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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF NEUROPEPTIDES USING RADIALLY COMPRESSED POLYTHENE CARTRIDGES

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SUMMARY

This study was designed to assess practically the suitability of different C_{18} reversed-phase radially compressed polythene cartridges (Radial-Pak, Waters Assoc.) in two types of radial-compression systems, for the separation and analysis of various neuropeptides at both high (< 5 μg) and low (> 100 pg) levels in biological extracts and to compare them with well established techniques using stainless-steel columns.

A solvent system fully compatible with both radially compressed and steel columns is described. The completely volatile mobile phase (acetonitrile gradient containing trifluoroacetic acid) allows ultraviolet detection below 215 nm, gives good resolution and is readily compatible with the further radioimmunoassay and bioassay of collected fractions.

The efficiency of radially compressed 5 and 10 μm "capped" and "non-capped" C_{18} silica supports and slurry-packed steel columns has been assessed by: (1) separation and recovery of a complex standard mixture of neuropeptides; (2) separation and subsequent identification of degradation products formed during the incubation of neurotensin with rat cortical synaptosomes; (3) analysis of α-melanotropin and corticotropin-(18—39) in tissue culture media containing varying amounts of foetal calf serum; and (4) characterization of corticotropin-like immunoreactivity in human cerebrospinal fluid.

The Z-module fitted with the capped 10-μm irregular C_{18} silica cartridge gave better resolution than with the μBondapak steel column but the selective retention was similar. The back-pressures in the Z-module are much reduced (approximately 13 bar at 1 ml/min); therefore, flow-rates may be increased and analysis times greatly reduced. In order to obtain good resolution with the RCM-100 module which uses a non-capped stationary phase, a salt must be added (e.g. 15 m*M* sodium chloride) to the mobile phase to reduce polar interactions between the peptide and the free silanol groups on the stationary phase. This makes the solvent non-volatile and therefore less useful.

INTRODUCTION

The potential of reversed-phase high-performance liquid chromatography

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(RP-HPLC) in the separation and characterization of neuropeptides has been demonstrated by numerous investigators (see, for example, refs. 1—5). In the vast majority of cases separations are achieved using C_{18} microparticulate (3—10 μm) silica supports slurry packed in stainless-steel columns. After introduction to the column the peptides are eluted selectively according to their hydrophobicity by an aqueous-to-organic solvent gradient. Apart from hydrophobic interactions there are various ion-pairing effects [6—8] and column modifications [9] which will selectively affect retention.

An important criterion in HPLC column efficiency is packed-bed uniformity [10]. This is difficult to achieve in conventional slurry-packed steel columns. However, by applying radial compression to a dry-packed polythene cartridge, heterogeneity of packing in the stationary phase is minimized, especially around the column wall and end fittings. The potential advantages of this chromatographic procedure are: increased sample capacity, shorter analysis times, improved resolution, reduced back-pressures and greater economy. Peptides are not ideal candidates for the assessment of column kinetics as they display both polar and non-polar interactions with the stationary phase in RP-HPLC [11]. However, other studies have shown using non-polar solutes with good thermodynamic properties that radially compressed beds display greater uniformity than slurry-packed columns (assessed by log height versus log volume plots) [11].

Three chromatographic systems have been compared: the μBondapak stainless-steel column slurry packed with 10-μm capped irregular C_{18} silica; the RCM-100 radial-compression module using 5 and 10-μm spherical non-capped C_{18} silica cartridges radially compressed at approximately 170 bar; and, finally, the Z-module using 10-μm irregular C_{18} capped silica cartridges radially compressed at approximately 13 bar.

The efficiency of these systems has been assessed by their ability to separate and resolve a standard mixture of neuropeptides ranging in both size and hydrophobicity. The Z-module has been further evaluated by its use in the separation and identification of products formed during the incubation of neurotensin with nerve endings isolated from rat brain, by monitoring the stability of α-melanotropin (α-MSH) and corticotropin-(18—39) (CLIP) in tissue culture media containing varying concentrations of foetal calf serum and characterizing the corticotropin-like immunoreactivity (ACTH-IR) present in human cerebrospinal fluid.

MATERIALS AND METHODS

Equipment

A Waters HPLC system was used comprising two 6000A pumps, a Model 720 system controller, a WISP automatic sample injector, a 450 variable-wavelength detector, RCM-100 and Z-module radial-compression systems, 5- and 10-μm spherical C_{18} Radial-Pak cartridges, μBondapak 10-μm C_{18} Radial-Pak cartridges (all 10 cm × 8 mm I.D.) and a μBondapak 10-μm C_{18} stainless-steel column (Waters Assoc., Northwich, U.K.). Fractions were collected using an LKB Ultrorac 2070 collector controlled by a microprocessor, allowing collection of any part of the column effluent defined by its elution time [12].

Chemicals

Acetonitrile (HPLC S-Grade), was obtained from Rathburn Chemicals (Peebles, U.K.). Sodium chloride (AnalaR), trifluoroacetic acid (TFA, spectroscopic grade), acetic acid (chromatographic grade), phosphoric acid (AnalaR) were obtained from BDH (Poole, U.K.). Water was deionized, double glass-distilled and passed through a Porapak Q column. Tissue culture media and foetal calf serum (non-heat-inactivated) were obtained from Gibco (Paisley, U.K.).

Peptides

Neurotensin, thyrotropin-releasing hormone (TRH), TRH-OH, luteinizing hormone–releasing hormone (LH–RH), α-MSH, substance P and somatostatin were obtained from Peninsula Labs. (San Carlos, CA, U.S.A.). ACTH-(18–39) (CLIP) was obtained from Universal Biologicals (Cambridge, U.K.).

Methods

A 20-μl volume of the standard neuropeptide mixture containing TRH, TRH-OH, LH–RH, neurotensin, somatostatin and substance P (each 5 μg) was introduced to each system. The separations were achieved using linear gradients of 3% to 70% of solvent B over 10 and 20 min at flow-rates of 1 and 2 ml/min, respectively. Solvent A = 11 m*M* TFA–2.6 m*M* acetic acid. Solvent B = 70% acetonitrile containing 11 m*M* TFA. In the case of the RCM-100 system, sodium chloride (15 m*M*) was added to the aqueous phase (to counter free silanol groups on the stationary phase).

Metabolically active synaptosomes from rat cortex were prepared using the rapid method of Dodd et al. [13]. The synaptosome pellet (one cortex equivalent) was resuspended in 2.5 ml of Krebs phosphate buffer and incubated at 37°C with 50 μg of synthetic neurotensin. At varying time intervals 100-μl aliquots were removed, acidified with 10 μl of 10% TFA and centrifuged prior to analysis by HPLC.

Amounts of 25 μg of CLIP and 25 μg of α-MSH were incubated in 600 μl of tissue culture media containing 0, 1, 5 and 30% foetal calf serum at 37°C for 24 and 48 h. The incubations were terminated by adding 60 μl of 10% TFA followed by centrifugation prior to HPLC. The incubations containing CLIP were also run on HPLC using heptafluorobutyric acid (HFBA) in place of TFA as the acid modifier [14].

For analysis of cerebrospinal fluid, 7 ml were first concentrated on Sep-Pak cartridges (Waters Assoc.); the fraction containing the ACTH-IR was dried under vacuum and reconstituted in 200 μl of 0.1% TFA prior to injection.

The Radial-Pak cartridges were washed with acetonitrile (25 ml) prior to use. All the chromatography was carried out at ambient temperature, the solvents being filtered and degassed immediately before use.

RESULTS

Good resolution of a standard mixture was obtained on the μBondapak steel column (30 × 0.39 cm) (Fig. 1a) using a solvent system previously described [1]. A gradient of 3% to 70% of solvent B (i.e. 2.1% to 49% aceto-

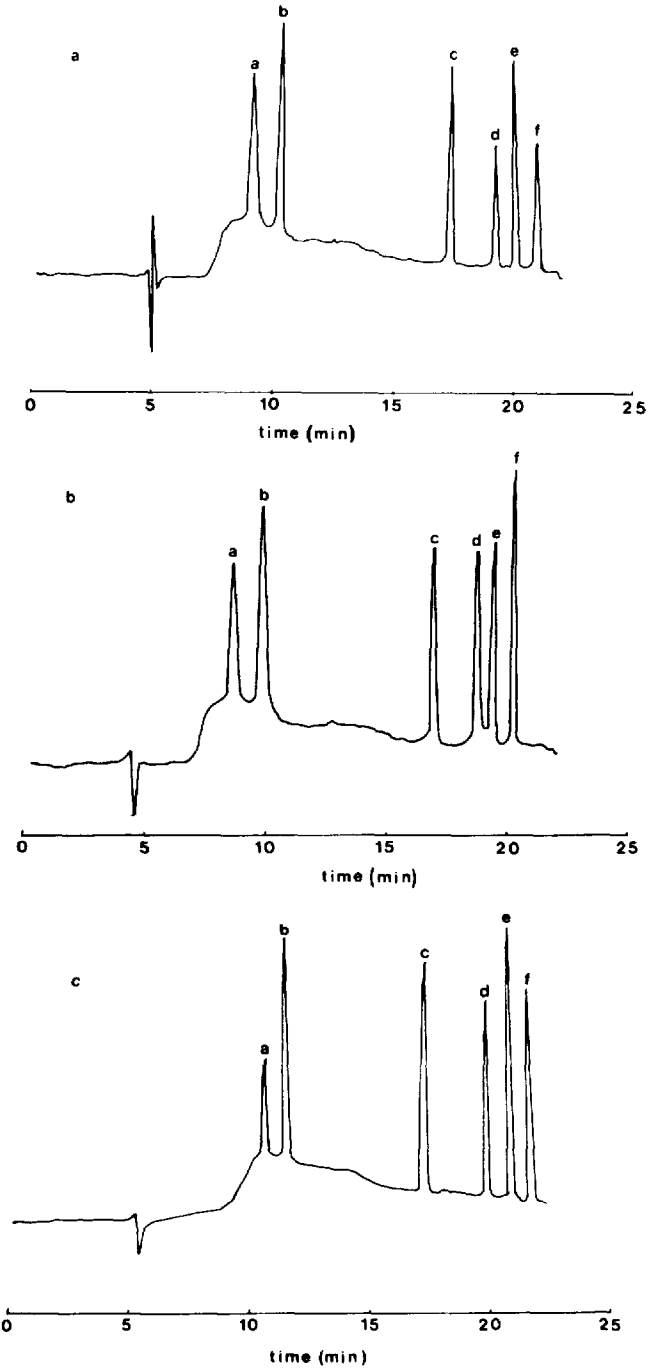

Fig. 1. (a) Separation of neuropeptide standards on the μBondapak steel column using an acetonitrile gradient. Solvent A = 11 m*M* TFA—2.6 m*M* acetic acid; solvent B = 70% acetonitrile containing 11 m*M* TFA; 20-min linear gradient, 3% to 70% solvent B at 1 ml/min. Peaks: a = TRH, b = TRH-OH, c = LH—RH, d = neurotensin, e = substance P, f = somatostatin (all 5 μg); 0.4 a.u.f.s. at 206 nm. (b) Elution conditions and standard as in Fig. 1a except the aqueous phase also contains 15 m*M* sodium chloride; the column used is the RCM-100 containing the 5-μm non-capped C_{18} Radial-Pak cartridge. Peaks as in (a). (c) Elution conditions and standard as in Fig. 1a. The column used is the Z-module containing the 10-μm capped C_{18} Radial-Pak cartridge. Peaks as in (a).

nitrile) in the presence of 11 m*M* TFA (pH 2.1) over 20 min at 1 ml/min is sufficient to resolve TRH, TRH-OH, LH—RH, neurotensin, somatostatin and substance P. Using this gradient a wide range of neuropeptides varying both in hydrophobicity and molecular weight can be separated. Small peptides such as the tripeptide TRH and its metabolites His-Pro diketopiperazine and TRH-free acid (TRH-OH) are well resolved in the early part of the chromatogram. The larger and more hydrophobic peptides (e.g. LH—RH, substance P and somatostatin) are retained longer but are equally well resolved.

The same standard was injected into the RCM-100 system using both 5- and 10-μm non-capped cartridges and the same solvent gradient. Only TRH and its metabolites were eluted and then as broad tailing peaks. However, by introducing a salt (in this case 15 m*M* sodium chloride) into the aqueous solvent A to act as a counter-ion to suppress the ionic interactions between the

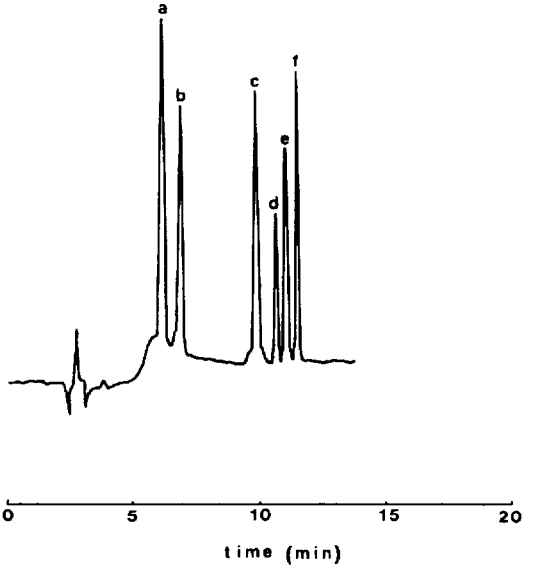

Fig. 2. Separation of the neuropeptide standards using the Z-module. Solvents as in Fig. 1a; 10-min linear gradient of 3% to 70% solvent B at 2 ml/min; peaks a—f as in Fig. 1a (a, 10 μg; b—f, 5 μg); 0.4 a.u.f.s. at 206 nm.

TABLE I

PEPTIDE RETENTION TIMES (min)

Elution conditions as in Fig. 1a.

Peptide	Z-module			μBondapak steel		
	$\bar{x}$	S.D.	*n*	$\bar{x}$	S.D.	*n*
TRH	9.17	0.067	10	5.56	0.041	10
LH—RH	19.81	0.058	10	18.51	0.052	10
Neurotensin	20.29	0.059	10	20.26	0.059	10
Substance P	22.06	0.053	10	21.60	0.050	10

free silanol groups on the stationary phase and the ionic amino acid side-chains in the peptide, all the peptides eluted as discrete sharp peaks (Fig. 1b).

The Z-module containing the 10-μm irregular capped Radial-Pak cartridge separated all the peptides in the standard under the same elution conditions used on the μBondapak steel column (Fig. 1c). Because of the low back-pressure with the Z-module (13 bar at 1 ml/min, 20% of that in the steel column), it was possible to reduce the analysis time by both increasing the flow-rate to 2 ml/min and reducing the gradient time to 10 min. The components of the standard mixture were again well resolved (Fig. 2).

Repeated injections of the standard were made onto both the Z-module and the μBondapak steel column in order to assess the reproducibility of each column type by comparing the variation in the peptide elution times in each system (Table I).

PRACTICAL APPLICATIONS

The products formed from the incubation of synthetic neurotensin with rat cortical synaptosomes were well separated using the Z-module (Fig. 3). The peaks (1—6) were collected and hydrolysed (6 *M* hydrochloric acid, 20 h, 110°C) prior to amino acid analysis. The amino acid ratios for each peak show that peak 1 is neurotensin-(12—13), peak 2 is neurotensin-(1—8), peak 3 is neurotensin-(1—10), peak 4 is neurotensin-(1—11), peak 5 contains both neurotensin-(1—12) and -(9—13) eluting together and, finally, peak 6 corresponds to synthetic neurotensin.

As part of a study on the characterization of α-MSH and CLIP-like peptides released by cultured pituitary cells, we examined the stability of the two peptides in tissue culture medium using the Z-module. The HPLC traces (Fig. 4) demonstrate the rates at which both α-MSH and CLIP are degraded in medium containing varying amounts of foetal calf serum (0—30%). α-MSH was shown to be degraded fairly rapidly without forming any visible stable products. The 18—39 fragment of ACTH, however, formed a stable product

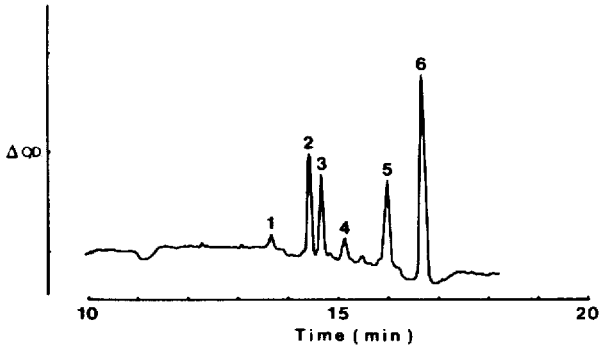

Fig. 3. HPLC profile of neurotensin degradation products formed on incubation with rat cortical synaptosomes. Elution conditions as in Fig. 1a. Peaks: 1 = neurotensin-(12—13), 2 = neurotensin-(1—8), 3 = neurotensin-(1—10), 4 = neurotensin-(1—11), 5 = both neurotensin-(1—12) and neurotensin-(19—13) eluting together, and 6 = synthetic neurotensin. The equivalent of 10 g of peptide was injected onto the Z-module.

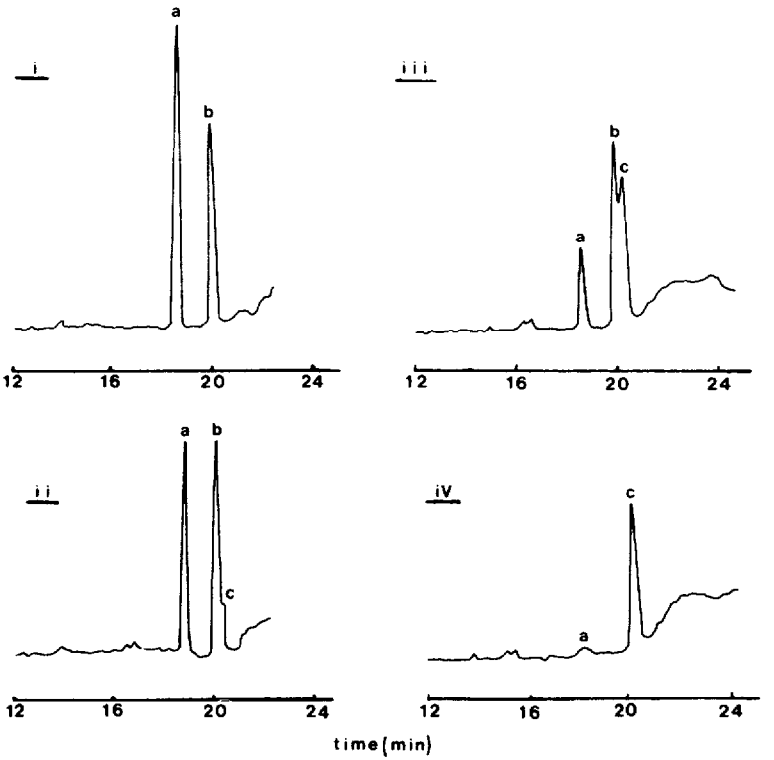

Fig. 4. HPLC profiles, using the Z-module, of α-MSH and CLIP incubated for 48 h at 37°C in tissue culture media containing 0% (i), 1% (ii), 5% (iii) and 30% (iv) foetal calf serum. Elution conditions and solvents are as in Fig. 3. Peaks: a = α-MSH, b = CLIP [ACTH-(18—39)], c = ACTH-(19—39). The equivalent of 20 μg of peptide was injected onto the column.

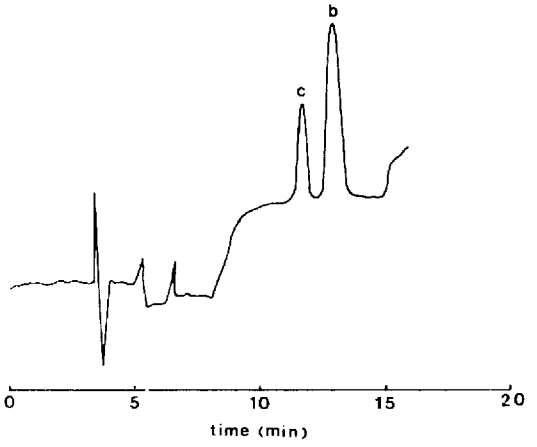

Fig. 5. The separation, using the Z-module, of peaks b and c (see Fig. 4) enhanced by substituting HFBA for TFA in the mobile phase. Linear gradient 45% to 60% solvent over 10 min at 1 ml/min; solvent A = 10 m*M* HFBA, solvent B = 70% acetonitrile in 10 m*M* HFBA; monitored at 220 nm, 0.4 a.u.f.s. The equivalent of 10 μg of peptide was injected onto the column.

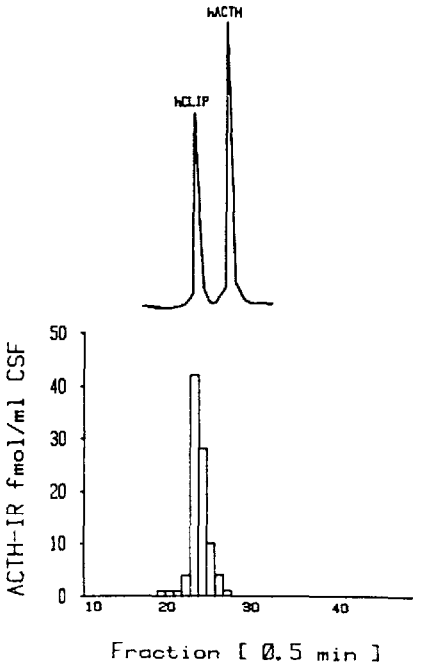

Fig. 6. HPLC trace of 5 μg of standard human ACTH and human CLIP over radioimmunoassay profile or human cerebrospinal fluid extract. Elution conditions are as in Fig. 5.

eluting very close to the parent peptide (peak c). In order to identify this product the separation was enhanced by substituting HFBA (10 m*M*) for TFA as the acid modifier in the mobile phase (Fig. 5), which will increase peptide retention depending on the number of basic amino acid residues present in the structure [6, 14]. The degradation product was then isolated and shown by amino acid analysis to be ACTH-(19—39), i.e. loss of the N-terminal arginine had occurred.

Human cerebrospinal fluid contains a significant amount of ACTH-IR [15]. To determine the molecular nature of this immunoreactivity we subjected a concentrated fraction of cerebrospinal fluid to HPLC again using HFBA in the mobile phase. The radioimmunoassay (using an antiserum directed against the C-terminus of ACTH) profile of the collected fractions (Fig. 6) shows that the ACTH-IR is not due to ACTH itself but is more likely to be CLIP, the 18—39 fragment of ACTH.

DISCUSSION

In slurry-packed steel columns the inside diameter of the column is limited in order to minimize the wall effect (i.e. when the dispersion of the mobile phase around the column casing is greater than that in the core). It has been claimed that in a column of 5 mm I.D. containing 20-μm particles, the peripheral area affected by the wall effect may be as great as 40% [16]. In a radially compressed flexible cartridge this effect may be much reduced as the column wall is able to mould around the column packing giving a more homo-

geneously packed bed thereby potentially increasing column efficiency. It is therefore possible to use a shorter column with a wider bore, the advantages being reduced back-pressures, improved resolution and greater sample capacity. The reduction in back-pressure will allow increased flow-rates resulting in shorter analysis times. As sample capacity is much increased (up to 20 mg) the system may be used in a preparative mode for the purification and isolation of neuropeptides.

In this report we have tried to assess the suitability of radial compression for the separation and isolation of neuropeptides and for the first time compared several C_{18} packing materials in different compression systems. Previous reports have successfully demonstrated the separation of proteins and peptides derived from the partial hydrolysis of human lipoproteins using a non-capped C_{18} 10-μm spherical packing radially compressed at 170 bar, the mobile phase being an acetonitrile or isopropanol gradient against a triethylammonium phosphate buffered (pH 3.2) aqueous phase [10, 17]. The neuropeptides angiotensin, and α-, β- and γ-endorphin have also been separated using the 10-μm spherical packing (Radial-Pak A) with triethylamine formate and ammonium bicarbonate as counter-ions [18]. The Z-module having a lower compression rating (13 bar) fitted with the 10-μm irregular C_{18} packing which has undergone a secondary derivatization or end-capping process (to ensure maximal coverage of the silica core) may be used successfully to separate a variety of peptides without the addition of any salts to the mobile phase (water—TFA—acetonitrile; Fig. 1c). This mobile phase is particularly useful by being ultraviolet-transparent below 220 nm to allow the sensitive detection of peptides containing non-aromatic residues, and by being totally volatile, collected fractions may be dried for further analysis without the problems associated with interference from residual salts [1]. Other solvents systems containing triethylamine formate or ammonium bicarbonate with acetonitrile are also volatile and ultraviolet-transparent but are much less convenient to use.

Hearn et al. [18] have demonstrated differences in selectivity for several peptides between radially compressed packings (Radial-Pak A) and conventional steel columns. However, using the Z-module fitted with the capped 10-μm irregular C_{18} packing we found that the selectivities for all the peptides we tried were similar to those obtained with the μBondapak steel column. It is also our experience that these columns have a much longer life than steel columns, especially when used for the analysis of large numbers of relatively crude biological extracts.

Three examples of the practical applications of radial compression using the Z-module are described briefly and full details will appear elsewhere. The putative neurotransmitter neurotensin has been shown to be degraded by specific peptidases present in soluble and particulate fractions prepared from rat brain [19]. Using HPLC we have identified a potential inactivation pathway for neurotensin at the nerve terminal using metabolically active rat cortical synaptosomes incubated with synthetic neurotensin (Fig. 4), the collected peaks being positively identified by amino acid analysis following acid hydrolysis.

One of the problems associated with the monitoring of peptide release from cultured cells is the peptidase activity present in tissue culture media containing foetal calf serum (normally 20—30%). In order to optimize the conditions

necessary for maximal cell growth and peptide stability we have used HPLC to look at the rate at which α-MSH and CLIP are degraded in tissue culture media containing varying amounts of foetal calf serum (Fig. 5). By substituting HFBA for TFA in the mobile phase we were able to isolate and identify a major degradation product formed from CLIP (Fig. 6). We have recently demonstrated that cells isolated from rat intermediate lobe may be successfully cultured in media containing 1% foetal calf serum where peptide breakdown is minimal (Hughes and Smith, unpublished results).

By subjecting a concentrated sample of cerebrospinal fluid to HPLC, we have been able to show that the immunoreactivity in collected fractions does not correspond to ACTH but is more likely to be CLIP, the 18—39 fragment of ACTH.

In conclusion, the 10-μm, irregular, capped C_{18} packing radially compressed in the Z-module compares favourably with steel columns slurry packed with similar material. The major advantages of this system are speedier analysis and increased sample capacity, longer life and therefore greater economy.

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REFERENCES

1 J.R. McDermott, A.I. Smith, J.A. Biggins, M. Al-Noaemi and J.A. Edwardson, J. Chromatogr., 222 (1981) 371.
2 J. Rivier, J. Liquid Chromatogr., 1 (1978) 343.
3 E.C. Nice and M.J. O'Hare, J. Chromatogr., 162 (1979) 401.
4 W.S. Hancock, C.A. Bishop, R.C. Prestridge, D.R.K. Harding and M.T.W. Hearn, Science, 200 (1978) 1168.
5 H.R. Morris, A.T. Etienne, A. Dell and R. Albuquerque, J. Neurochem., 34 (1980) 574.
6 H.J.P. Bennett, C.A. Browne and S. Solomon, J. Liquid Chromatogr., 3 (1980) 1353.
7 M. Schöneshöfer and A. Fenner, J. Chromatogr., 224 (1981) 472.
8 J.L. Meek, Proc. Nat. Acad. Sci. U.S., 77 (1980) 1632.
9 I. Molnár and Cs. Horváth, J. Chromatogr., 143 (1977) 391.
10 W.S. Hancock, J.D. Capra, W.A. Bradley and J.T. Sparrow, J. Chromatogr., 206 (1981) 59.
11 J.S. Landy, J.L. Ward and J.G. Dorsey, J. Chromatogr. Sci., 21 (1983) 49.
12 A.I. Smith, J.R. McDermott, J.A. Biggins and R.J. Boakes, J. Chromatogr., 236 (1982) 489.
13 P.R. Dodd, J.A. Hardy, A.E. Oakley, J.A. Edwardson, E.K. Perry and J.P. Delaunoy, Brain Res., 226 (1981) 107.
14 A.I. Smith, A.B. Keith, J.R. McDermott, J.A. Biggins and J.A. Edwardson, Neurosci. Lett., 30 (1982) 133.
15 J.P. Allen, J.W. Kendall, R. McGilvra and C. Vancura, J. Clin. Endocrinol. Metab., 38 (1974) 586.
16 J.H. Knox (Editor), High Performance Liquid Chromatography, Edinburgh University Press, Edinburgh, 1978, p. 82.
17 W.S. Hancock and J.T. Sparrow, J. Chromatogr., 206 (1981) 71.
18 M.T.W. Hearn, B. Grego and C.A. Bishop, J. Liquid Chromatogr., 4 (1981) 1725.
19 J.R. McDermott, A.I. Smith, J.A. Edwardson and E.C. Griffiths, Regul. Peptides, 3 (1982) 397.