 ml of water and the protein concentration was accurately determined by measuring the absorbance at 281.5-nm using an $A^{1\%}_{281.5\ nm}$ value of 26.4 [48].

Peptide synthesis

Peptides were synthesized on an Applied Biosystems peptide synthesizer (Model 430A) using 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) for the coupling of Boc-amino acids as described elsewhere [49].

RESULTS AND DISCUSSION

Capillary column performance

For the efficient operation of capillary columns ($\leqslant$0.3 mm I.D.), both the flow-rate and detector volumes have to be substantially reduced compared with microbore and large-bore liquid chromatography. The capillary liquid chromatographic system used in this study was designed to achieve accurate low-flow-rate (3–5 μl/min) and to minimize the extra column volume (see Fig. 1). Whereas the detector was the same as that used for the operation of the 2.1 and 4.6 mm I.D. columns (either a Spectra-Physics Spectra Focus Detector System (Part No. SF102-0122) or an Applied Biosystems Model 120A detector), the 4.5-μl standard flow cell in both of these detectors was replaced with a longitudinal 0.075 mm I.D. fused-silica capillary flow cell (LC Packings). The path length of this U-shaped axial-beam capillary flow cell is 6 mm and the illuminated volume is 26.5 nl.

The accuracy of the flow-rate, under gradient elution conditions, over six consecutive chromatographic runs (calculated from the variance in retention time for lysozyme) was $\pm$0.26%. For protein and peptide separations, prolonged capillary column usage (*e.g.*, 20–30 consecutive chromatographic runs) often resulted in a 25% reduction in the flow-rate (*i.e.*, form 4 to 3 μl/min). This was presumably due to "dirty" samples which caused partial column/frit blockage and, in the first instance, could be readily corrected by increasing the total flow-rate of the liquid chromatograph (say, from 200 to 266 μl/min). With prolonged usage, this problem was corrected by either replacing the top column frit (for the commercial glass-lined stainless-steel columns) or by cutting 2 mm off the top of the column (for the fused-silica tube columns that we packed).

In PTH-amino acid separations, the samples were much "cleaner" and the potential problem of frit plugging was less pronounced, thereby extending the column lifetime.

Mass sensitivty achieved with capillary columns

It is established that to operate microbore columns (1–2 mm I.D.) at linear flow velocities equivalent to those used with larger bore columns ($\geqslant$4.6 mm I.D.), the flow-rate must be decreased in proportion to the square of the reduction in column inside diameter. Provided that the microbore columns are packed with similar efficiency to that of largerbore columns, and are not overloaded, their sample peak volumes will be proportionally decreased in comparison with those from larger bore columns [3,4].

The advantages of narrow-bore and capillary columns with respect to enhanced sensitivity of protein detection and reduced peak volumes are shown in Fig. 2. All columns were packed with the same support (Brownlee RP-300) and operated at equivalent linear flow velocities. A comparison of the 4.6 and 2.1 mm I.D. columns revealed that a fivefold increase in sensitivity of protein detection was achieved by using the 2.1 mm I.D. column. As the extra-column volumes and detector cell geometry (Hewlett-Packard Model 1090A diode-array cell, 6 mm path length, 4.5 μl volume) were identical for the operation of both the 2.1 and 4.6 mm I.D. col-

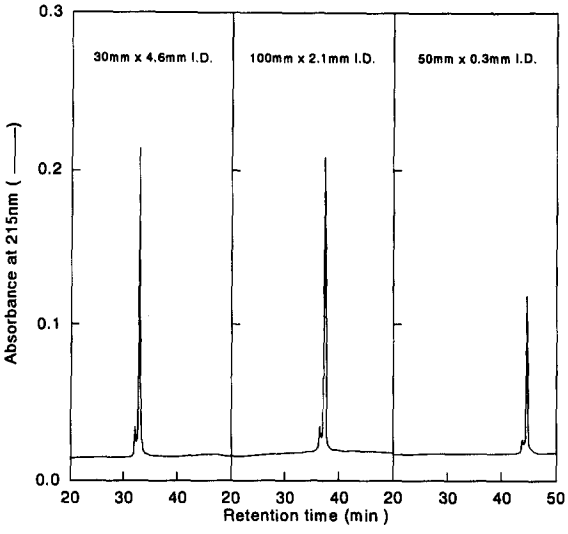

Fig. 2. Effect of column side diameter and flow-rate on detector sensitivity. Column support, Brownlee RP-300 C_8 (7-μm dimethyloctylsilica, pore size 300 Å, 30 × 4.6 mm I.D. and 100 × 2.1 mm I.D., Applied Biosystems, and 50 × 0.32 mm I.D. packed in-house; see Experimental); linear 60-min gradient from 0 to 100% B, where eluent A is 0.1% (v/v) trifluoroacetic acid and eluent B is acetonitrile–water (60:40) containing 0.1% (v/v) trifluoroacetic acid; column temperature, 45°C; detection, 215 nm; flow-rates, 1 ml/min, 200 μl/min and 4 μl/min for 4.6, 2.1 and 0.32 mm I.D. columns, respectively. Sample, lysozyme (4.6 mm I.D. column: 10 μg; 2.1 mm I.D. column: 2 μg; 0.3 mm I.D. column: 0.04 μg).

umns, such an increase in mass sensitivity is expected. The small variance in retention times between the two columns is due to the variation in column length and to the precolumn instrumental dead volume (about 300 μl) for the instrument used in this experiment (Hewlett-Packard Model 1090A liquid chromatograph).

When the 0.32 mm I.D. capillary column was compared with 2.1 and 4.6 mm I.D. columns, 25- and 125-fold increases in sensitivity of protein detection were achieved, respectively (Fig. 2). The constant peak band widths, as a function of time, for the three columns shown in Fig. 2 indicated that comparable column efficiencies can be achieved with capillary liquid chromatography compared with conventional liquid chromatography. Peak recovery volumes for the 0.3, 2.1 and 4.6 mm I.D. columns were 2.5, 133.5 and 660 μl, respectively.

Linearity of detector response

The linearity of the output response of the Applied Biosystems Model 120A detector, fitted with a 6-mm longitudinal capillary flow cell, was examined using various concentrations of ribonuclease A. For this experiment, ribonuclease A (2.0 mg/ml) was dissolved in acetonitrile–water (40:60) containing 0.1% (v/v) trifluoroacetic acid. This stock solution was serially diluted to the required concentrations and 200-μl aliquots were injected directly into the capillary flow cell and the absorbance at 215 nm

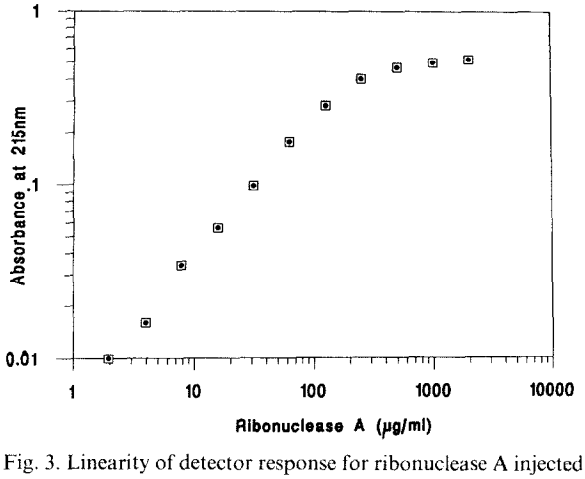

Fig. 3. Linearity of detector response for ribonuclease A injected direclty into the longitudinal capillary flow cell. Calibration graph presented in log–log format.

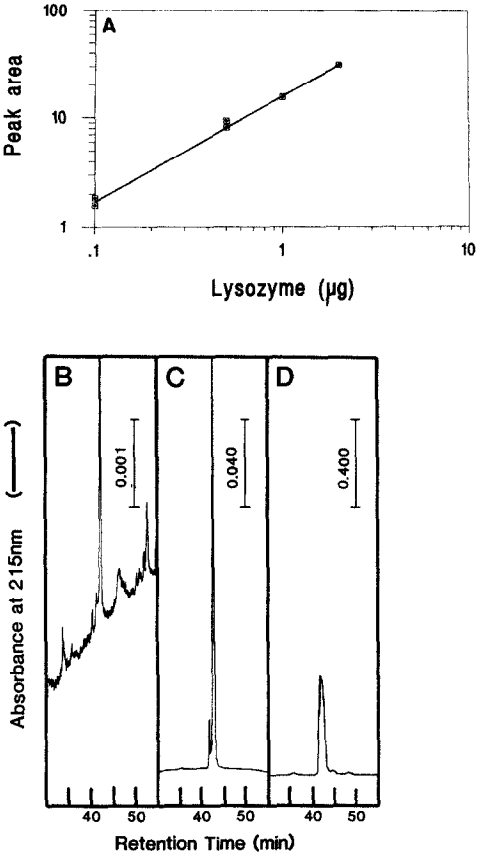

Fig. 4. Linearity of detector response for lysozyme chromatographed on a 50 × 0.32 mm I.D. Brownlee RP-300 column (LC Packings). The liquid chromatograph system 1 described under Experimental was used. Elution conditions as for the 0.32 mm I.D. column in Fig. 2. Progressively larger amounts of lysozyme were chromatographed in duplicate and average peak-area values (arbitrary units) at each concentration were plotted (A). Representatives chromatograms for (B) 500 pg, (C) 100 ng and (D) 2 μg of lysozyme are shown so that the practical limits of sensitivity can be assessed.

was measured. It can be seen in Fig. 3 that the output signal of the detector appears to be linear up to about 0.5 absorbance; this corresponds to a protein concentration of about 125 μg/ml.

To determine the extent to which the detector deviates from linearity, the data in Fig. 3, up to and including absorbance values for protein concentrations of 125 μg/ml, were fitted to the equation $y = Ac^r$ reported by Scott [50], where y is the detector response, c is the solute concentration, A is a con-

stant and r is the response index. For ribonuclease A in Fig. 3, $r = 0.86$ and the correlation coefficient for the curve is 1.00. Although this value of r indicates that the detector deviates slightly from true linearity (according to Scott, [50], true linearity can only be assumed for values $0.98 \geqslant r \geqslant 1.02$), calibration graphs can still be used with reasonable accuracy.

The linearity of the detector response using our packed 0.32 mm I.D. capillary column, operated under gradient conditions at a flow-rate of 5 μl/min, was investigated using the liquid chromatograph configuration described for system I (see Experimental). The linearity of the detector was determined by plotting the peak area (arbitrary units) against the amount (micrograms) of lysozyme injected on to the column. Fig. 4 shows that the dynamic linear range of the 6-mm longitudinal capillary flow cells for lysozyme extends to 2 μg. Representative chromatograms for lysozyme in the range 500–2000 ng are shown in Fig. 4B–D. For the 6-mm pathlength capillary flow cell and the equipment used for generating low solvent delivery rates (3–5 μl/min) (see system 1 in Fig. 1 and Experimental), it appears that the detection limits are 50–100 pg. We estimate that the minimum detectable amount (MDA) of lysozyme at 215 nm, using the capillary liquid chromatography described, which produces a peak that is twice the baseline noise (signal-to-noise ratio = 2) is *ca.* 50 pg (3.6 fmol). The MDA for ribonuclease A at 215 nm was estimated to be *ca.* 100 pg (7.3 fmol).

Resolution of proteins

Fig. 5 illustrates the separation efficiency that can be achieved for proteins on a reversed-phase capillary column. This chromatogram was obtained with a 50 × 0.32 mm I.D. RP-300 column, packed by us (see Experimental). Using gradient elution between 0.1% (v/v) aqueous trifluoroacetic acid and acetonitrile–0.1% (v/v) aqueous trifluoroacetic (60:40) at a flow-rate of 3.6 μl/min, the resolution of this set of proteins (Fig. 5) is as good as that obtained on microbore and largerbore columns packed with the same support. For the 0.32 mm I.D. columns, proteins were recovered in peak volumes of 2–8 μl, which are small enough to allow efficient interfacing with electrospray mass spectrometry [47,51].

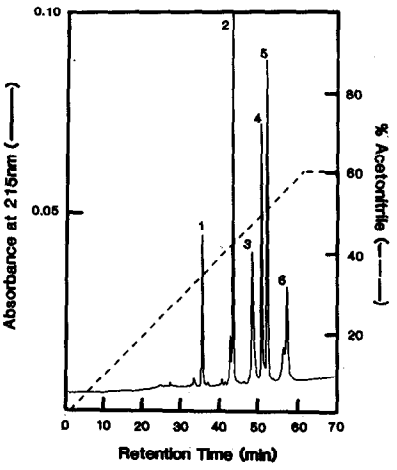

Fig. 5. Separation of protein standards on a Brownlee RP-300 column (50 × 0.32 mm I.D.). This column was slurry-packed in-house as described under Experimental. The column was developed with a linear 60-min gradient from 0 to 100% B, where solvent A was 0.1% (v/v) trifluoroacetic acid and solvent B was acetonitrile–water (60:40) containing 0.1% (v/v) trifluoroacetic acid. Column temperature, 45°C. Flow-rate, 3.6 μl/min. Protein standards: 1 = ribonuclease A; 2 = lysozyme; 3 = bovine serum albumin; 4 = carbonic anhydrase; 5 = myoglobin; 6 = ovalbumin. Sample load, 50 ng in 0.5 μl of water.

Load capacity

The effect of the mass of lysozyme on peak width for a representative 50 × 0.32 mm I.D. Brownlee RP-300 column is shown in Fig. 6. For protein loads of 50–500 ng there was very little variation in band width (measured by the peak width half-height) or band shape. With amounts of protein in excess of 1 μg the band width increased significantly (Fig. 6) and the band profile became distorted (see Fig. 4D), in a similar manner to that described by Snyder *et al.* [52]. Hence it appears that the optimum working range for a column of this dimension is < 1 μg and that beyond this, mass overload and detector saturation (see Fig. 3) conditions are encountered.

Peptide mapping

Peptide mapping by reversed-phase liquid chromatography is an extremely powerful technique that is now widely applied in the structural analysis of proteins. Some important applications include the localization of post-translational modifications in proteins (*e.g.*, deamidation, oxidation, glycosyla-

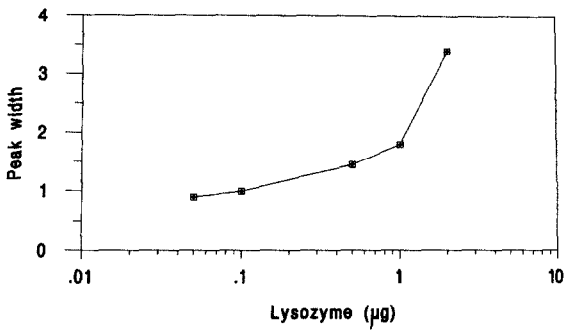

Fig. 6. Effect of protein load on peak width of eluted lysozyme for a 50 × 0.32 mm I.D. column (Brownlee RP-300). Values are averages of two experiments. Chromatographic conditions in Fig. 5. Peak widths (mm) were measured at half-height.

tion, phosphorylation, sulphation, methylation, blocked N-terminus, truncated N- and C-termini), the localization of disulphide bonds in proteins, the identification of genetic variants and the analysis of purity and quality control of genetically engineered protein products. The power of this technology is considerably enhanced when it is integrated with classical biochemical techniques such as the Edman degradation procedure [53] and mass spectrometric analysis (21,22,25,28,51,54,55], which allow amino acid sequence and accurate molecular weight determinations, respectively. This latter information is crucial for establishing the nature of post-translational modifications, many of which are critical to the biological activity of a molecule. Such modifications are not revealed by DNA sequences obtained from gene cloning experiments.

An example of a high-sensitivity peptide map obtained with capillary liquid chromatography is given in Fig. 7. Recombinant mIL-6 (200 ng) was digested with *S. aureus* V8 protease and the resultant digest chromatographed on 0.32 mm I.D. capillary columns, packed by us (Fig. 7A) or obtained commercially (LC Packings) (Fig. 7B), using a trifluoroacetic acid–acetonitrile gradient elution system. Fig. 7. indicates that the chromatographic efficiency of our packed column (A) compares favourably with that of a commercial capillary column (B), and both with that of a commercial microbore (2.1 mm I.D.) column (C). All three columns were packed with the same support (Brownlee RP-300). The flow-rates for the capillary columns (A and B) were

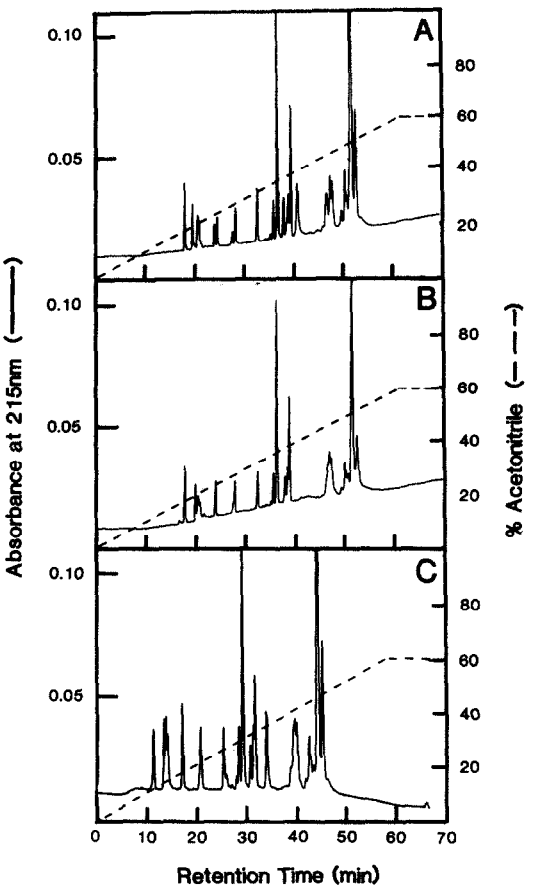

Fig. 7. High-sensitivity peptide mapping of recombinant mIL-6 using 0.32 mm I.D. reversed-phase capillary columns. Support, Brownlee RP-300 (7-μm dimethyloctyl silica, pore size 300 Å). Elution conditions were as in Fig. 5. (A) 50 × 0.32 mm I.D. fused-silica column packed in-house (see Experimetnal); (B) 50 × 0.32 mm I.D. glass-lined column packed by LC Packings; (C) 30 × 2.1 mm I.D. cartridge (Applied Biosystems). Sample load (A and B) 200 ng of digest, 10 pmol; (C) 5 μg of digest, 250 pmol. Flow-rate: 0.32 mm I.D. columns (A and B), 3.6 μl/min; 2.1 mm I.D. column (C), 178 μl/min.

3.6 μl/min whereas that for the microbore column (C) was 178 μl/min. Average peak volumes of 2–8 μl were obtained for the capillary columns (A and B) compared with 100–300 μl for the microbore column (C).

The sensitivity of peptide mapping on 0.32 mm I.D. capillary columns, demonstrated in Fig. 7, where 200 ng (*ca.* 10 pmol) of mIL-6 digest was chromatographed, is ideally suited to the peptide mapping of proteins resolved by two-dimensional

gel electrophoresis (2-DE). We have reported elsewhere [56,57] that protein spots from six to eight identical 2-DE gels can be readily revealed by high-resolution dynamic imaging [58] and the recovered from the gel by passive elution. For peptide mapping, protein (1–2 μg) can be recovered from the eluate, free of gel-related contaminants, by desalting using either reversed-phase or "inverse-gradient" reversed-phase liquid chromatography [59–62].

Multiple-wavelength detection

A multiple-wavelength spectrophotometric absorbance monitor (Spectra-Physics, Spectra Focus) was adapted for use in the capillary liquid chromatography system shown in Fig. 1. The geometry of this monitor, based on a rapidly moving grating, uses a forward-optics system [63].

We were interested in establishing whether this multiple-wavelength detector, fitted with a 6-mm optical path length capillary flow cell, would be useful for monitoring the absorbance of the column eluate at 195 nm rather than at 215 nm. This interest was based on the finding by Rosenheck and Doty [64] that the maximum UV absorption for polypeptides occurs at *ca.* 195 nm. Fig. 8 shows the peptide map for an *S. aureus* V8 digest of mIL-6. Two observations were made: first, the detector signal for peptides was 2–6 times higher at 195 nm than 215 nm (compare peaks 1 and 2), and second, the noise in the baseline at 195 nm is only twice that observed at 215 nm (see insets E and F in Fig. 8B).

In order to determine whether the multiple-wavelength detector would be useful for identifying aromatic amino acid-containing peptides, two synthet-

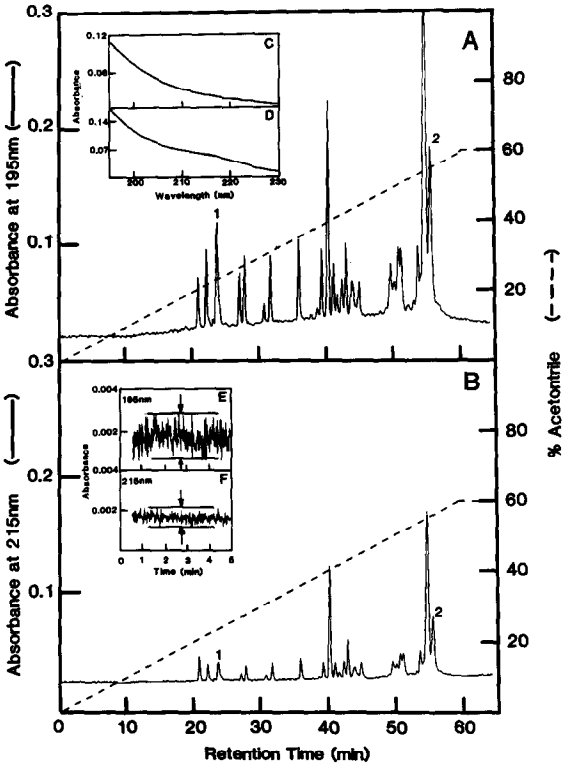

Fig. 8. Multiple-wavelength detector performance. Sample: *S. aureus* V8 protease digest of mIL-6 (200 ng). Column: 100 × 0.32 mm I.D. Brownlee RP-300 packed in-house (see Experimental) and operated as described in Fig. 5. Detector: Spectra-Physics Spectra Focus fitted with a 6-mm optical path length longitudinal capillary flow cell (LC Packings). (A and B) detection at 195 and 215 nm, respectively; (C and D) spectral analysis of peaks 1 and 2, respectively; (E and F) baseline noise at 195 and 215 nm, respectively.

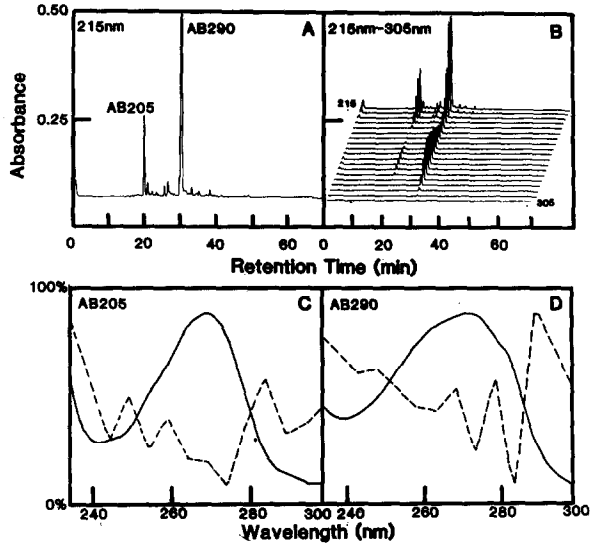

Fig. 9. Spectral analysis of aromatic amino acid-containing synthetic peptides chromatographed by reversed-phase capillary liquid chromatography. Spectral data were obtained using a multiple-wavelength detector (Spectra-Physics) fitted with a 6-mm optical path length longitudinal capillary flow cell (LC Packings). Chromatographic conditions as in Fig. 8. (A) 0.32 mm I.D. capillary column profile of synthetic peptides AB205 (SRDMYMESEGGDGY) and AB290 (DLAWWELR); (B) multiple signal plot of data shown in A; (C and D) zero-order (solid lines) and second-order derivative spectra (dashed lines) of synthetic peptides AB205 and AB290, respectively. Sample load, 250 ng.

ic peptides were chromatographed by reversed-phase capillary liquid chromatography (Fig. 9A): peptide AB205 (SRDMYMESEGGDGY), a tyrosine-containing fourteen-residue synthetic peptide, and peptide AB290 (DLAWWELR), a tryptophan-containing eight-residue peptide. A multiple signal plot of the data shown in Fig. 9A is given in Fig. 9B. Inspection of these spectra reveals major absorption peaks in the range 270–290 nm which are indicative of the aromatic amino acids tyrosine and tryptophan.

Analysis of the spectra by second-order derivative spectroscopy reveals characteristic minima at 287 ± 5 nm for AB290 and 277 ± 5 nm for AB205 (Fig. 9C and D). Previously, it has been shown [65–67] that minima at 290 ± 2 nm) and 278 ± 2 nm are characteristic of tryptophan residues, while a single minimum at 280 ± 2 nm is characteristic of tyrosine residues.

High-sensitivity phenylthiohydantoin-amino acid analysis

With the introduction of the gas-phase sequencer in 1981 [5], N-terminal amino acid sequence analysis of proteins and peptides can now be routinely performed on as little as 10 pmol of material. Since this development in sequencing technology. There has been a concerted research effort in many protein chemistry laboratories towards increasing the sensitivity of amino acid sequence analysis even further [68–71].

The development of more sensitive sequence analysis methods can be realized in two ways: one is to explore new Edman-type reagents and the other to increase the sensitivity for detection of the PTH-amino acids. New Edman-type reagents that have been investigated include 4-N,N-dimethylamino-azobenzene 4′-isothiocyanate (DABITC) [69,72], fluorescein isothiocyanate (FITC) [69,73] and more recently dansylamino-PITC (dimethylaminonapthylsulphonylamino phenylisothiocyanate) [69–70, 74]. In the last instance, dansylamino-PTH-amino acid detection is possible at the 200-fmol level by reversed-phase HPLC [68]. A number of other Edman-type coupling reagents for N-terminal sequence analysis have been documented (for a review, see ref. 75); however, these reagents have not yet gained general applicability owing to problems such as poor coupling yields and serious side-reactions. Another exciting prospect for increasing the sensitivity of the Edman degradation procedure was reported by Tsugita and co-workers [71,76]. Using conventional Edman degradation chemistry, the phenylthiocarbonyl (PTC)-protein is cleaved with trifluoroacetic acid and the resulting anilinothiazolinone derivative, rather than being converted to the stable UV-detectable PTH derivative, is sensitized by reaction with the fluorescent reagent 4-aminofluorescein. The amino acid derivatives sensitized with 4-aminofluorescein were separated by reversed-phase HPLC at 0.1–0.2-pmol levels [71,76].

We report here a method for increasing the sensitivity of detection of PTH-amino acids that relies on the use of reversed-phase capillary liquid chromatography. Fig. 10 illustrates the separation efficiency for PTH-amino acids that can be achieved by capillary liquid chromatography. The chromatogram was obtained with a 150 × 0.32 mm I.D. column packed with Applied Biosystems PTH-C_{18} support (LC Packings). The resolution in Fig. 10 is very similar to that achieved with a commercial 220

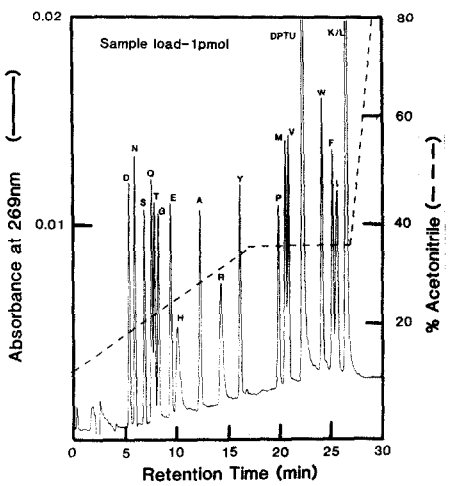

Fig. 10. Separation of phenylthiohydantoin-amino acids by reversed-phase capillary liquid chromatography. Column, 150 × 0.32 mm I.D. Applied Biosystems PTH-C_{18} (packed by LC Packings). Solvent A, 8.3 m*M* sodium acetate–5% (v/v) tetrahydrofuran (pH 4.1); solvent B, acetonitrile. Column temperature, 55°C. Flow-rate, 5 μl/min. Sample load: mixture of 1 pmol each of PTH-amino acids in 0.5 μl of 2% (v/v) aqueous acetonitrile. Capillary liquid chromatography configuration, system 1 (see Fig. 1 and Experimental). PTH-amino acid notation is shown using the one-letter code for amino acids; DPTU, diphenylthiourea.

× 2.1 mm I.D. cartridge obtained from Applied Biosystems (Foster City, CA, USA) [56]; however, the sensitivity of detection of PTH-amino acids using the 0.32 mm I.D. capillary column is, conservatively, 25 times higher (25 fmol) than that achievable with the 2.1 mm I.D. column.

The enhanced mass sensitivity of PTH-amino acid analysis by capillary liquid chromatography, like capillary electrophoresis (data not shown), offers great potential for low-femtomole protein sequence analysis. The obvious drawback for interfacing capillary liquid chromatography with existing sequencer hardware is the problem of sample volume limitations. Given that low concentrations of organic solvent are required to solubilize all of the PTH derivatives, this restricts the volume that can be applied to a capillary column (*ca.* $\leqslant 1$ μl). The technical challenge for the future is to design miniaturized sequencer hardware that allows accurate, reproducible sub-microlitre solvent deliveries that will permit its interfacing with capillary column ($\leqslant 0.5$ mm I.D.) liquid chromatography.

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