HIGH PRESSURE LIQUID CHROMATOGRAPHY IN THE ANALYSIS OF UNDERIVATISED PEPTIDES USING A SENSITIVE AND RAPID PROCEDURE*

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1. Introduction

High pressure liquid chromatography (HPLC) has been used in the analysis of PTH amino acids [1,2] PTC-peptides [3] some growth promoting peptides [4] and peptide antibiotics [5–7]. A major improvement would be achieved if no derivatisation of the peptides was necessary and a rapid, sensitive, low cost method could be employed. To achieve this HPLC was tested on an analytical scale with the possibility that methods developed could be scaled up to a preparative system. Because of the relative complexity of even small peptide molecules in terms of partition coefficient, polarity, hydrophobicity, and bulkiness [8] a series of column supports were investigated with a view to finding a system which both retained samples and allowed their ready elution. The reverse phase mode (liquid–liquid chromatography) was found to best satisfy these criteria. The most common and economical polar solvents used for reversed phase liquid–liquid chromatography, water, methanol and acetonitrile have the additional advantage of allowing spectrophotometry in the range 205–225 mm. In this range the peptide bond has sufficient absorption to allow quantitative detection in the nmol range.

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It is the purpose of this report to show that a variety of peptides can be readily analysed by this procedure.

2. Materials

2.1. *Apparatus*

A Waters high-pressure liquid chromatograph system was used which included two M-6000 solvent delivery units, an M-660 solvent programmer, a U6K universal liquid chromatograph injector and a column heater block, coupled to a Cecil 212 variable wavelenght ultraviolet monitor.

Stainless steel columns (61 cm X 2 mm) were purchased in a prepacked form from Waters Associates.

Bondapak C_{18}-Corasil and Bondapak Phenyl-Corasil (Waters Associates) with particle size rang 37–50 μm were used as column packings. These columns have a monomolecular layer of octadecyl silane and diphenyl silane, respectively, chemically bonded to the pellicular silica packing. Sample injections were made with a Pressure-Lok liquid syringe, series B-110, from Precision Sampling Corporation.

2.2. *Chemicals*

Organic solvents were Analar grade with methanol supplied by Mallinckrodt Chemical Works. Water was glass distilled and deionised. Samples 1–3 (table 1)

Table 1
The retention times of peptides on HPLC

Sample	Retention time (min)							
	Solvent system H_2O			50% CH_3OH : 50% H_2O			70% CH_3OH : 30% H_2O	
C_{18}-Corasil	I[a]	II[b]		I	II		I	II
1	0.8	1.4						
2	0.8	1.6	2.4(s)[c]					
3	0.8	1.8						
4				0.8	5.4		0.9	
5				0.85	9.5		1.0	
6				0.8	13.4		0.9	5.6
7				0.85	8.4[d]		0.9	5.6
8				0.9	7.2		0.95	5.0
Phenyl-Corasil								
2				1.0	6.3			
3				0.8	2.1			
4				0.8	1.25(s)	3.2	0.8	1.1(s) 4.2
5				0.9	7.8		0.9	
6				0.8	7.8		0.9	4.0
7				0.8	13.0		0.9	5.8
8				0.9	28		0.9	8.1
6 and 8								3.6 7.8[e]

[a] I, Peak at void volume attributed to impurities
[b] II, Peptide peak
[c] See fig.2
[d] See fig.1
[e] See fig.3
(s) Refers to a minor impurity peak.

Samples: 1, Pyroglutamic acid–His–Pro; 2, Val–Tyr–Ile–His–Pro–Phe; 3, Tyr–ile–His–Pro–phe; 4, Gly–Gly–Ala–NH_2; 5, Gly–Leu–Tyr; 6, Met–Arg–Phe–Ala; 7, Leu–Trp–Met–Arg; 8, Leu–Trp–Met–Arg–Phe.

were produced in this laboratory by the solid-phase method [9].

Gly–Gly–Ala–amide was purchased from Cyclo Chemical, Gly–Leu–Tyr from Sigma, Met–Arg–Phe–Ala, Leu–Trp–Met–Arg and Leu–Trp–Met–Arg–Phe from Research Plus Laboratories Inc. All amino acids were of the L-configuration except for glycine.

3. Methods

A flow rate of 1.5 ml/min was used for each column, which was maintained by a pressure of 1100 ψ for the C_{18}-Corasil column, and 2000 ψ for the phenyl-Corasil column. All tests were carried out at room temperature (approx. 22°C). Sample sizes varied between 0.25–10 ng of peptide material with volumes of 1–25 μl. Detection was in the range 212–218 nm. All peptides were taken up in deionised, distilled water and, prior to injection, made up in the eluting solvent. All solvents were degassed separately with stirring by means of a water aspirator with the following times being used; water for at least 30 min, methanol for 1.5 min. The solvents were mixed as required, degassed for 1.5 min, and then equilibrated to room temperature. This precise procedure was important for reproducibility. All solvents were stirred magnetically throughout equilibration and elution. All columns were equilibrated to new solvents for at least 30 min.

4. Results and discussion

The results of the analysis of eight peptides on the C_{18}- and phenyl-Corasil columns are shown in table 1. As the peptides vary in size from a tripeptide to a hexapeptide the results are a good test of the ability of HPLC to separate closely related compounds. In all cases a significant absorbance peak was observed at the void volume of the column (0.8–1.1 min) and was attributed to impurities in the sample. A typical elution profile is shown in fig.1 and corresponds to the peptide Leu–Trp–Met–Arg on the C_{18}-Corasil column. In fig.2 the analysis of a synthetic angiotensin fragment Val–Tyr–Ile–His–Pro–Phe, also on a C_{18}-Corasil column, is shown, with peak II corresponding to a deletion product Val–Ile–His–Pro–Phe. Figure 3 shows the separation of a mixture, Met–Arg–Phe–Ala and Leu–Trp–Met–Arg–Phe on phenyl-Corasil. This result suggests that it is possible, with the use of this reversed phase system, to succesfully separate small peptides varying in sequence by as little as one amino acid.

Retention times of both pure peptides and peptide mixtures can be altered markedly by changes in the nature of the eluant, whereby increasing solvent polarity increases retention time (see table1). It appears that 50% methanol/50% water is a suitable starting solvent and with small alterations in the methanol content marked flexibility in resolution can be gained. More polar samples, which are retained longer, can be eluted within time limits of 10 min by use of a 50–80% methanol/water gradient. Significant differences were noted between the highly non-polar C_{18}-column and its less non-polar counterpart the phenyl-column e.g. Met–Arg–Phe–Ala has retention times of 13.4 and 7.8 min respectively. We would recommend, therefore, that peptide mixtures be routinely examined on both column systems.

A feature of this HPLC procedure is the facile separation of underivatised peptides by reversed phase chromatography using cheap, readily available solvent mixtures. Several publications [1–3] have demonstrated that derivatised amino acids or peptides can be separated on HPLC, but these procedures are not applicable to all peptides and involve lengthy sample preparation and quantitation studies. Small peptides have been detected in HPLC at 254 nm [4] but can only be applied to peptides which contain tyrosine, tryptophan or other chromophores. If reversed phase liquid chromatography is used, however, solvents

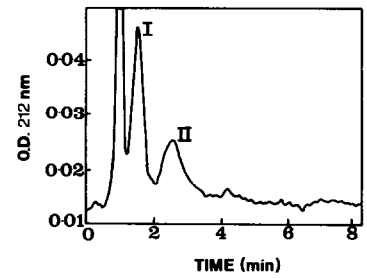

Fig.2. The elution profile of the synthetic peptide Val–Tyr–Ile–His–Pro–Phe (I) on a C_{18}-Corasil column with water as the eluant. The break-through peak is not labelled, while peak II corresponds to the deletion peptide Val–Ile–His–Pro–Phe.

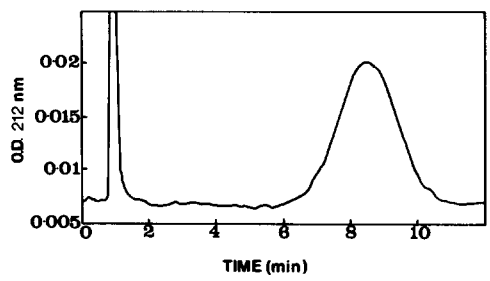

Fig.1. The elution profile of Leu–Trp–Met–Arg on a C_{18}-Corasil column with 50:50 methanol/water. The first peak corresponds to impurities which emerge in the break-through volume, and the second peak to the peptide.

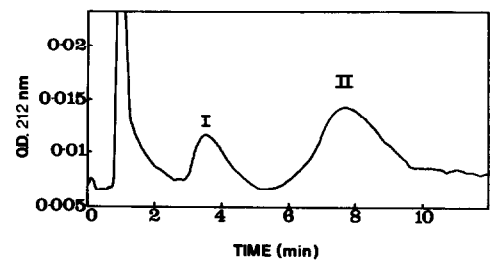

Fig.3. The elution profile of a mixture of peptides on phenyl–Corasil with 70:30 methanol/water as the eluant. The break-through peak is not labelled, peak I corresponds to Met–Arg–Phe–Ala and peak II to Leu–Trp–Met–Arg–Phe.

such as methanol and water can be used which are transparent at 212 nm. At this wavelength all peptides examined show significant absorption thus allowing ultraviolet detection to be used as a sensitive universal detector for these compounds. At a convenient sensitivity (0.02 units for full scale deflection) 2 nmoi of a typical peptide can be detected. This sensitivity compares favourably with that obtained for HPLC separation of PTH amino acids [1]. Increased sensitivity can be achieved by operating at 187 nm (λ max for the amide bond) [10], but the slight increase in sensitivity (5-fold) would not be adequate, in most cases, to warrant the expensive equipment required.

A universal sensitive method for analysing the purity of peptides should make significant contributions to peptide chemistry in general for example in the analysis of products from solid phase peptide synthesis, peptide mixtures from protein enzymatic digestions, and various peptide hormone derivatives. In addition the method uses relatively low pressures (1000–2000 ψ) and readily available, inexpensive solvents which allows considerable savings in operation costs.

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References

[1] De Vries, J. X., Frank, R. and Birr, C. (1975) FEBS Lett. 55, 65.
[2] Bollet, C. and Caude, M. (1976) J. Chromatogr. 121, 323.
[3] Waters Associates Bulletin.
[4] Pickart, L. R. and Thaler, M. M. (1975) Prep. Biochem. 5, 397.
[5] Rzeszotarski, W. J. and Mauger, A. B. (1973) J. Chromotogr. 86, 246.
[6] Tsuji, K., Robertson, J. H. and Back, J. A. (1974) J. Chromotogr. 99, 597.
[7] Tsuji, K., and Robertson, J. H. (1975) J. Chromotogr. 112, 663.
[8] Jones, D. D. (1975) J. Theor. Biol. 50, 167.
[9] Merrifield, R. B. (1963) J. Am. Chem. Soc. 85, 2149; Hancock, W. S., Prescott, D. J., Marshall, G. R. and Vagelos, P. R. (1972) J. Biol. Chem. 247, 6224.
[10] Woods, A. H. and O'Bar, P. R. (1970) Science 167, 179.