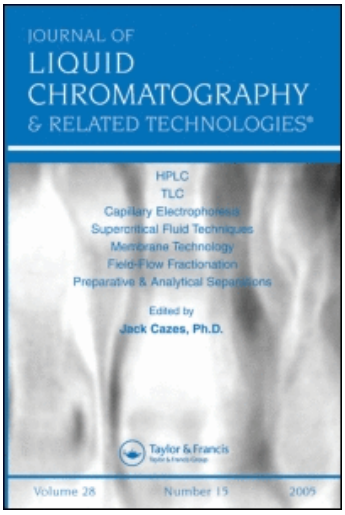


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# APPLICATION OF REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY IN SOLID PHASE PEPTIDE SYNTHESIS.

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HIGH PRESSURE LIQUID CHROMATOGRAPHY OF AMINO ACIDS PEPTIDES AND PROTEINS XIII. PART XII REF. 2.

## ABSTRACT

The application of ion-pair partition reversed phase high performance liquid chromatography to the analysis and purification of peptides produced by solid-phase synthesis is described. This approach permits very rapid recognition of deletion or partial deprotection products which are often generated during the synthesis and, depending on their molecular characteristics, allows these components to be resolved. The use of hydrophobicity parameters has been examined and found useful in the prediction of the relative elution order of these closely related peptides. The separation of a series of synthetic preparations, including the angiotensins, leu-enkephalin amide, a fragment of β-TSH, and linear antamanide, illustrates the speed of the method which can be used to assess the homogeneity of the reaction mixtures and also provide, preparatively, highly purified peptides.

## INTRODUCTION

During the last five years a rapidly expanding number of peptides have been discovered which elicit profound biological responses. Many of these compounds are highly potent, inducing diverse biochemical events at membrane or extra-cellular sites, at the nano- or pico-molar level, but are available naturally in only minute quantities. Peptide synthesis by conventional procedures in solution or by solid-phase technique has the potential to provide large quantities of these molecules as well as structural variants which would allow their biological characteristics, including structure-activity relationships, to be established. The solid-phase approach, initially pioneered by Merrifield, has several advantages for synthesis of peptides since it is rapid and lends itself to automation[1]. In this method, the growing peptide chain is attached throughout the synthesis to an insoluble polymeric support with the side-chain functionality of the reagent amino-acids and growing peptide appropriately protected to prevent uncontrolled amide bond formation. The final stages of the synthesis involve cleavage of the completed peptide and removal, often achieved concommitantly, of the protecting groups. Purification of the desired peptide from contaminants including peptidic deletion products and artefacts generated in the cleavage from the resin can then be undertaken. Open column chromatographic procedures based on gel permeation ion-exchange, adsorption or partition methods and counter current distribution have been extensively used in the purification of both protected and unprotected synthetic peptides. All these techniques are relatively slow and, depending on the nature of the components, can have low separation efficiencies. Furthermore, the isolated fractions need to be monitored by analytical methods to ascertain purity. Homogeneity of peptide preparation has conventionally been assessed by thin layer or paper chromatography or by electrophoretic methods. In a series of recent publications[2-5] we have reported methods which

permit the rapid analysis and purification of peptides by high performance liquid chromatography. We wish now to report our experiences using these methods which permit very rapid analysis of the crude products obtained from solid phase peptide synthesis. The application of these methods to preparative-scale purification of a variety of synthetic peptides is feasible with only minor changes in the composition of the mobile phase. The synthetic examples reported in this paper cover a range of amino acid compositions. A typical analytical chromatogram of a crude synthetic product can be completed within ca. 30 minutes and requires less than 5µg of material.

## EXPERIMENTAL

### Reagents.

All solvents were AnalaR grade. Methanol and acetonitrile, supplied by Fisher Scientific Co. were purified as reported earlier[5]. Orthophosphoric acid was from May and Baker Ltd. Water was de-ionised by reverse osmosis and distilled. The peptides were produced in this laboratory by the solid phase method using standard procedures. All amino acids, except glycine, were of the L- configuration.

### Apparatus.

A Waters high performance liquid chromatography system was used which included two M6000A solvent delivery units, an M660 solvent programmer and a U6K universal liquid chromatograph injector, coupled to a M450 variable wavelength UV monitor and a Rikadenki dual channel chart recorder. The µ-Bondapak $C_{18}$ and µ-alkyl Phenyl columns (10µm, 30cm x 4mm I.D.) were purchased from Waters Associates, Milford. The preparative Bondapak Phenyl-Porasil B (37-50µm, Waters Assoc.) columns (60cm x 7mm I.D.) were packed by the tap-fill method[6]. Sample

injections were made with a Pressure Lok liquid syringe, Series B110 from Precision Sampling (Baton Rouge, La., U.S.A.). Filtration of solvents was carried out using a pyrex filter holder (Millipore Corp., Bedford, Mass., U.S.A.) while peptide samples were filtered using a Swinney Filter (AP2500 Filters, Millipore Corp.).

## METHODS

All chromatograms were carried out at room temperature (ca. $20^0$). All peptides were made up in the eluting solvent. Bulk solvents were degassed separately and the appropriate mobile-phases prepared and equilibrated as reported previously[4,5]. All columns were equilibrated to new solvents for at least 30min. and after gradient elutions, the gradients were reversed and the columns then re-equilibrated for 15-30min, with the initial mobile phase. Flow rates were maintained between 1.0ml/min and 4.0ml/min as indicated in the text. Detection of the peptides was in the range 205-220nm depending on the nature of the sample and the mobile phase.



## RESULTS AND DISCUSSION

In 1974, Ward and Pelter[7] drew attention to the potential use of HPLC for the separation of protected peptides by adsorption phase techniques. Subsequently, other groups[8,9] have elaborated this approach using silicagel packing materials. The advent of chemically bonded octyl-, octadecyl-silane and related reversed phase packings has considerably expanded the scope of HPLC methods for the analysis and purification of both protected and unprotected peptides.

In our previous studies[2-5], it was noted that the polarity of purified unprotected peptides and proteins was significantly modified by the addition of phosphoric acid and phosphates to the mobile phase in reversed phase HPLC sep-

arations. Subsequently, conditions which permit modulation of the polarity of a wide range of unprotected peptides and proteins by hydrophilic, hydrophobic or composite ion-pairing and dynamic ion exchange with free amino- or carboxyl groups have been found[2]. These chromatographic conditions provide valuable alternatives to conventional methods for the analysis and separation of peptides and proteins with widely differing amino acid content and sequence. Mobile phases containing phosphoric acid or phosphates are particularly well suited for the separation and analysis of peptides on hydrophobic stationary phases[2-5,10]. These reagents permit the use of buffers that are UV transparent to less than 200nm and containing significantly lower concentrations of organic solvents in the mobile phase. In addition, marked alterations in retention times of underivatised peptides have been noted which are consistent with the formation *in situ* of hydrophilic ion-pair complexes. Finally, these conditions are compatible with *in vitro* and *in vivo* biological assay methods after removal of the organic solvent. Highly polar peptides may be insufficiently retained on reversed phase columns using these buffers to allow resolution. For these peptides, containing a large number of polar amino acids with ionogenic side chains, conditions involving hydrophobic ion-pairing reagents have been developed[4] which permit adequate resolution. However, the majority of peptides so far examined show reasonable retention on non-polar stationary phases. The examples discussed in this paper are certainly representative of this latter group.

Incomplete coupling of the reagent amino acid to the nascent peptide represents the commonest fault in solid-phase synthesis. This results in deletion products which may ultimately resemble the desired peptide very closely having only minor variations in the sequence. Commonly used methods[11] to monitor the synthetic products, for example BAWP paper or the silica gel chromatography, often lack the resolving power necessary to distinguish between these various components. The advantages of high speed,

efficiency and resolution noted with previous HPLC separations in the peptide field could potentially overcome these difficulties in resolution and provide both an analytical and preparative adjunct to assist in the purification. With the examples described below, suitable conditions were readily developed which allowed the direct monitoring of crude synthetic products and which had sufficient resolving power to provide analytically pure materials. Furthermore, the rapidity of these separations makes this a convenient method to follow the larger scale preparative purification by reversed phase or other types of chromatography.

A preparation of the tetrapeptide CAGY* a fragment common to the β-chain of the pituitary glycoprotein hormones,[13] cholera toxin B protein and trypsin, was obtained by solid phase synthesis, followed by cleavage and concommitant deprotection from the insoluble resin. The product was shown by thin-layer chromatography on Eastman chromatogram sheets (13181 silica gel with fluorescent indicator) in 2 solvent systems: BAW (n-butanol-acetic acid-water 4:1:5 v/v upper phase) and BPA (n-butanol-pryidine-0.1% aqueous acetic acid 5:3:11 v/v upper phase) to contain a major poorly resolved band consisting of at least two components and two minor contaminants. The homogeneity of this preparation was then assessed by gradient elution on a HPLC system. As can be seen from Figure 1, these conditions give excellent resolution and allow quantitation of structurally closely related peptides. Figure 2 shows the HPLC profile of the purified peptide CAGY after repeated gel permeation chromatography on G15 superfine and Bio-gel $P_2$. Several recent studies have shown[2,14] the elution order



* Footnote.
The single letter code for amino acid used in this paper is as suggested by Dayhoff[12], A=alanine, N=asparagine, R=arginine, F=phenyl alanine, P=proline, G=glycine, C=cysteine, V=valine, Y=tyrosine, H=histidine, L-leucine, I=isoleucine.

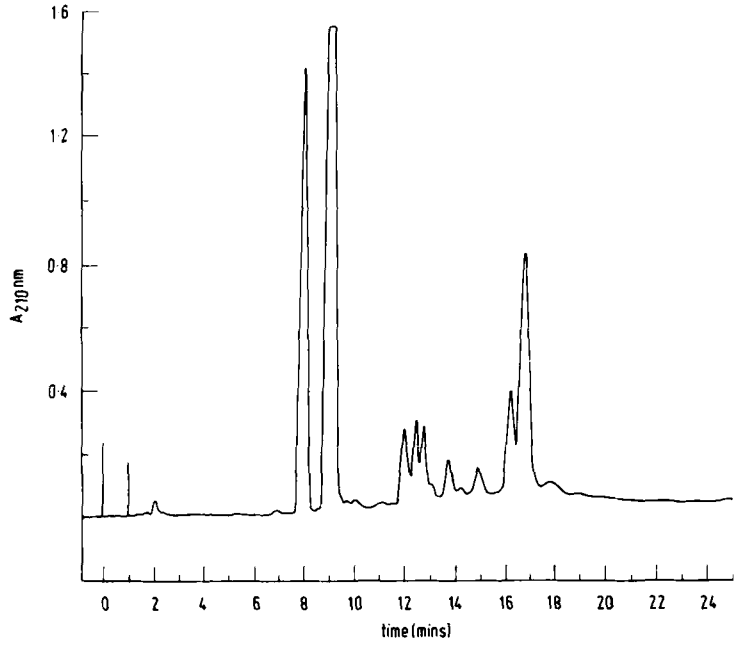



FIGURE 1.

Chromatogram obtained with CAGY peptide mixture prepared by solid-phase synthesis. Column μ-Bondapak $C_{18}$, flow rate 2.0ml/min. A 10 minute linear gradient of water-0.1% phosphoric acid to 40% methanol-water-0.1% phosphoric acid was used. The gradient was started one minute after injection of the sample.



of a series of related small peptides in acidic buffers, and particularly in phosphate mediated buffers, follows closely the order of the sum of the relative hydrophobicities of the side chains of the component amino acids. Illustrative of this phenomenon, is the elution behaviour on a reversed phase medium of a series of dipeptides related to the C-terminus of CAGY. As can be seen from the chromatogram shown in Figure 3, a good correlation exists between the retention times of these dipeptides and the average side chain hydrophobicity functions as proposed by Rekker[15]. Recently, Molnar and Horvath discussed[14] the use of this approach in general terms. For mixtures of closely

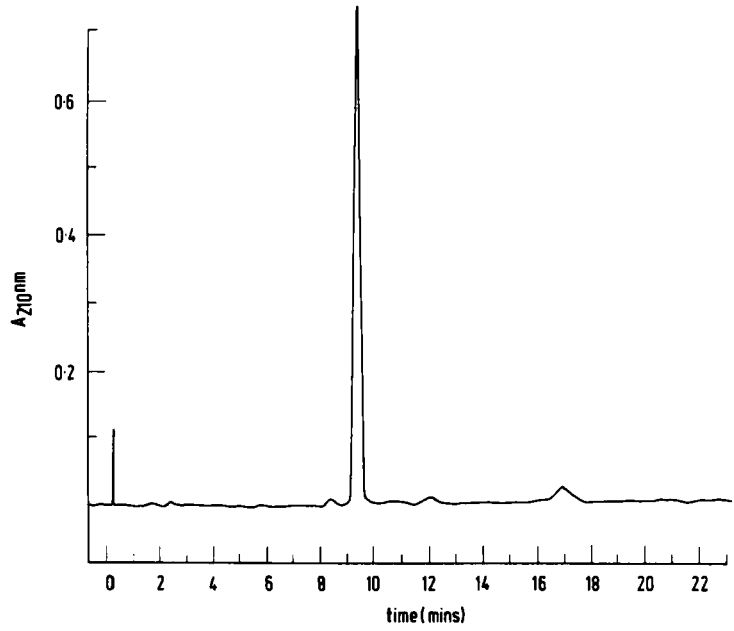



FIGURE 2.

Elution profile of purified CAGY on a μ-Bondapak $C_{18}$ column. Chromatographic conditions were the same as in Figure 1.



related peptides obtained from synthesis, the ability to predict from the retention times the likely sequence variants (desired product, deletions, transaminations etc.) would be particularly useful.

With hydrophobic peptides like linear antamanide[16], V-P-P-A-F-F-P-P-F-F, the value of hydrophilic ion pair partition HPLC methods for monitoring solid phase synthetic products is particularly pronounced. This peptidic preparation proved refractory to separation by most open column chromatographic techniques although partial purification could be achieved by chromatography on Sephadex LH-20 with methanol as an eluant. In contrast to the long times required to carry out this open column chromatography, the HPLC chromatogram of the crude

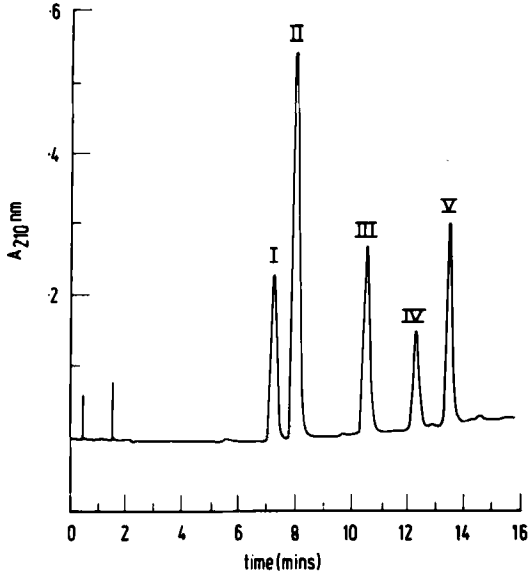



FIGURE 3.

Separation of C-terminal tyrosinyl peptides, related to the tetrapeptide CAGY, on μ-Bondapak $C_{18}$ column. Chromatographic conditions were the same as in Figure 1. The following dipeptides were used (I) A-Y, (II) P-Y, (III) L-Y, (IV) V-Y, (V) Y-Y-Y.



cleavage product can be obtained in the order of minutes (Figure 4). The major peak with apparent k´ 9.3 corresponds to the desired linear antamanide. These results also indicate the benefits of gradient elution compared to isocratic elution for the resolution of very similar hydrophobic peptides (Figure 5a,b).

Synthetic peptides related to angiotensin II have attracted[17] considerable attention in view of their pronounced peripheral and central neurogenic vasconstrictor responses *in vivo* and *in vitro*. As part of our studies in this area, a range of peptides of the angiotensin type have been synthesised. It was of interest to compare our HPLC methods with earlier techniques for analysis and purification of these molecules. Using phosphate mediated

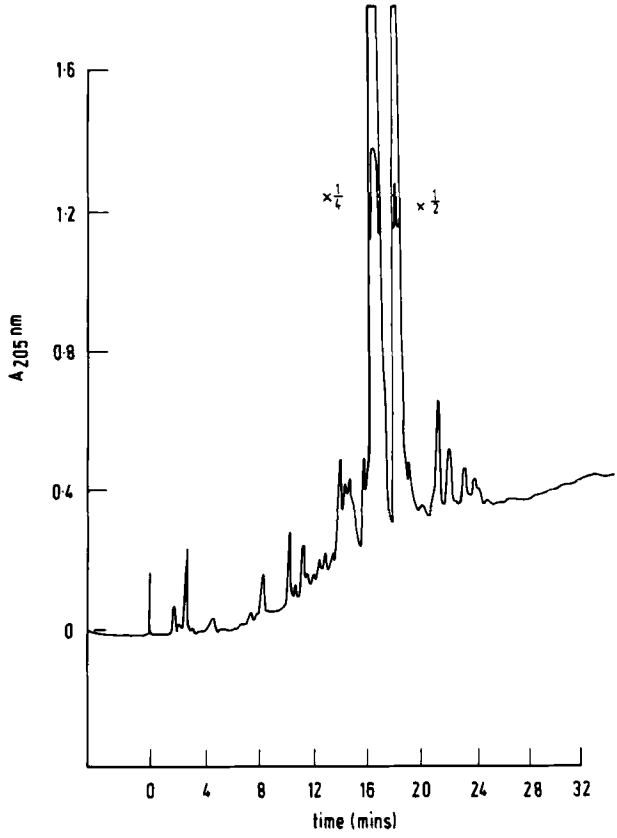


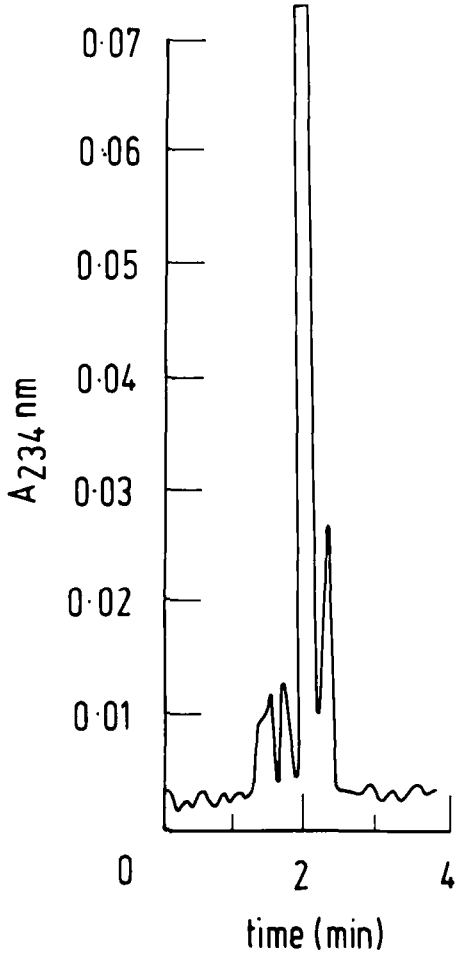

FIGURE 4.

Chromatogram obtained with a linear antamanide preparation Column: μ-Bondapak $C_{18}$, flow rate 2.0ml/min. A 30 minute linear gradient of 10% acetonitrile-water-0.1% phosphoric acid to 75% acetonitrile-water-0.1% phosphoric acid was used. The gradient was started at the time of injection of the sample.

ion-pairing HPLC on μ-Bondapak $C_{18}$ columns, the separation of the $(Ileu^5)$- angiotensins I, II and III whose amino acid sequences are given in the Table can be readily achieved with aqueous mobile phases containing either methanol or acetonitrile under isocratic or gradient elution conditions as shown in Figures 6 and 7. It is

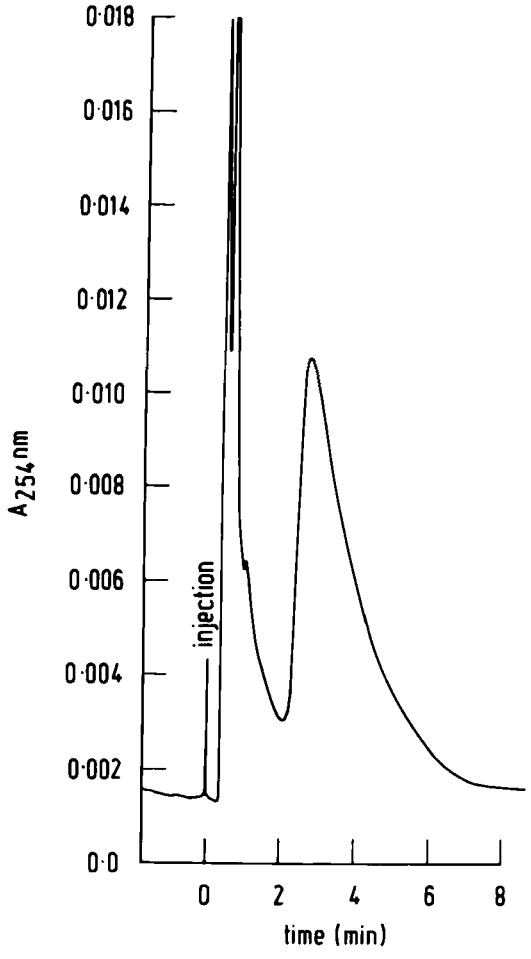
interesting to note that the relative retention values of these three peptidic hormones are again in agreement with relative hydrophobicity functions. Earlier studies[14] with (Val$^5$)-angiotensin I and II have observed a related effect under similar elution conditions with Lichrosorb RP-8 columns. Since the nature of the hydrocarbonaceous stationary phase can obviously have an important bearing on the elution sequence of peptides, it is a parameter worthy of further study. Figure 8 shows the chromatogram of the C-terminal hexapeptide, V-Y-I-H-P-F, which is contaminated with a significant amount of the valinyl and tryosinyl deletion products.

Partial deprotection during cleavage or subsequent work-up has been well documented in solid phase synthesis. Using a HPLC approach the extent of deprotection can often be readily assessed. Illustrative of this point are the results obtained with the endogenous opiate agonist Leu-enkephalin amide[18], a pentapeptide which has the amino acid sequence Y-G-G-F-L-$NH_2$. A preparation of the O-benzylether of Leu-enkephalin amide was purified by preparative reversed phase HPLC on a Phenyl Porasil B column using an acetonitrile-water-orthophosphoric acid (30:70:0.1%) eluant. In addition the crude product was examined by analytical HPLC on a μ-alkylphenyl column. Isocratic elution with acetonitrile-water-orthophosphoric acid (40:60:0.1%) of the crude product indicated the presence of five major components. Peaks $\underline{\text{IV}}$ and $\underline{\text{V}}$ (Figure 9) were readily shown by standard biochemical methods to correspond to the unprotected peptide and the O-benzyl protected peptide respectively. Furthermore, the interconversion of the protected peptide to leu-enkephalin amide on hydrogenation could be followed by analytical HPLC. Figure 10 illustrates the advantages which preparative reversed-phase HPLC can offer in the purification of peptides like leu-enkephalin amide. As can be seen, the separation was rapid, being completed in less than fourteen minutes, with good resolution.




(a)

The examples described in this paper demonstrate the value of reversed phase HPLC both as an analytical and semipreparative technique (loadings ca. 10mg) which should considerably simplify the analysis and purification of peptides prepared by solid-phase methods. Large scale preparative HPLC methods (with loadings > 100mg) using the peptides described in the present paper have been investigated and will be described[19] in detail elsewhere. These HPLC approaches are also applicable to solution phase synthesis.




(b)

FIGURE 5.

Isocratic elutions of crude linear antamanide on (a) a μ-Bondapak-$C_{18}$ reversed phase column using as a mobile phase 80% methanol-water-0.1% phosphoric acid at a flow rate of 4ml/min. and (b) a Bondapak-$C_{18}$ Corasil column using as a mobile phase 55% methanol-100mM potassium dihydrogen sulphate-0.1% phosphate acid at a flow rate of 2ml/min.

TABLE

Amino Acid* Sequence for Angiotensins

1. N-R-V-Y-I-H-P-F-H-L
2. N-R-V-Y-I-H-P-F
3. N-R-V-Y-I-H-P
4. V-Y-I-H-P-F
5. V-I-H-P-F
6. V-I-H-P-F

---

The code for amino acids is as used by Dayhoff[12].



In this case, the stepwise purification of protected intermediates which is routinely carried out during various stages of the synthesis, would result in a final product containing less contaminants and consequently not require the same level of resolution. Finally, it is noteworthy that recoveries obtained with the chromatographic elution systems described in this paper were consistently high. This has been noted previously[5,10] with phosphate buffers used for the elution of small peptides from reversed phase columns.

Because of their high selectivity and efficiencies, the fully porous reversed-phase packings are preferred for both analytical or preparative separations (cf Figure 5(a) and (b)). The pellicular packings, like $C_{18}$-Corasil or Phenyl-Corasil, generally show lower efficiencies for the compounds discussed in this paper than the fully porous packings due to the peaks showing pronounced tailing. Similar results have been noted with Phenyl-Corasil previously with purified peptides[5,20,21]. Peak tailing of peptides has been assumed to reflect residual silanol groups in the $C_{18}$- or Phenyl-Corasils.

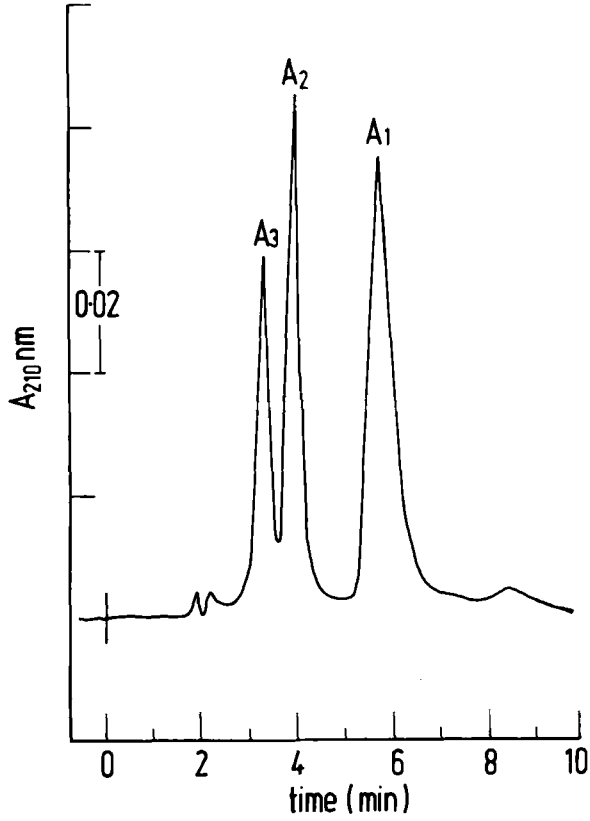



FIGURE 6

Separation of (Ile$^5$)-angiotensins I($A_1$), II($A_2$) and III($A_3$) on a μ-Bondapak-$C_{18}$ column under isocratic conditions. The mobile phase consisted of 35% methanol-water-0.1% phosphoric acid with a flow rate of 2ml/min. The loading was 5μg of each component.

However, similar elution profiles are found after extensive silylation of the packing material with hexamethyldisilazane in dry toluene. Obviously effects other than the presence of residual silanol groups must operate during the elution of peptides from reversed phase pellicular packings with phosphate buffers at ca. pH3.

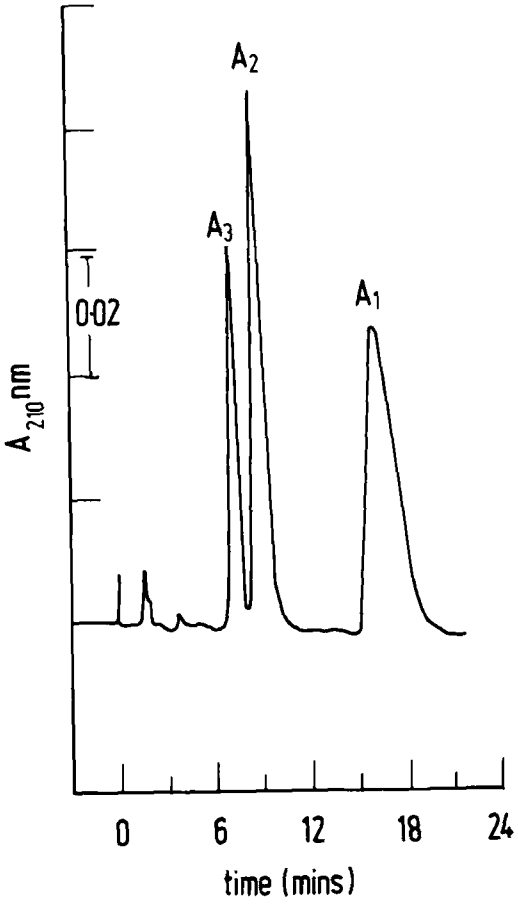



FIGURE 7.

Isocratic elution of (Ile$^5$)-angiotensins I($A_1$), II($A_2$) and III($A_3$) using a mobile phase of 15% acetonitrile-water-0.1% phosphoric acid at a flow rate of 2ml/min. Other conditions were the same as in Figure 6.

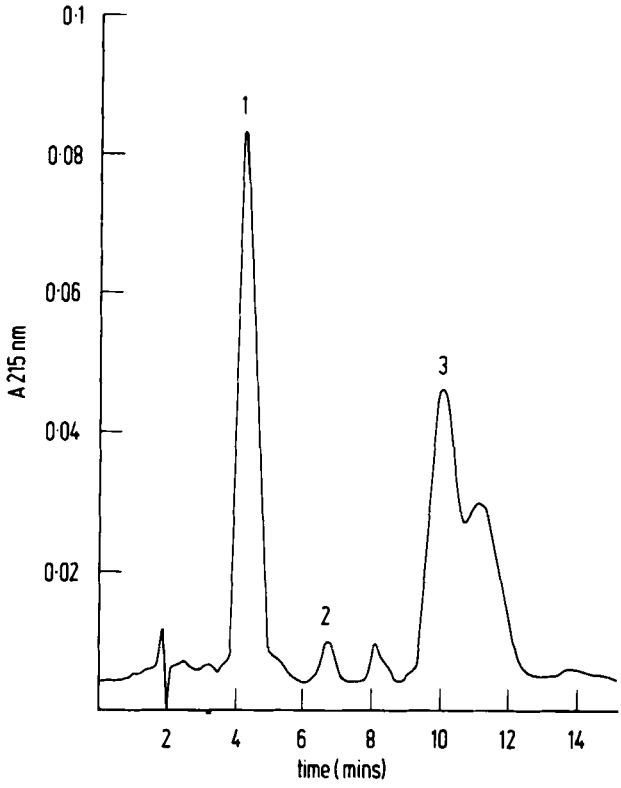



FIGURE 8.

Elution profile of synthetic preparation of the C-terminal (Ile$^4$)-angiotensin II hexapeptide, V-Y-I-H-P-F, on a μ-Bondapak-Fatty acid analysis column. The mobile phase consisted of 25% acetonitrile-water-0.1% phosphoric acid at a flow rate of 1.5ml/min. The peaks 1,2, and 3 correspond to the hexapeptide, the valinyl deletion- and the tyrosinyl deletion-pentapeptide respectively.

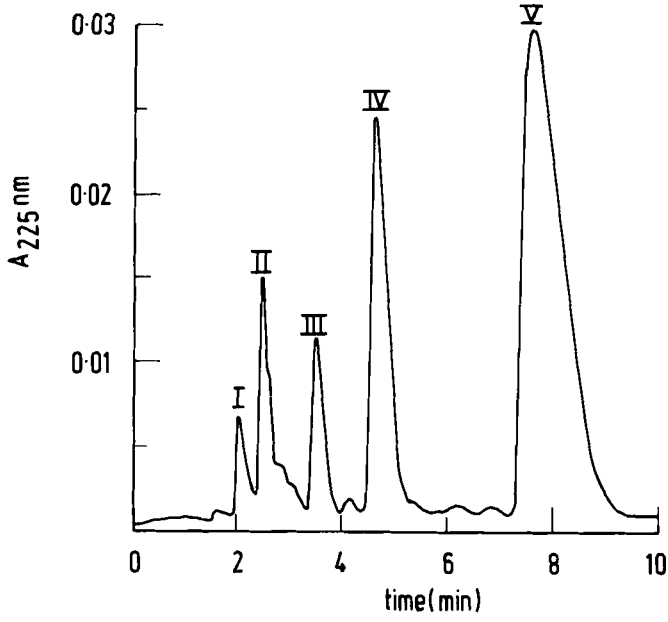



FIGURE 9.

Chromatogram obtained with synthetic preparation of leu-enkephalin amide O-benzyl ether, after gel filtration on Sephadex G10. Column: μ-Bondapak alkylphenyl, flow rate 2.0ml/min. Isocratic conditions were used for the elution with a mobile phase of 40% acetonitrile-water-0.1% phosphoric acid. Peaks IV and V correspond to the unprotected leu-enkephalin amide and the O-benzyl ether respectively.

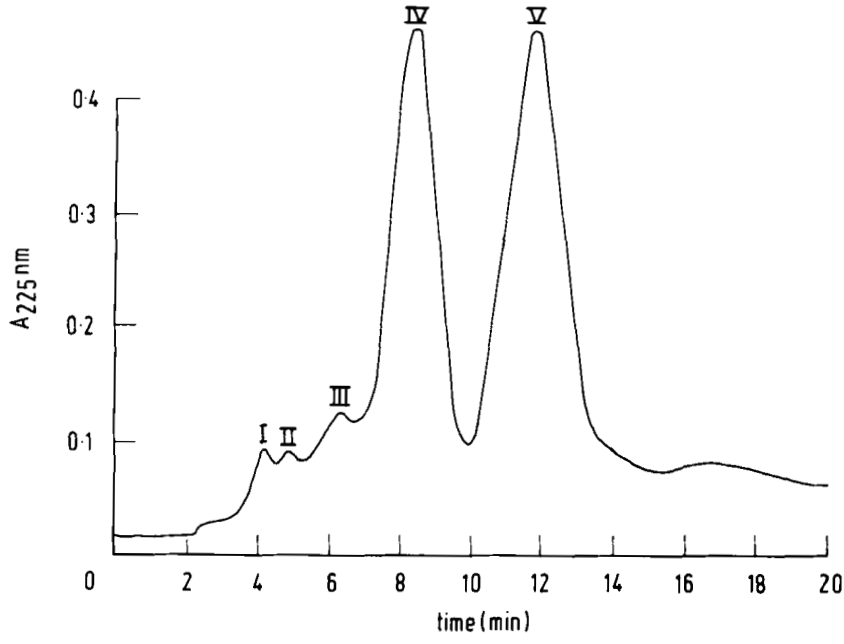



FIGURE 10.

Preparative separation of crude leu-enkephalin amide O-benzyl ether (V) and debenzylated product (IV) on a Phenyl-Porasil B column (60cm x 7mm I.D.) using 30% acetonitrile-water-0.1% orthophosphoric acid as mobile phase. The loading was 4mg and the flow rate 2ml/min.



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## REFERENCES


1. Marshall, G.R. and Merrifield, R.B., in 'Biochemical Aspects on Solid Supports', ed. G.R. Stark, Academic Press, New York, 1971, p.111.
2. Hearn, M.T.W. and Hancock, W.S. Trends in Biochem. Sci. (1978) in press.
3. Hancock, W.S., Bishop, C.A., Prestidge, R.L., Harding, D.R.K. and Hearn, M.T.W. Science, 200, 1168, (1978).
4. Hancock, W.S., Bishop, C.A., Meyer, L.J. Harding, D.R.K. and Hearn, M.T.W. J. Chromatogr. (1978) In press.
5. Hancock, W.S., Bishop, C.A., Prestidge, R.L., Harding, D.R.K. and Hearn, M.T.W. J. Chromatogr. 153, 391, (1978).
6. Santi, W., Huen, J.M. and Frei, R.W. J. Chromatogr. 115, 423, (1975).
7. Ward, R.S. and Pelter, A. J. Chromatogr. Sci. 12, 570, (1974).
8. Gabriel, T.F., Michalewsky, J. and Meienhofer, J. J. Chromatogr. 129, 287, (1976).
9. Pickart, J. and Thaler, M.M. Prep. Biochem. 5, 397, (1975).
10. Rivier, J.E. J. Liquid Chromatogr. 1, 347, (1978).
11. Stewart, J.M. and Young, J.D. in 'Solid Phase Peptide Synthesis' W.H. Freeman, San Francisco, 1969, p.49.
12. Dayhoff, M.O. 'Atlas of Protein Sequence and Structure' National Biomedical Research Foundation, Silver Spring, Maryland, U.S.A.
13. Kohn, L. in 'Clinical Reports in Medicinal Chemistry' ed. Shen, T.Y. (1977).
14. Molnar, I. and Horvath, C. J. Chromatogr. 142, 623, (1977).
15. Rekker, R.F. in 'The Hydrophobic Fragmental Constant' Elsevier, Amsterdam, (1977), p.301.
16. Wieland, T., Dungen, A. and Birr, C. F.E.B.S. Letts. 14, 299 (1971).
17. Peach, M.J. in 'Chemistry and Biology of Peptides' ed. Meienhofer, J. Ann Arbor Science Publishers Inc. Michigen (1972) p. 471.

18. Hughes, J., Smith, T.W., Kosterlitz, H.W., Fothergill, L.A., Morgan, B.A. and Morris, H.R. Nature 258, 577 (1975).

19. Hearn, M.T.W. and Hancock, W.S. Manuscript in preparation.

20. Hansen, J.J. Greibrokk, T., Currie, B.L., Johansson, K.N. and Folkers, K. J. Chromatogr. 135, 155, (1977).

21. Hancock, W.S., Bishop, C.A. and Hearn, M.T.W. F.E.B.S. Letters, 72, 139, (1976).