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THE ROLE OF SHORT MICROBORE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COLUMNS FOR PROTEIN SEPARATION AND TRACE ENRICHMENT

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SUMMARY

The use of short microbore columns (7.5 cm × 2.1 or 1.0 mm I.D.) for the separation of proteins is described. The loading capacity for protein standards of such columns has been investigated and shown to be compatible with the purification of sufficient amounts of protein in a form directly suitable for automated amino acid sequence analyses (pmole or nmole levels). The trace enrichment potential of these columns for proteins is demonstrated by the ability to load large sample volumes (greater than 2 ml) recovered from analytical (4.6 mm I.D.) high-performance liquid chromatography columns onto the microbore columns and recover the protein quantitatively in eluent volumes as small as 25 μl. In addition it is shown how the increased levels of detectability (*ca.* five-fold for the 2.1 mm I.D. column), arising from the increased solute concentration, may be used to quantitate ng quantities of high specific activity radioiodinated proteins.

INTRODUCTION

The role of high-performance liquid chromatography (HPLC) in the separation and purification of proteins is now widely accepted[1]. Such methods have been shown to offer unrivalled advantages in terms of speed, resolution, sensitivity and recovery when used for the purification of small (ng–μg) quantities of highly potent biological substances, e.g. growth factors, from bulk biological samples[2]. Even so, the chromatographic efficiency associated with analytical HPLC columns means that proteins are typically recovered in eluent volumes of *ca.* 1 ml. The resultant protein concentration (μg/ml) of these eluents is not ideal for subsequent microsequencing analysis or radioiodination. Increased chromatographic efficiency for proteins and hence increased protein concentration, can be obtained by the use of flow-rates lower than those normally associated with high efficiency reversed-phase HPLC columns[3]. This is presumably due to improved mass transfer. However low flow-rates have been shown to be associated with the poor recovery of a number of more hydrophobic proteins[4,5]. Although the reason for this phenomenon is unclear, it is necessary to maintain a suitable linear flow velocity to avoid excessive losses of proteins.

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An alternative method of concentrating proteins eluted from reversed-phase columns is to use volatile chromatographic solvents which can be removed by subsequent evaporation. However, solvent evaporation can lead to the chemical modification of some proteins even when performed in an inert atmosphere[6] and often results in low recoveries due to non-specific adsorption onto the walls of the collection vessel when the organic solvent is removed[7].

A microbore chromatographic concentration process whereby recovery was effected in small (less than 100 μl) volumes would appear to offer considerable advantages with respect to both overall recovery, since further manipulations could be avoided, and sensitivity of detection. It has been shown that peak volume is inversely proportional to the square of the column diameter and directly proportional to the column length[8]. If the overall chromatographic efficiency of the system is maintained then the use of low flow-rates associated with short (less than 10 cm) microbore columns should allow recovery of samples at higher concentration (with increased sensitivity of detection)[9] compared with standard analytical columns (25 cm × 4.6 mm I.D.) operated at the same linear flow velocity.

Typically microbore columns are limited to injection of small sample volumes (less than 1 μl)[10,11] to avoid extra-column band broadening. At first sight this would appear to be the limiting factor in their use for protein purification where samples may only be available in larger volumes at very low concentration. Takeuchi and Ishii have recently demonstrated the use of a short micro precolumn for the concentration of samples present in trace quantities, prior to separation on microbore columns[12]. Since proteins exhibit very large capacity factors (k') on reversed-phase or ion-exchange HPLC packings below critical secondary solvent compositions[13], trace enrichment of large sample volumes directly on the analytical column itself is possible before recovery of proteins by gradient elution[14].

In this paper it will be demonstrated how microbore (2.1 and 1 mm I.D.) columns can be used to enrich protein samples and then to recover them at much higher concentration. The protein capacity of the microbore columns has been investigated to demonstrate their usefulness for the trace enrichment of samples prior to automated amino acid sequence analysis at the pmole level. In addition, we have used microbore columns to accurately determine the specific activity of radiolabelled proteins for use in receptor binding studies. A recent publication[15] has demonstrated the use of conventional analytical columns (25 cm × 4.6 mm I.D.) to both purify and determine the specific activity of radiolabelled biochemicals including triiodothyronine, tyroxine and estriol. However, the much lower extinction coefficients of proteins requires an increased detection sensitivity which is obtainable using the short microbore columns.

EXPERIMENTAL

Chemicals

Acetonitrile (Unichrom grade) was from Ajax Chemicals, Sydney, Australia. Sodium chloride (Aristar grade), hydrochloric acid (Aristar grade) and trifluoroacetic acid (TFA, Spectrosol grade) were purchased from BDH Chemicals, Australia. Heptafluorobutyric acid (HFBA, Sequanal grade) was from Pierce, Rockford, IL, U.S.A. All water used was from a Millipore Super Q polishing system. Sources of protein

standards used were as follows: insulin, crystalline (cat. no. 44061) from BDH Biochemicals, Poole, U.K.; cytochrome *c* Type VI from horse heart (cat. no. C-7752) from Sigma, St. Louis, MO, U.S.A.; myoglobin, 2 × cryst, from horse heart (cat. no. 1155) from Mann Research Laboratories, New York, U.S.A.; lysozyme (cat. no. 32-012) from Mile-Seravac, Maidenhead, U.K.; and bovine pancreatic ribonuclease A was from P. L. Biochemicals, Milwaukee, WI, U.S.A. Standard test solutions of those proteins were prepared in the primary chromatographic solvents.

Purification of epidermal growth factor ($EGF\alpha_1$)

EGFα1 was purified from murine salivary glands by HPLC as described elsewhere[16,17]. Standard solutions of EGFα1 were quantitated by measuring their absorbance at 280 nm and using the published extinction coefficient[18] of 30.9 ($E^{1\%}_{1\,cm}$, 280 nm) or by direct weighing after lyophilisation from volatile solvents.

Iodination of $EGF\alpha_1$

$EGF\alpha_1$ was radioiodinated as described previously[17]. Separation of ^{125}I-$EGF\alpha_1$ from free ^{125}I was performed on Waters C_{18} Sep-pak cartridges (Waters Assoc., Milford, MA, U.S.A.). All recovered fractions, transfer syringes and vessels used during the radioionization were monitored for radioactivity using an ECCO N559 medical probe with a 1 in. crystal connected to a Nucleic Enterprises SR5 scale-ratemeter. This enabled overall recoveries and labelling efficiency to be determined.

Chromatographic apparatus

Separations were performed on a Beckman-Altex Model 324-40 HPLC system consisting of a Model 421 microprocessor controller, two Model 100 pumps, a 1.8-ml dynamic high-pressure mixer and an Altex 210 injection valve equipped with a 2-ml injection loop (Beckman Instrument Division, Berkley, CA, U.S.A.). Tryptophan-containing proteins were detected by their endogenous fluorescence using a Kratos Model FS950 fluorometer (215 nm excitation, 340 nm emission) equipped with a 25-μl flow cell. A Hewlett-Packard Model 1040 scanning diode array detector (Hewlett-Packard, Palo Alto, CA, U.S.A.) equipped with a 4.6-μl flow cell with a 6-mm optical path length was used in experiments to determine the column protein capacity and also to confirm the integrity of tryptophan residues after radioiodination. Chromatograms were recorded on either a Kipp & Zonen BD40 recorder (Beckman Instrument Division), or a computer controlled data acquisition system developed at the Melbourne Branch of the Ludwig Institute. Quantitation was either by direct measurement of peak height or from computerised determination of the peak area. Eluent fractions were recovered using a Pharmacia FRAC100 collector (Pharmacia, Uppsala, Sweden) connected to the detector outlet with carefully calibrated low dead volume PTFE tubing (0.35 mm I.D.) so that recovered biological activity or radioactivity in the fractions could be accurately correlated with the recorder trace.

Gradient elution with the 2.1-mm columns was achieved by forming the gradient at low flow-rates within the 1.8-ml Beckman mixing chamber in the following manner: following injection, the flow-rate was maintained at 1 ml/min for 2 min with primary solvent alone. During this time the protein sample was cleared from the 2-ml injection loop and trace enriched onto the head of the microbore column. The flow-rate was then programmed to 0.1 or 0.2 ml/min and the secondary solvent

composition was simultaneously adjusted to that desired at the end of the gradient. In this way an exponential gradient was formed as the volume of solvent in the mixing chamber was slowly displaced. By adding a UV absorbing material (HFBA) to the secondary solvent and monitoring the rise in A_{210} the exact gradient profile could be determined.

The 1 mm column was also used for trace enrichment of proteins followed by step-wise elution of retained material as follows: samples were loaded onto the head of the column via the 2-ml injection loop at a flow-rate of 1 ml/min. The loaded column was then removed from the apparatus, the secondary solvent changed to the desired eluting composition and the instrument flushed with this solvent. Alternatively a low dead volume solvent purge valve could be fitted between the injection valve and the column to facilitate this manipulation. The column was then reinserted into the chromatograph, and retained solutes were eluted with this solvent at a flow-rate of either 20 or 40 μl/min. To minimise dilution due to intrinsically high post-column dead volumes relative to these low flow-rates, peak volume and recovery data were also obtained by manual collection of eluted ^{125}I radiolabelled proteins from a 1-cm length of 0.35 mm I.D. PTFE tubing inserted directly into the column outlet fitting.

A Hewlett Packard Model 1090 chromatograph, designed for low dispersion chromatography, was purchased during the course of these studies and the results of protein microbore chromatography using this machine, which was compatible with direct programming of gradients at low flow-rates, were compared with those from the Beckman chromatograph, designed primarily for use with conventional analytical columns.

Chromatographic columns

Stainless-steel tubing (7.5 cm long, 1/4 in. O.D., 2.1 mm I.D. or 7.5 cm long, 1/8 in. O.D., 1 mm I.D.) was from Sugiyama, Tokyo, Japan. The end fittings for the 1-mm I.D. column were modified Swagelok reducing fittings fitted with 0.5 μ frits from Mott Metallurgical, Farmington, CN, U.S.A. For the 2-mm I.D. column standard Swagelok end fittings with Beckman 2-μm frits were used. These empty columns were a gift from Beckman Instrument Division, Berkeley, CA, U.S.A. The columns and fittings were extensively cleaned by sonication in acetone and acetonitrile prior to use. They were packed from 2% (w/v) slurries of Hypersil ODS (Shandon Southern, Runcorn, U.K.) in 2-propanol at 10,000 p.s.i., and then further consolidated at 25,000 p.s.i., using a Haskell Engineering Model DSTV122 air driven fluid pump with "Sno-Trik" valves and unions for connecting purposes. After the completion of packing columns were equilibrated with acetonitrile–water (25:75) and their chromatographic efficiencies at optimised linear flow velocities were determined using a test mixture of acetophenone, nitrobenzene, benzene and toluene.

The suitability of commercially available microbore guard columns (3 cm × 2.1 mm I.D.) for the chromatography of proteins (Brownlee Labs., Santa Clara, CA, U.S.A.) was also investigated.

Determination of column protein loading capacity

The loading capacity of the columns was determined in two ways: a test protein solution (cytochrome *c* or lysozyme, 0.5 mg/ml in 0.9% sodium chloride, pH 2.1) was loaded onto the column, (previously extensively washed with acetonitrile–0.9%

sodium chloride pH 2.1 (1:1), and then requilibrated with 20 ml of the aqueous primary solvent), at a flow-rate of 1 ml/min directly via the HPLC pumps until a rapid rise in the baseline absorption at 280 nm indicated that saturation of the column with the protein had occurred. The total volume of the standard protein solution required to achieve this was noted. To correct for system dead volume the protein solution was then replaced with the aqueous primary buffer and the volume required to displace the residual unadsorbed protein solution was determined. From the difference in these two values the total mass of protein adsorbed on the column packing could be quantitated. The total trace enrichment capacity and the subsequent recovery of proteins from these columns could be confirmed by eluting the retained protein with acetonitrile–0.9% sodium chloride pH 2.1 (1:1), and quantitating the amount of protein recovered by measuring the A_{280} of the eluate fraction.

Having determined the maximum load capacity of the columns, the practical limiting load above which apparent chromatographic efficiency diminished rapidly (*i.e.* peak volumes increased) was determined by single injections of increasing protein load under aqueous loading conditions. Proteins were recovered by gradient elution and the extrapolated width at the peak base of the resultant chromatographic trace was used to quantitate peak volume.

Determination of specific acitivity

Specific activities of ^{125}I radiolabelled proteins were calculated by direct correlation of protein mass, as determined from the absorption profile with the recovered radioactivity in corresponding eluate fractions. Radioactivity was determined using a Packard Auto-Gamma 500C programmed for automatic efficiency determination (Packard Instrument, IL, U.S.A.).

RESULTS AND DISCUSSION

Although it is over 5 years since Ishii *et al.*[19] first described the use of packed microbore columns for HPLC separations, such technology has seen little use for the separation of proteins. The role of HPLC in the extraction and purification of proteins from crude biological samples prior to chemical and structural analysis is now widely accepted[20]. We now attempt to further extend the potential of HPLC in this area by the use of short microbore columns.

It has been the aim of this study: (a) to evaluate the potential of short microbore columns for the chromatography of proteins with high efficiency, resolution and recovery; (b) to show that the total protein load capacity of these columns is sufficient for subsequent analysis of recovered proteins by automated amino acid sequence analysis; and (c) to show that the increased eluate concentration associated with microbore chromatography improves the levels of protein detectability and thus allows direct calculation of the specific activity of ng quantities of radiolabelled proteins.

Columns and packings

The microbore columns used in these studies were packed in the laboratory with Hypersil-ODS, a packing which has previously been used successfully for the efficient chromatography of a wide range of polypeptides and proteins[13] and is readily available as a bulk packing. The details of the columns and packings used are given in Table I.

TABLE I

DETAILS OF COLUMNS AND PACKINGS EVALUATED

Packing	*Particle size (μm)*	*Pore diameter (Å)*	*Surface area (m^2/g)*	*Ligand*	*Column length (cm)*	*Column diameter (mm)*	*Source**	*Efficiency (plates/metre)***
Hypersil ODS	5	120	200	C_{18}	7.5	4.6	LP	63,330
					7.5	2.1	LP	40,257
					7.5	1.0	LP	29,000
Ultrasphere RPSC	5	300	100	C_3	7.5	4.6	PP	66,480
Aquapore RP-300	10	300	NA***	C_{18}	3.0	4.6	PP	23,025
					3.0	2.1	PP	14,958

* LP = packed in the laboratory; PP = packed by manufacturer.
** Efficiencies were determined as described in Experimental.
*** NA = not available.

It can be seen that the chromatographic efficiencies of the microbore columns were lower than that shown by the 4.6-mm I.D. analytical column. However, absolute column efficiency does not appear to play a significant role in gradient elution RP-HPLC of proteins, elution and separation being predominately achieved by selective desorption when a critical concentration of organic modifier is achieved[13,21]. The efficiencies achieved with the 1 mm column or the 3 cm × 2.1 mm I.D. Brownlee guard column were therefore considered more than adequate for the purposes of this study.

Gradient elution with microbore columns

A more serious limitation was considered to be the pre- and post-column dead volumes associated with the Beckman 324-40 HPLC system used for the majority of these studies. In particular, the relatively large volume (1.8 ml) of the Beckman mixing chamber would represent a delay of *ca.* 36 min in the formation of programmed gradients at a flow-rate of 100 μl/min compatible with a 2.1-mm I.D. column. It was therefore decided to form the gradient within the mixing chamber by displacement with secondary solvent (see Experimental). Such a system has been shown to form an exponential gradient in which the early portion approaches a linear gradient[22]. The actual gradient profile formed in this manner at a flow-rate of 100 μl/min was monitored by adding a UV absorbing material to the secondary solvent. The profile obtained is illustrated in Fig. 1. The delay of *ca.* 20 min before the gradient profile starts to change may be attributed to the pre-column volume of the injection loop (2 ml) used to load the sample.

It should be emphasised at this point that a 2-ml injection loop was used throughout these studies. This was possible because large volumes of protein solutions can be enriched and retained on either reversed-phase or ion-exchange packings at concentrations of secondary solvent below those required to cause selective desorption[13,14,20]. Under trace enrichment conditions chromatographic concentration of large sample volumes was achieved without significant band broadening, thereby

overcoming one of the potential limitations of microbore chromatography, namely small injection volumes.

Fig. 1 demonstrates the potential of this system to separate a number of protein standards on the 2.1-mm I.D. column using gradient elution between 0.9% sodium chloride (pH 2.1) and acetonitrile–0.9% sodium chloride (pH 2.1) (60:40) at a flow-rate of 100 μl/min. At first sight the relative inflexibility of this method of gradient formation would appear to be a limitation. However, the narrow range of secondary solvent composition over which protein elution occurs allows separation of compounds of only slightly differing hydrophobicity with gradients of this shape. As would be predicted, the separations achieved are similar to those obtained in previous studies with 4.6-mm I.D. columns packed with the same support[13]. Despite the simplicity of the method for producing the gradient, reproducible chromatography was achieved. Using the elution conditions shown in the legend to Fig. 1, 5 consecutive chromatograms of an EGFα_1 standard yielded retention times of 35.47 ± 0.22 min allowing accurate peak identification in more complex chromatograms. Absolute recovery was monitored from the recovery of a radiolabelled protein, ^{125}I-EGFα_1, and shown to be quantitative (greater than 95%) within the range tested (500 pg to 10 μg). Similar results were obtained with the 3 cm × 2.1 mm I.D. Brownlee microbore guard columns or when a similar gradient profile was generated with the Hewlett-Packard 1090 chromatograph.

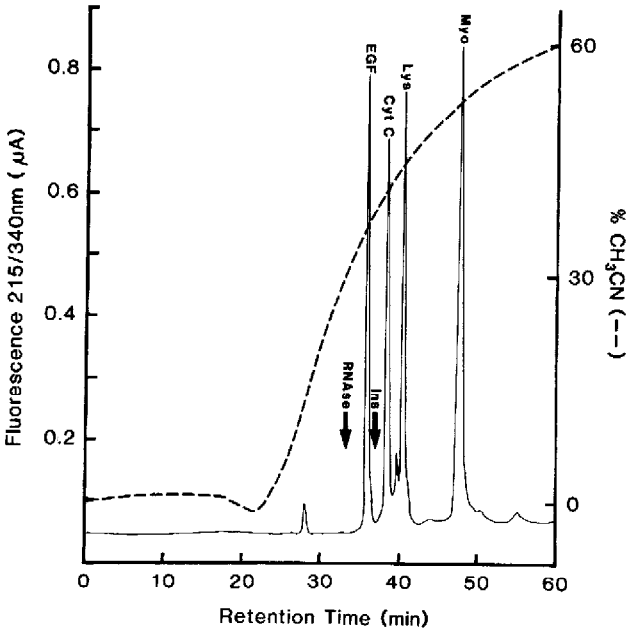

Fig. 1. The separation of protein standards on a 7.5 cm × 2.1 mm I.D. microbore column, packed with Hypersil ODS, using the Beckman HPLC system. Gradient elution between a primary solvent of 0.9% sodium chloride (pH 2.1) and acetonitrile at a flow-rate of 100 μl/min was achieved as described in Experimental. The resultant gradient profile is illustrated (– – –). The protein standards are epidermal growth factor (180 ng), cytochrome *c* (450 ng), lysozyme (550 ng) and myoglobin (750 ng) injected in a volume of 2 ml. Detection was by endogenous tryptophan fluorescence (215 nm excitation, 340 nm emission). The relative retention positions of ribonuclease A and insulin standards are indicated by arrows.

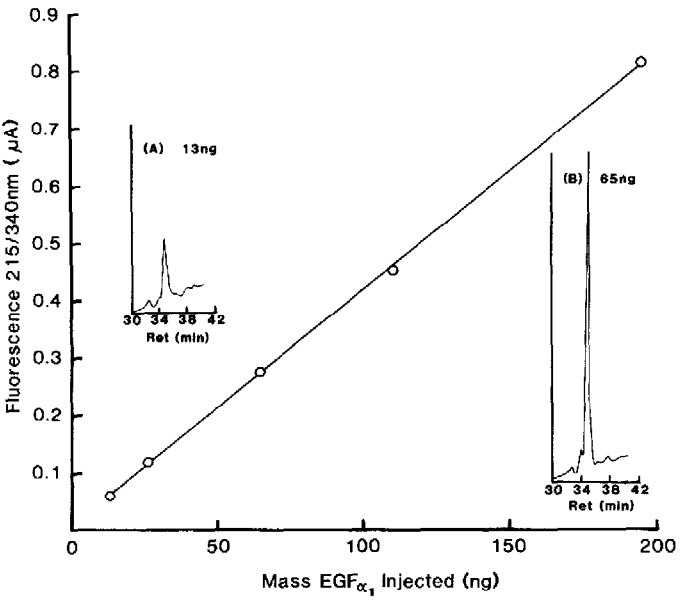

Fig. 2. The linearity of detector response for epidermal growth factor chromatographed as described in Fig. 1. The chromatograms obtained with (A) 13 ng and (B) 65 ng standards are shown (insert) so that detection limits may be estimated.

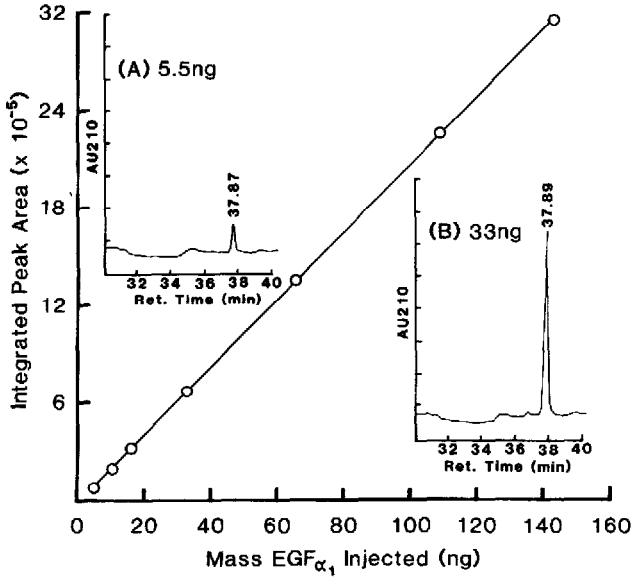

Fig. 3. The linearity of detector response for epidermal growth factor (EGF) chromatographed on a 7.5 cm × 2.1 mm I.D. column packed with Hypersil ODS using the Hewlett-Packard 1090 chromatograph. Elution conditions were programmed so that they were similar to those obtained with the Beckman HPLC (Fig. 1). Representative chromatograms for (A) 5.5 ng and (B) 33 ng of EGF are shown so that the limits of sensitivity may be calculated. These chromatograms were recorded at an attenuation of 0.008 a.u.f.s.

Sensitivity of detection

The linearity of detector response and the limits of sensitivity (defined as a signal-to-noise ratio of 5) using the 2.1-mm I.D. column under gradient elution conditions at a flow-rate of 100 μl/min were investigated using protein standards on both the Beckman 324-40 system equipped with the fluorescence detector and the Hewlett-Packard 1090 chromatograph with detection by ultraviolet absorption at 210 nm. Examples of the chromatograms and the resultant detector calibration curves are shown in Figs. 2 and 3. Using the Beckman 324-40 system with the fluorescence detector, 5 ng can be routinely detected with the equipment configuration used (Fig. 2). For the ultraviolet detector (HP1040) and using equipment designed specifically for low dispersion chromatography (HP1090), *ca.* 1 ng would appear to be a conservative estimate of the limits of detectability (Fig. 3) (greater than 5× baseline noise).

Our experience with conventional analytical columns (7.5 cm × 4.6 mm I.D.) operating at 1 ml/min where losses of apparent chromatographic efficiency due to post-column band broadening are reduced, demonstrated that at least a 5-fold increase in sensitivity could be expected from the fluorescence detector (Fig. 4). The failure to realise this increase in practice when using microbore columns and when comparing data from the Beckman system with the fluorescence detector (cell volume 25 μl) with those obtained from the HP1090 with the ultraviolet absorption detector (cell volume 4.6 μl, 6-mm path length) is almost certainly a reflection of the detector geometry. The flow cell in the fluorescence detector (Kratos FS950) is a 1-mm I.D. quartz tube held in position with 0.3 mm to 1 mm I.D. tapered Kel-F fittings. Inspite of the relatively large volume of this flow cell (25 μl) its contribution to increase in

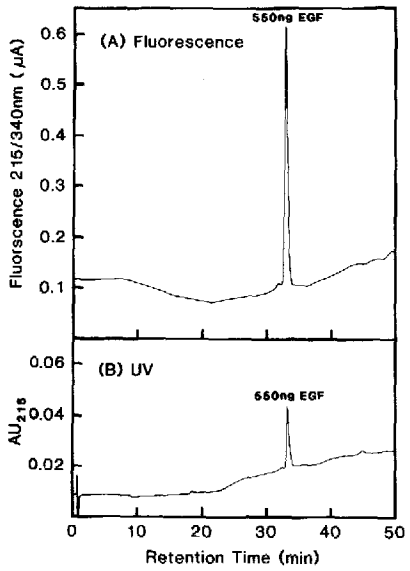

Fig. 4. The relationship between fluorescence (215 nm excitation, 340 nm emission) and ultraviolet absorption (215 nm) detection of epidermal growth factor. Chromatograms were obtained on a 7.5 cm × 4.6 mm I.D. Ultrasphere RPSC column using the Beckman HPLC system to generate a linear 1.5%/min gradient between 0.9% sodium chloride (pH 2.1) and acetonitrile at a flow-rate of 1 ml/min.

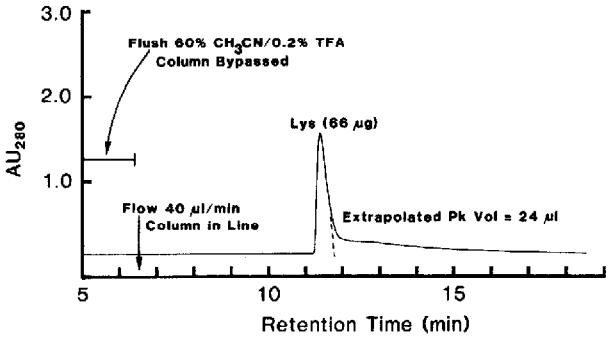

Fig. 5. Stepwise gradient elution of lysozyme (66 μg) from the 7.5 cm × 1 mm I.D. microbore column packed with Hypersil ODS. The sample was loaded in a 2-ml volume. Elution was with acetonitrile–0.2% TFA (60:40) as indicated on the diagram and in Experimental.

the peak volume was tolerable. Using the 7.5 cm × 2.1 mm I.D. column at a flow-rate of 100 μl/min and under comparable gradient conditions (*ca.* 1% acetonitrile/min), the peak volume obtained with the fluorescence detector was *ca.* 75 μl (Figs. 2 and 7) whereas that obtained with the lower volume cell in the ultraviolet absorbance detector was *ca.* 50 μl (Fig. 3). This would suggest that the use of a fluorescence detector with a flow cell volume compatible with the low flow-rates used in microbore chromatography and perhaps with an increase in lamp excitation energy (*e.g.* laser excitation)[23,24] giving improved quantum yields of fluorescence would easily allow detection and quantitation of pg quantities of a polypeptide, such as EGF.

The practical importance of the results obtained with the 2.1-mm I.D. column and the fluorescence detector with the standard Beckman HPLC should not be overshadowed by the results obtained with the Hewlett-Packard equipment which was designed specifically for microbore chromatography. Firstly, there was a significant increase in the level of detectability (× 5) compared with the use of 4.6-mm I.D. columns of the same length in the same equipment (*cf.* Fig. 2, Fig. 4). Secondly, it must be remembered that the increases in sensitivity observed with proteins will also hold true for background contaminants arising from sample workup or due to the chromatographic solvents themselves particularly in work at low wavelengths (210 nm). The fluorescence detector, being more selective, is less susceptible to background perturbations. Thirdly and perhaps most importantly, samples loaded in a volume of 2 ml or greater can be recovered after chromatography on the 2.1 mm column in a volume of less than 100 μl, representing a *ca.* 20-fold increase in concentration demonstrating the trace enrichment potential of these columns.

Since many workers will be limited to using microbore techniques on equipment, designed primarily for analytical and preparative HPLC, results obtained with the Beckman HPLC system are likely to be representative of the capabilities of other modified microbore systems. However, since the fluorometric detector is not ideally suited to handling high protein concentrations, the Hewlett-Packard 1040 scanning diode array detector was used to monitor protein elution for the column protein capacity experiments.

Protein loading capacity and recovery

Having established that efficient separation, recovery and detection of low levels of protein is possible with short microbore columns the next objective was to determine whether the protein load capacity of such columns was compatible with the mass required for the subsequent automated amino acid sequence analysis of purified eluate fractions. Recent publications[7,25,26] show that, working with the gas phase sequenator, sequence data can be obtained from 50 to 100 pmol (*i.e.* ng–μg quantities) of protein. We, and other workers, have demonstrated the potential of conventional 4.6-mm I.D. columns for the chromatography of milligram quantities of protein standards and have successfully used such columns in the preparation of up to 10 mg of highly purified proteins from biological samples[16,27]. The protein load capacity will depend on the structure of the chromatographic packing[28] (proportional to the surface area), the dimensions of the column[28] (proportional to the volume) and the nature of the protein itself. It therefore seemed likely that the protein mass load capacity of the microbore columns would be easily sufficient to chromatograph the μg quantities needed for sequencing studies.

The protein load capacity of the columns was evaluated by pumping a standard solution of cytochrome *c* or lysozyme onto the column until a significant rise in the detector baseline indicated that protein was no longer being retained by the column. After correcting for the dead volume of the system (see Experimental) the total mass of protein loaded onto the packing was calculated. To confirm this load capacity, to determine the protein recovery and the maximum concentration of protein achievable in eluate fractions, the enriched protein was recovered by elution with acetonitrile–0.9% (w/v) aqueous sodium chloride pH 2.1 (60:40). The results obtained are shown in Table II. It can be seen that even the 1 mm I.D. column, packed with the 100 Å C_{18} reversed-phase support (surface area 200 m^2/g) has a total capacity of 4.55 mg for cytochrome *c*, enriched from a sample volume of 9.1 ml. This protein load was

TABLE II

TRACE ENRICHMENT CAPACITY OF COLUMNS EVALUATED

ND = not determined.

Packing	*Column length (cm)*	*Column diameter (mm)*	*Calculated protein*		*Eluate volume (ml)*	*Eluate protein concentration (mg/ml)*
			Loaded (mg)*	*Recovered** (mg)*		
Hypersil ODS	7.5	4.6	100	ND	ND	ND
		2.1	21	22.38	1.6	13.98
		1.0	4.7	4.55	1.5	3.03
Ultrapore RPSC	7.5	4.6	50.55	53.14	4.8	11.07
Aquapore RP-300	3.0	4.6	17.0 (16.8)***	17.7 (17.3)***	2.5	7.08
		2.1	4.8 (3.97)***	4.17 (3.93)***	1.4	2.97

* Calculated from the volume of cytochrome *c* loaded before protein break through occurs.

** Calculated from the A_{280} of the protein recovered in a 60% acetonitrile eluent fraction.

*** Figures in parentheses are for lysozyme.

TABLE III

CONCENTRATION OF PROTEINS RECOVERED FROM HPLC COLUMNS OF VARYING I.D.

Column packing	*Flow (ml/min)*	*Column dimensions*	*Mass protein loaded (μg)*	*Protein load concentration (mg/ml)*	*Eluate volume (μl)*	*Eluate protein concentration (mg/ml)*	*Concentration factor*
Hypersil ODS	0.20	7.5 cm × 2.1 mm I.D.	100	0.05	200	0.50	10.0
			50	0.025	160	0.31	12.4
			20	0.01	110	0.18	18.0
Hypersil ODS	0.04	7.5 cm × 1 mm I.D.	66	0.33	25	2.64	80.0
			20	0.01	25	0.80	80.0
Aquapore RP300	0.20	3.0 cm × 2.1 mm I.D.	500	0.25	500	1.00	4.0
			100	0.05	300	0.33	6.6
			20	0.01	120	0.18	18.0
			10	0.005	100	0.10	20.0
			0.2	0.0001	100	0.02	20.0
Ultrasphere RPSC	1.00	7.5 cm × 4.6 mm I.D.	10,000	5.0	4400	2.27	0.45
			5500	2.75	2600	1.92	0.7
			2500	1.25	2000	1.25	1.0
			1000	0.5	1000	1.00	2.0
			500	0.25	1000	0.5	2.0

recovered essentially quantitatively in 1.5 ml at a resultant concentration of 3.03 mg/ml. The loading achieved with 2.1-mm I.D. columns was proportionately greater. Recent publications have shown the superiority of large pore size[4] (300 Å) silica packing materials with chemically bonded short-chain (C_3) alkyl silicas for the efficient chromatography and recovery of larger, more hydrophobic proteins[4,29]. The reduced surface area of such supports (see Table I) will of course result in a comparatively lower protein mass load capacity. On the positive side, a recent study[30] has shown that short-alkyl-chain packings have a significantly increased load capacity compared with the corresponding long ($> C_{12}$)-alkyl-chain packings. Since the 300 Å C_3 materials are not yet readily available commercially, either as packed short microbore columns or as loose bulk packings, the potential performance of these supports has been extrapolated from results obtained with a 7.5 cm × 4.6 mm I.D. analytical column (details of the packing material are given in Table I). This column exhibited a trace enrichment potential of 53.14 mg, from which we would conclude that a 7.5 cm × 1 mm I.D. microbore column, packed with the same material would have a protein capacity of *ca.* 2 mg, again compatible with subsequent sequence analysis. It can be seen from Table II that all the columns tested had protein capacities in excess of 1 mg. These quantities could be recovered virtually quantitatively at concentrations which would have allowed sequence data to be obtained if aliquots (25–30 μl) were spotted directly on to the polybrene-treated glass fibre disc used in the gas phase sequenator[7]. However, such quantities are hardly representative of the amounts likely to be encountered in the isolation of biologically active proteins from bulk biological samples. We therefore investigated the relationship between protein mass and peak volume for the 2.1-mm and 1-mm I.D. columns operated under gradient elution conditions. When analytical (ng) quantities of protein were chromatographed peak volumes of 50–100 μl were obtained (Figs. 1, 2, 3 and 7). However, the practical criterion for the suitability of this technique was that pmole quantities of protein could be loaded onto the microbore column in large eluent volumes (*i.e.* at least 2 ml: equivalent to a 1-ml fraction recovered from an analytical column and then diluted 1:1 for subsequent trace enrichment onto the microbore column) and recovered at *ca.* mg/ml concentration, allowing the total sample to be directly loaded into the gas phase sequenator. The relationship between the mass loaded and the resultant eluent recovery volume after chromatography for protein standards was investigated on the 2.1-mm and 1-mm I.D. columns using gradient elution conditions between 0.2% TFA and acetonitrile–0.2% TFA (50:50) at flow-rates of 200 μl and 40 μl/min, respectively (a similar linear flow velocity to that used with 4.6-mm I.D. columns for the optimum recovery of all proteins[4,5]). Salt-containing mobile phases were not used for these studies as they interfere with subsequent sequence analysis[7]. The results obtained are presented in Table III. Clearly the 1-mm I.D. column, operated at a flow-rate of 40 μl/min allows recovery of proteins at eluite concentrations which are directly compatible with their use in subsequent automated amino acid sequence analyses (greater than 0.8 mg/ml) at a mass load which is representative of that likely to be encountered in the purification of biologically important proteins present in bulk biological samples at trace concentration, e.g. hemopoietic colony stimulating factors[31], transforming growth factors[32], or platelet-derived growth factor[25]. Fig. 5 shows the chromatogram obtained with 66 μg of lysozyme on the 1-mm I.D. column at a flow-rate of 40 μl/min. Elution conditions are

given in the figure legend. It should be noted that at the loading flow-rate of 1 ml/min this column develops a back pressure of *ca.* 8000 p.s.i. Although this is compatible with the Beckman Model 100 pumps, lower loading flow-rates would be needed with other pumping systems. In spite of these high pressures, no problems with column lifetime have been noted to date (greater than 50 injections). In spite of some peak asymmetry, possibly related to the lower efficiency of this column, the majority of the peak could be recovered in a volume of *ca.* 25 μl at a resultant concentration of 2.64 mg/ml. The 2.1-mm I.D. columns, while not offering the recovery concentration of the 1-mm I.D. columns, offer significant advantages over 4.6-mm I.D. columns *viz.* eluate protein concentration for protein loads less than 500 μg.

An important practical point is that under the gradient conditions used, the 2.1-mm I.D. column can achieve further purification of closely related proteins (*e.g.* Figs. 1 and 5), whereas the 1-mm I.D. column was operated in a manner giving only batchwise elution of proteins of similar hydrophobicity. It was felt that linear gradient elution at flow-rates lower than 100 μl/min with the equipment configuration used was not practical although a recent modification (data not presented) where the volume of the dynamic mixer was reduced by the use of a PTFE insert would have allowed this. The lack of further resolution using stepwise elution would not be of consequence where proteins have already been purified on conventional HPLC columns, particularly if the use of a number of different selectivities (*e.g.* reversed phase, ion exchange, chromatofocussing or size exclusion) has already been exploited to achieve maximum purification from possible contaminants and chromatographic concentration prior to sequence analysis or chemical manipulation is the only objective.

At higher mass loads (greater than 500 μg) and under the gradient conditions used, the microbore columns offer no great advantage over conventional (4.6 mm I.D.) columns operated at flow-rates of 1 ml/min. The results in Table III show that with 4.6-mm I.D. columns, eluate protein concentrations of greater than 1 mg/ml are achievable for protein loads of 1–10 mg. Similar results were obtained by Pearson *et al.*[30] in studies on protein load capacities of a 5 cm × 4.1 mm I.D. column packed with a 330 Å, C_8 support, where bovine serum albumin and ovalbumin were used as test proteins.

Chromatography of radiolabelled proteins

The use of radiolabelled proteins of high specific activity as eluent volume markers, when operating in the stepwise gradient elution mode as described above, may prove useful in the chromatographic concentration of uncharacterised proteins prior to sequence analysis. The use of radiolabelled proteins allows the determination of eluent protein volumes while avoiding extra-column peak broadening due to the detector geometry, which becomes significant at the low flow-rates associated with the 1-mm I.D. columns. Eluted radioactivity can be collected manually from a 10-mm length of 0.35 mm I.D. PTFE tubing inserted directly into the column outlet (volume 0.96 μl). To facilitate collection the end of the PTFE tube is cut at *ca.* 45° so that the eluent stream may be run down the side of the collecting vessel. The radioactivity histogram obtained with a ^{125}I-EGFα_1 standard is shown in Fig. 6. ^{125}I-EGFα_1 was purified by analytical HPLC on a 4.6-mm I.D. reversed-phase column as described previously[17]. Unlabelled EGFα_1 (10 μg) was added to a 1-ml eluate

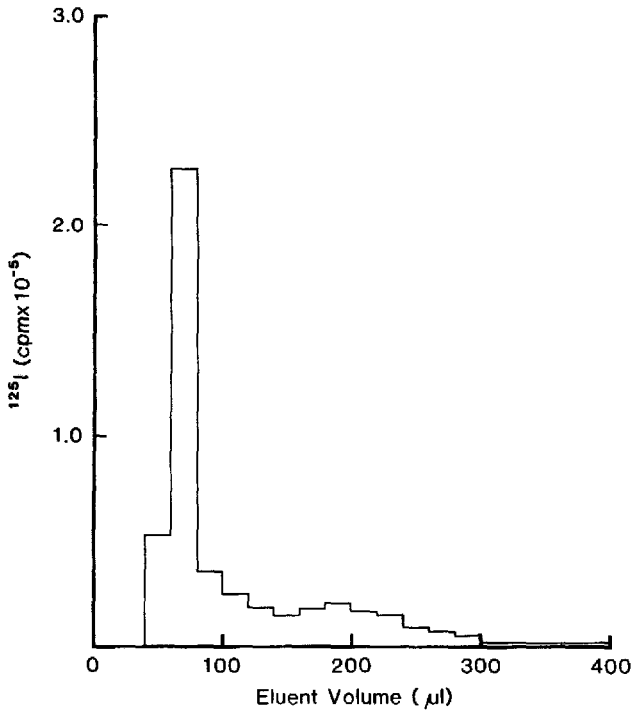

Fig. 6. Stepwise gradient elution of EGFα_1 (10 μg) and ^{125}I-EGFα_1 from the 7.5 cm × 1 mm I.D. microbore column packed with Hypersil ODS. Gradient elution conditions were as in Fig. 5, except a flow-rate of 20 μl/min was used. Eluted radioactivity was recovered manually from a short length of 0.35-mm I.D. PTFE tubing inserted into the column outlet.

fraction containing the ^{125}I-EGFα_1, which was then diluted to 2 ml with aqueous primary buffer (0.2% TFA) and loaded onto the 1-mm I.D. microbore column at 1 ml/min via the injection loop. The proteins were then eluted with acetonitrile–0.2% TFA (60:40) at a flow-rate of 20 μl/min as described in Experimental. Fractions were collected manually at 1-min intervals. The recovery of total radioactivity loaded was essentially quantitative. It can be seen that greater than 60% of the radioactivity was recovered in one 20-μl fraction. Under these conditions where batchwise elution of proteins of similar hydrophobicity is obtained radiolabelled proteins of high specific activity and of relatively low hydrophobicity could be added to highly purified proteins for sequence analysis, prior to their microbore trace enrichment as described herein, as markers to optimise recovery in minimal volumes.

It should be noted at this point that the use of automated fraction collection equipment poses operational problems when low flow-rates are used. All connecting tubing between the detector flow cell and the fraction collector needs to be carefully calibrated and of small volume. The spacial design of commercially available fraction collectors means that even with tubing of 0.35-mm I.D. post-detector volumes of *ca.* 50 μl must be tolerated. The limiting collection volume becomes the drop size (*ca.* 8 μl) formed at the tube outlet which is sufficiently large to give rise to volume

innaccuracies if collecting fractions in the timed mode. Additionally, the loss of even a single drop in between tube changes can result in a large proportion of the sample being lost. However a fraction collector of the type described recently for the collection of microbore samples prior to laser mass spectrometry should overcome these problems[33].

In the course of these studies it became apparent that the increase in detector sensitivity associated with the microbore protein separations was sufficient to enable quantitation of the protein content of ^{125}I-EGFα_1 preparations with high specific activity which had been radioiodinated using iodogen as described previously[17]. A typical result obtained using the Beckman HPLC system with the fluorescence de-

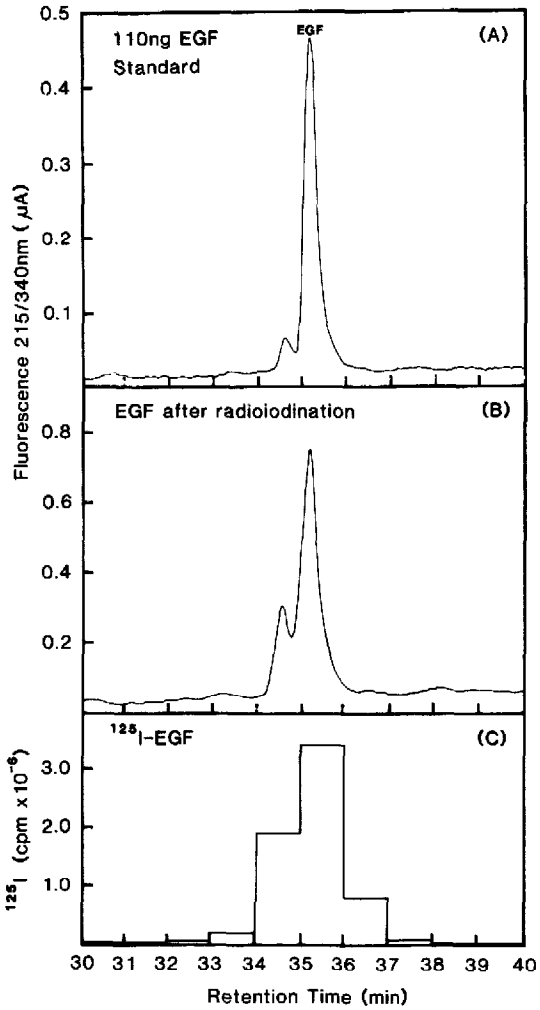

Fig. 7. The determination of the specific activity of ^{125}I-EGFα_1 using microbore HPLC. Chromatographic conditions were as in Fig. 1. (A) Chromatogram obtained with 110 ng EGFα_1 standard; (B) chromatogram obtained with an aliquot of ^{125}I-EGFα_1 after separation from the ^{125}I on a Waters C_{18} Sep-Pak cartridge (see Experimental); (C) the recovered radioactivity in 1-ml eluent fractions associated with (B).

tector is shown in Fig. 7. The specific activity of this preparation may be calculated as 19 mCi/mg. Similar results were obtained with the Hewlett-Packard 1090 using detection by ultraviolet absorption at 210 nm. Oxidation of tryptophan under the oxidising conditions used for radioiodination can occur[34], and could have interfered with quantitation by endogenous tryptophan fluorescence. The peak eluting slightly earlier (retention time = 34.5 min) is possibly due to methionine oxidation and has therefore been quantitated for the purpose of the specific activity calculation. It is of interest to note that considerable batch-to-batch variation in specific activity was noted for samples iodinated under nominally identical conditions, demonstrating the need for this type of analysis.

CONCLUSION

The results obtained in this study demonstrate the potential of short microbore columns for the chromatography and chromatographic concentration of proteins available in ng–μg quantities. Although it is more time consuming than batch concentration techniques the ability to recover proteins in high yield at high concentration in small (20–50 μl) volumes prior to subsequent structural analysis or chemical manipulation will be of use. In particular the considerable losses, due to non-specific absorption, which are often experienced when handling proteins at the ng–μg level with concentration methods such as lyophilisation, Speedy-Vac concentration or dialysis, will be avoided. In addition, the increased detector sensitivity obtainable with short microbore columns will be advantageous for analytical purposes, *e.g.* the measurement of the specific activity of radiolabelled proteins. Although optimum results will be achieved with equipment designed specifically for use with microbore columns, useful separations may be achieved on machines designed primarily for use with conventional analytical columns with only minimal modification, provided that pump and detector performance are adequate[35]. With most equipment configurations the use of a low dead volume mixer[35] will allow direct programming of the gradient profile increasing the flexibility of the method described herein.

It is obviously mandatory that the protein be compatible with HPLC purification for this technique to be applicable. However, the recent development of a number of new chromatographic supports for the HPLC of proteins[1,5,21] has already greatly increased the range of proteins for which this will be the case.

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