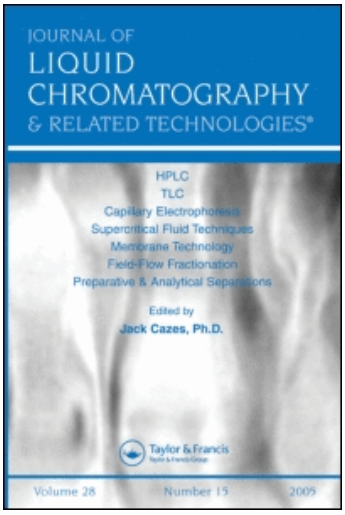

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The Preparative Separation of Synthetic Peptides on Reversed-Phase Silica Packed in Radially Compressed Flexible-Walled Columns.

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THE PREPARATIVE SEPARATION OF SYNTHETIC PEPTIDES ON REVERSED-PHASE SILICA PACKED IN RADIALLY COMPRESSED FLEXIBLE-WALLED COLUMNS.+

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ABSTRACT

The rapid and efficient separation of multigram amounts of unprotected synthetic peptides on octadecylsilica, packed in flexible walled polyethylene columns, is described. The mobile phase used was 0.05% trifluoroacetic acid with methanol as the organic modifier. An advantage of this eluant system was that the salt free peptide could be isolated simply by lyophilisation of the sample after chromatography. The following peptides were purified with this system: glycylgly-

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cine ethyl ester, glyclyglycylglutamic acid, glycylglycyllysine, pyroglutamylhistidinylglycine, prolyprolylproline, leucine-enkephalin and methionine-enkephalin, in amounts ranging from 50mg to 5gm.

INTRODUCTION

The use of peptides for research and clinical purposes has increased dramatically in the past five years, particularly in the field of neuro-endocrinology where a number of peptide mediated processes have been elucidated [1,2]. The peptides used in these studies have either been isolated from biological sources or prepared by solution or solid phase chemical synthesis techniques. Purification of peptides from both synthetic and biological sources has presented significant problems, as an extremely high degree of purity is required for biological studies. Recently 'ion-pair' reversed phase HPLC has been used effectively in the analysis of a variety of peptides [3-11]. This technique uses an aqueous mobile phase which contains small amounts of an ionic modifier which can ion-pair with the analyte and/or modify the organic coating on the silica particles of the stationary phase. The theoretical basis of the interactions which occur between the analyte, mobile phase and stationary phase are now beginning to be understood more clearly [12-19].

In an early study we described the use of radially compressed polyethylene cartridges containing octadecylsilane bonded silica particles in the purification of 10gm amounts of a model underivatised synthetic peptide [20]. In this case a homogeneous product was obtained with an elution time of twenty minutes using an aqueous solvent containing a hydrophilic ion-pairing reagent [13]. It is the purpose of this report to extend this study and demonstrate that preparative high performance liquid chromatography with radially compressed columns can be used to purify the following synthetic peptides - the pro-

tected dipeptide glycylglycyl-OEt, the tripeptides glycylglycyl-glutamic acid, glycylglycyllysine, pyroglutamylhistidinylglycine, and prolylprolylproline and the pentapeptides Leu-enkephalin and Met-enkephalin with separation times less than 60min.

MATERIALS AND METHODS

Apparatus: A Waters Assoc. (Milford, Mass., U.S.A.) HPLC system was used for the analytical separations. This consisted of two M6000A solvent delivery units, an M660 solvent programmer and U6K universal liquid chromatograph injector, coupled to an M450 variable wavelength UV Spectrophotometer and an Omniscribe two-channel chart recorder (Houston Instruments, Austin, Texas, U.S.A.). Waters μBondapak C_{18} columns (10μm, 30cm x 4mm, I.D.) were used for all analyses. Sample injections were made using a Microliter 802 syringe (Hamilton, Reno, Nev., U.S.A.).

The preparative separations were carried out on a Waters Assoc. Prep LC/System 500 instrument with a built in refractive index detector connected in series with the UV spectrophotometer and coupled to an Omniscribe two-channel recorder (Houston Instruments). A Waters Assoc. Prep PAK-500 C_{18} cartridge (75μm mean particle size, 30cm x 5.7cm) was used for the purification. Before use and after each chromatographic separation the cartridges were washed with 4 litres of methanol and the 4 litres of methanol:water (1:1) at a flow rate of 50ml/min to remove any adsorbed contaminants. Sample injections were made using a Gastight 101W syringe (Hamilton).

For both analytical and preparative work solvents were filtered using a Pyrex filter holder (Millipore, Bedford, Mass., U.S.A.) while peptide samples were filtered using a Swinney Filter (Millipore). Millipore HA grade, 0.45μm filters were used at all times for solvent and sample preparation, except

for filtration of the methanol when a Millipore FH grade filter was used.

Chemicals: Water was glass-distilled. Methanol was drum-grade (ICI, Wellington, New Zealand) and distilled before use. Trifluoroacetic acid (TFA) (Halocarbon Products, Hackensack, N.J., USA) was also distilled before use. Phosphoric acid (AR) was obtained from May and Baker. Synthetic peptides were prepared by standard solution techniques which will be described elsewhere. All amino acids were of the L-configuration except glycine.

Methods: Analytical HPLC was carried out at a flow rate of 1.5ml/min using 0.05% TFA in water, pH 2.3, as mobile phase. All chromatography was carried out at room temperature (ca. 22^{o}). Peptides were dissolved in the mobile phase at a concentration of 5mg/ml and 10-25µl volumes injected onto the columns. Similarly, 25µl aliquots from the preparative HPLC runs were analysed under identical elution conditions.

For the preparative separations, a flow-rate of 100ml/min was maintained (back pressure 100psi). The mobile phase was degassed completely by vacuum aspiration or by a helium gas purge. The crude sample was loaded in amounts between 50mg and 10g in 10ml of the eluting solvent. Immediately after collection each fraction was neutralised to pH 7 with ammonium hydroxide, concentrated on a rotory evaporator and lyophilised.

RESULTS

Figure 1 shows the analysis and preparative scale purification of a crude sample of the ethyl ester of glycyl-glycine, a simple dipeptide containing a single amide group and no side chains. Part A shows the analysis of 50µg of the peptide on a µBondapak C_{18} column using a mobile phase consisting of a 0.05% solution of trifluoroacetic acid (TFA) in water, with UV detection at 210nm. Figure 1B and 1C respectively show the purification of 50 and 200mg of the dipeptide

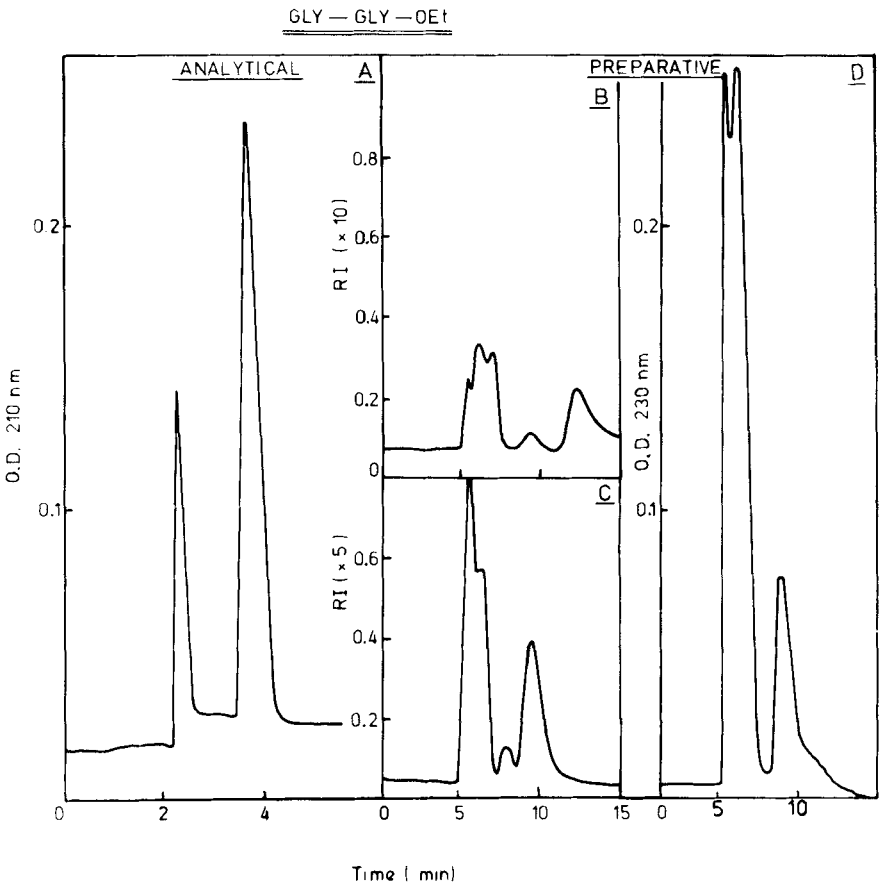


Figure 1.

The purification of Gly-GlyOEt. In part A a 50µg sample of Gly-GlyOEt was analysed on a µBondapak-C_{18} column with 0.05% TFA as the mobile phase and a flow rate of 1.5ml/min. In parts B to D the purification was carried out on a C_{18} cartridge in the Prep 500 Liquid Chromatograph with 0.05% TFA as the mobile phase and a flow rate of 50ml/min. The loadings were 0.05g and 0.2g in parts B and C respectively and the solute was monitored with a RI detector. Part D shows the monitoring of the effluent from the RI detector used in part C with an UV detector connected in series.

on the Prep 500 liquid chromatograph, using a C_{18} cartridge. The mobile phase was 0.05% trifluoroacetic acid in water, and detection was by means of a differential refractometer. The flow rate was 50ml/min. Figure 1D shows the same analysis as depicted in Figure 1C but with detection by means of a UV detector connected in series after the refractive index detector. In this separation the first peak(s) were attributed to salts and derivatives of glycine, while the later eluting peak (retention time of 4min in part A and 10min in part D) was the desired product Gly-GlyOEt, recovered in a 93% yield. The identity of this peak was confirmed by the chromatography of an authentic sample of the dipeptide.

Figure 2 shows the purification of 5gm of a crude synthetic sample of the tripeptide pyroglutamylhistidinylglycine, the

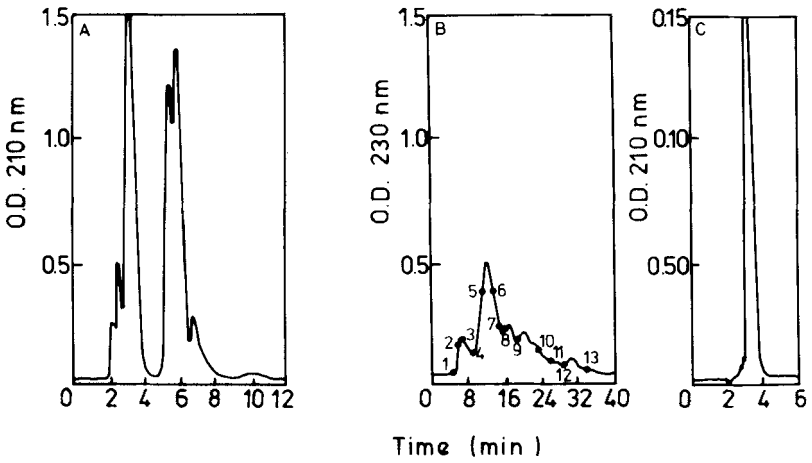


Figure 2.

The purification of Pyr-His-Gly. Parts A and C show the analytical elution profile of the crude mixture and the purified peptide respectively. The analytical system and preparative system were the same as described in Figure 1. The loading of crude mixture was 5g, and the elution profile for the preparative separation is shown in part B.

proposed anorexigenic peptide [21], with analysis before and after the purification (parts A and C). The mobile phase in each case was 0.05% TFA in water. Figure 2A shows the analytical HPLC profile of the crude sample with detection at 210nm. Figure 2B shows the preparative purification of 5gm of crude product on a C_{18} cartridge with UV detection at 230nm using a flow rate of 100ml/min. Figure 2C shows the analysis of the collected fraction which corresponded to the required product, numbered five on the chromatogram, again with detection at 210nm. Fractions 4 and 6 also contained significant amounts of the required peptide and could readily be concentrated and rechromatographed, using the recycle facility to improve their degree of purification. Following this procedure 2gm of the purified peptide was recovered and shown to be homogeneous by amino acid analysis of a hydrolysate and by analytical HPLC (Figure 2C). In addition after three lyophilisations the sample was found not to have any residual TFA, an important factor since the peptide was to be used in animal studies.

Figure 3 shows the elution profiles of the relatively polar tripeptide glycylglycylglutamic acid using a 0.05% TFA in water as the mobile phase. Figure 3A shows the analysis of crude synthetic product on the μBondapak-C_{18} column with 0.05% TFA as mobile phase. Figure 3B and 3C show the analysis of the crude and purified materials, using the same column but with 5mM perfluoropropionic acid as the mobile phase. Due to the polar nature of the peptide, little retention was obtained on the reversed phase column and therefore the center of the main peak (see Figure 3D) was recycled to improve resolution. Subsequent analysis of each fraction by analytical HPLC (see runs 1 to 7 in Figure 3) showed that the centre of this peak (fraction 5) was essentially homogeneous.

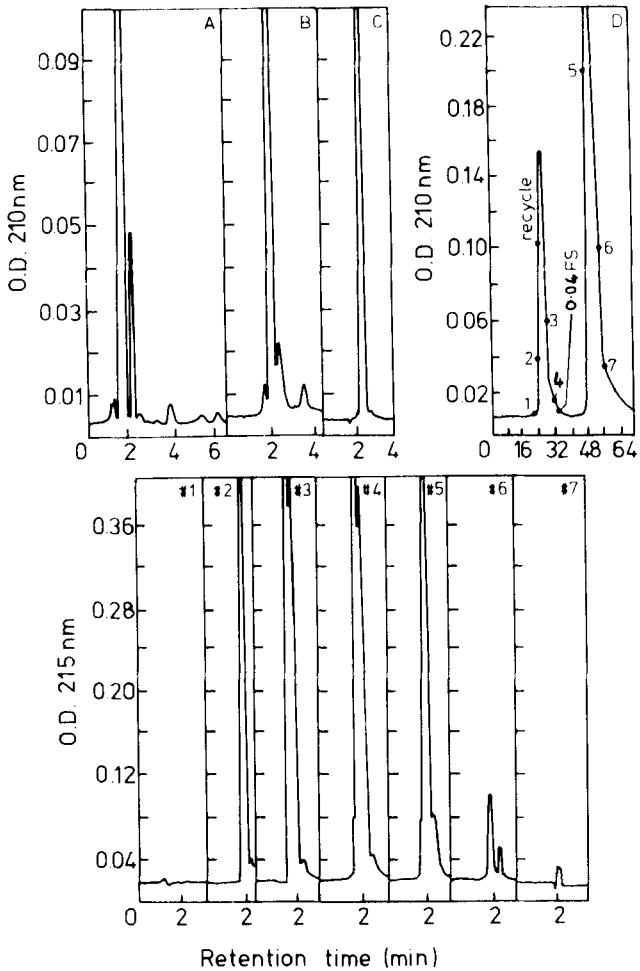


Figure 3.

The purification of Gly-Gly-Glu. Parts A and B show the analytical elution profile of the crude mixture and Part C the profile for the purified peptide. The analytical procedure was the same as described in Figure 1 except that the mobile phase used in Part A was 0.05% TFA and in Parts B and C perfluoro-propionic acid (5mM). The preparative run (loading 4gm) is shown in Part D and the same conditions as Figure 1 were used. The centre of the peak was recycled and the elution profile of the recycled peak is shown at a greater OD sensitivity. The amount of material in the major fractions was as follows, 2, 1.1gm; 5, 1.28gm; 3 and 4, 0.6gm. The analytical HPLC of the fractions is shown in the bottom part of the figure using the conditions as described in Figure 1.

Figure 4 shows the analysis and purification of HPLC of the tripeptide glycylglycyllysine using 0.05% TFA in water as eluant. Figure 4A and 4C show the analysis of the crude sample and the purified product respectively. The product was analysed after fraction 3 from the preparative run had been neutralised, concentrated and lyophilised. Figure 4B shows the UV trace obtained during the preparative run of 4gm of the crude sample at a flow rate of 100ml/min. The numbers on the chromatogram refer to fraction numbers collected. The profiles numbered 1-4 are analytical HPLC chromatograms obtained by analysing the fractions as they were collected during the preparative run. In each case a 25µl sample was withdrawn and injected directly onto the analytical HPLC system in the same solvent.

Figure 5 shows the analysis and purification of the tripeptide prolylprolylproline. Figure 5A is the analysis of 0.5mg of the crude sample whilst Figure 5B is the preparative scale separation of 5gm of the crude sample obtained with a flow rate of 100ml/min. The mobile phase used in this separation was 20% methanol-water, 0.05% TFA. Again the numbers refer to the collected fractions, while the solvent eluted between fractions 3 and 4 was diverted to waste. The numbered chromatograms relate to the collected fractions from the preparative run, and were achieved by direct injection of aliquots from the relevant fraction, prior to neutralisation. Identical conditions were used for the analysis of both crude and purified fractions. The desired peptide was identified in the last eluting peak (t_R 4min and 22min in the analytical and preparative runs respectively) by quantitative amino acid analysis.

Figure 6 shows the analytical and preparative chromatogram of the pentapeptide tyrosylglycylglycylphenylalanylmethionine, methionine-enkephalin. In this separation 0.4gm of the crude

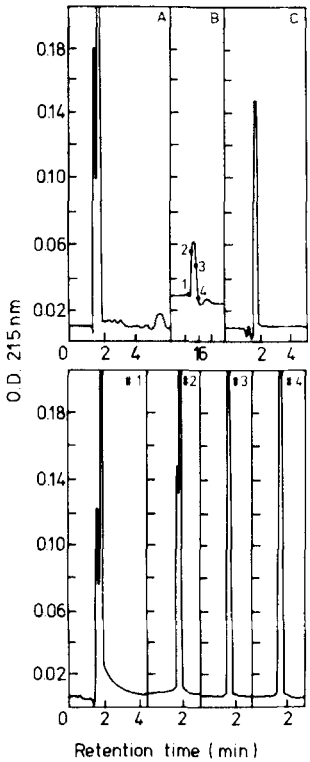
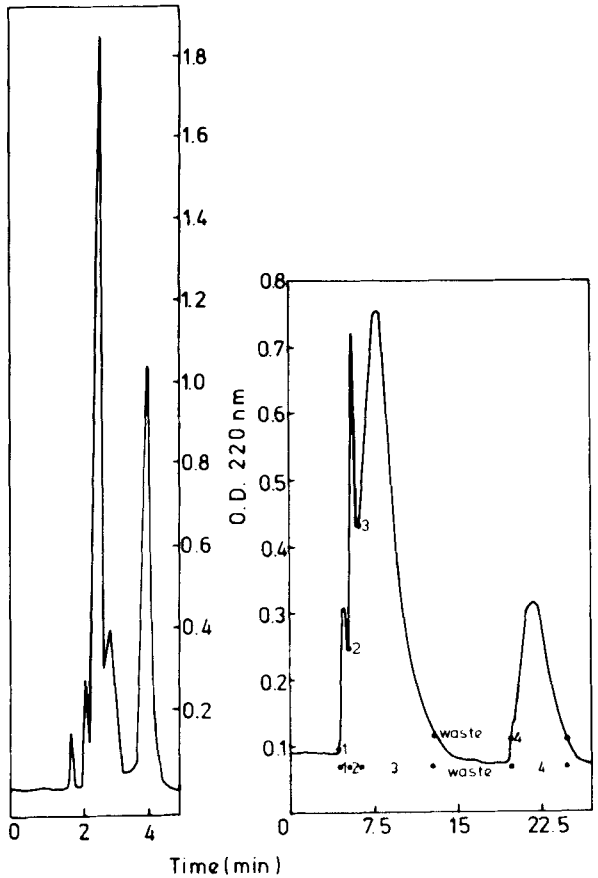


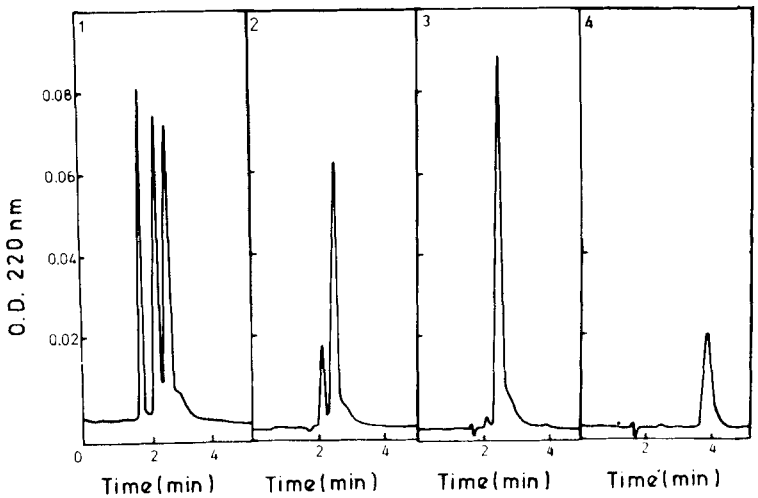
Figure 4.

The purification of Gly-Gly-Lys. Parts A and C show the analytical elution profile of the crude mixture and the purified peptide respectively, while part B shows the profile obtained for the preparative run. All chromatographic conditions were the same as described for Figure 2. The analysis of the fractions from the preparative run is shown in the bottom Figure. The loading for the preparative run was 4gm.

Figure 5.

The purification of $(Pro)_3$. Part A shows the elution profile of a 0.5mg samples of the crude product on a μBondapak-C_{18} column with a mobile phase of 0.05% TFA/H_2O-20% CH_3OH at a flow rate of 1.5ml/min. Part B shows the corresponding preparative run which was carried out on a 5gm sample and with a mobile phase of 0.05% TFA/H_2O-25% CH_3OH and a flow rate of 100ml/min. The bottom part of the Figure shows the analysis of fractions from the preparative run.





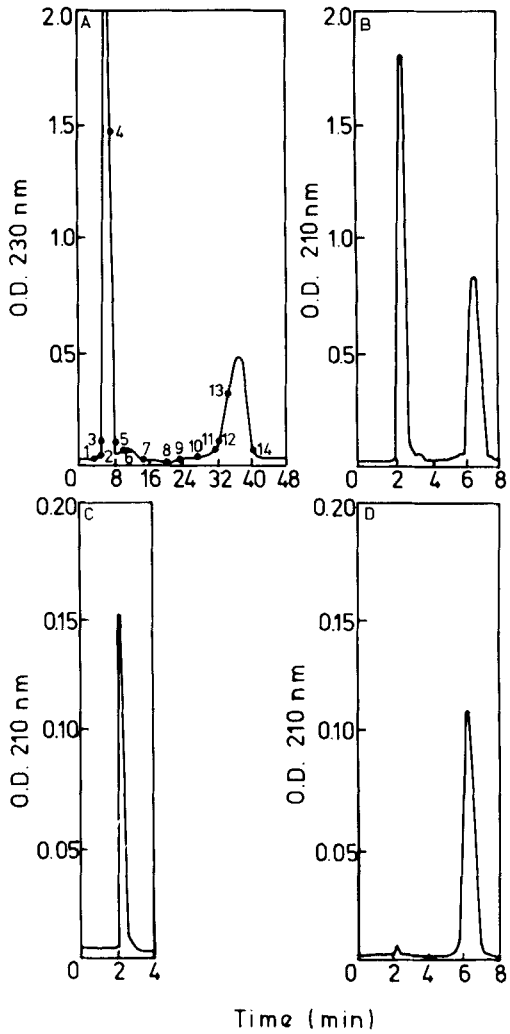


Figure 6.

The purification of methionine-enkephalin (0.4gm). The chromatographic conditions were as described for Figure 2 except that 0.05% TFA/H_2O-25% CH_3OH was used as the mobile phase. Part A shows the elution profile for the preparative run, Part B, C, D are analytical profiles of the crude mixture and the two major fractions from the preparative run.

sample was loaded in 28ml of the mobile phase, which was 25% methanol-water, 0.05% TFA. Figure 6A represents a UV trace of the preparative separation carried out at 100ml/min. The numbers represent fractions collected. Figure 6B shows the analytical separation of the crude sample. Figure 6C is the analysis of 25µl of fraction 4 and Figure 6D the analysis of 25µl of fraction 13. The analyses were carried out immediately after collection and prior to neutralisation. Again the analysis of the crude and purified fractions were carried out in the same mobile phase, namely 25% methanol-water, 0.05% TFA. Amino acid analysis of an acid hydrolysate of an aliquot of each peak identified the desired pentapeptide as the late eluting peak.

Figures 7A and 7B show the preparative scale purification of a crude sample of the pentapeptide, tyrosylglycylglycyl-

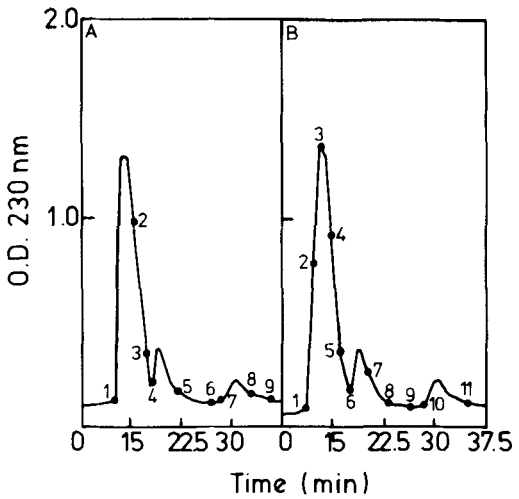


Figure 7.

The purification of leucine-enkephalin. The chromatographic conditions were as described for Figure 2, except that the mobile phase was 0.01% phosphoric acid/H_2O-25% CH_3OH. Parts A and B show the elution profiles obtained for two duplicate preparative runs on 2gm of crude mixture.

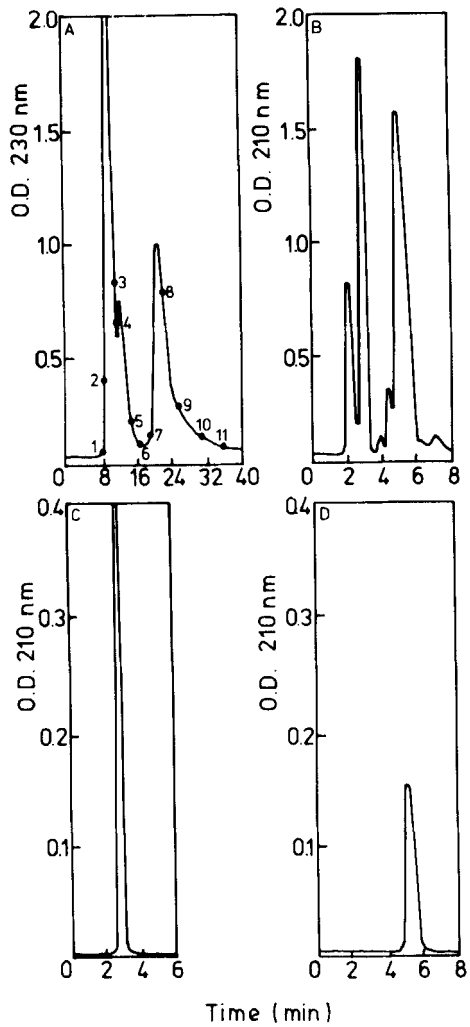


Figure 8.

The purification of leucine-enkephalin. The chromatographic conditions used were as described in Figure 2 except that the mobile phase was 0.05% TFA/H_2O-25% CH_3OH. Part A shows the preparative run, while parts B, C, D are analytical runs on the crude mixture and the two major fractions from the preparative run.

phenylalanylleucine, leucine-enkephalin, with a mobile phase consisting of 25% methanol-water, 0.1% phosphoric acid. In these separations 2gm of the sample was loaded in 10ml of the mobile phase. Again the numbered fractions were collected and an aliquot of each analysed by HPLC in 20% methanol-water, 0.1% phosphoric acid prior to neutralisation, concentration and lyophilisation.

Figure 8A shows the preparative separation of Leu-enkephalin using 30% methanol-water, 0.05% TFA as mobile phase. 1gm of the crude sample was loaded in 10ml of the mobile phase, and the flow rate maintained at 100ml/min. Figure 8B shows the analysis of a sample of the crude product while Figures 8C and 8D show analysis of fractions collected during the preparative run which corresponds to the major peaks in Figure 8B. Amino acid analysis studies of the late eluting peak (retention time of 23min in part A and 5.3min in parts B to D) showed that the desired pentapeptide was contained in this peak.

DISCUSSION

Recently there has been increasing interest in the use of preparative high performance liquid chromatography for the isolation of compounds of biological or synthetic origin [22]. It is now clear that the capacity of a particular column can vary considerably for different biological compounds. At this stage of development, three categories of column dimensions are used for peptide purification by HPLC: (1) sample size up to 1mg, analytical columns, e.g. 25 x 0.4cm I.D.; (2) sample size between 1-100mg, semipreparative columns, e.g. 60 x 0.7cm I.D. stainless steel columns or 10 x 0.8cm I.D. radial compression columns, and (3) sample size 100mg-1gm and upwards, preparative columns, e.g. 30 x 5.7cm I.D. With current technology, nearly all columns available either commercially or

packed in-house would fit in these ranges. The great majority of analytical columns function most efficiently in the microgram to low milligram range. Recently, some manufacturers have introduced colums with greater internal diameter and length, packed with materials essentially identical to those available for analytical chromatography [23]. Such columns probably have a maximum capacity of approximately 100-500mg for solutes such as peptides which contain a number of ionisable groups. Fully preparative HPLC has effectively extended this range, as is clearly demonstrated in the present report on the rapid and high efficiency purification of eight synthetic peptides. The good resolution obtained with the radially compressed, flexible-walled cartridges can be seen in preparative separations shown in Figures 1 to 8, where the elution profile of the preparative run is similar to that of the analytical run. It should be remembered that these preparative cartridges are packed with 75μm particles and the analytical columns with 10μm particles. The improvement in column efficiency on radial compression of flexible cartridges has been related to the formation of highly efficient chromatographic beds without wall effects [24].

As described in a previous report on the preparative separation of a model tetrapeptide [21], trifluoroacetic acid (TFA) was chosen as the hydrophilic ion pairing reagent on account of its volatility and transparency at the low wavelengths required to monitor elution of these solutes. The effectiveness of this volatile ionic modifier can be assessed in the comparative study shown in Figures 7 and 8 where leucine-enkephalin is purified with a mobile phase which consists of 0.1% phosphoric acid and 0.05% TFA respectively. Clearly the volatile acid allows an equally effective separation as the involatile phosphoric acid. The volatility of TFA allows the facile isolation of the peptide by lyophilis-

ation, after the pH of the sample is adjusted to 7 with ammonium hydroxide. It has been our experience that it is important to carry out the neutralisation step as soon as possible after collection since some peptides are unstable when exposed to the acidic aqueous-organic solvent mixtures for extended periods of time.

At the higher sample loadings used in preparative separations, refractive index detection can be used to monitor the purification of ca. 50mg of a simple peptide (see Figure 1). Also, as is apparent by comparison of parts C and D of Figure 1, the use of a UV detector linked in series with the refractometer can identify certain differences in the chromatogram. In addition UV detection in the preparative separation can be more readily related to the analytical system, where UV detection is often the method of choice due to the relative insensitivity of the refractometer to peptides.

Figure 7 shows that the preparative separation is extremely reproducible as in this Figure two identical loadings of leucine-enkephalin gave exactly the same elution profile when chromatographed under the same chromatographic conditions. Similar reproducibility was obtained with mobile phases containing either trifluoroacetic or phosphoric acid. In the separations described in this report the loading limit of the purification was not established, as the amount of sample available was always less than the expected maximum capacity of the cartridge. In a previous study however, it was found that 10gm of a model tetrapeptide could be chromatographed without loss of separation efficiency [20]. The samples used in this new study were deliberately limited to crude synthetic products to test the resolving power of the chromatographic techniques. In each case it was possible to

demonstrate that the isolated pure peptide was the major component of the crude mixture. For example, in the purification of Pyr-His-Gly (Figure 2), 5gm of a crude sample were loaded with 2gm of pure product being obtained despite the fact that fraction 5 was a conservative cut. In our earlier study it was demonstrated that rechromatography of a purified tetrapeptide gave essentially quantitative recovery [20].

In conclusion, this paper as well as several related studies [20,26-29] have shown that preparative reversed phase HPLC, when used in conjunction with radially compressed columns in the Prep 500 Liquid Chromatograph or with conventional HPLC columns, is a promising technique for the large scale purification of synthetic peptides, under conditions which allow the facile and rapid isolation of the desired compound in excellent yield. As a consequence, these reversed phase HPLC methods should gain wide popularity for the large scale isolation of peptides from natural and synthetic sources and compliment existing capabilities [30,31] for the preparative separation of protected peptides by liquid-solid adsorption HPLC.

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