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Note

High-performance liquid chromatography of amino acids, peptides and proteins

XXI*. The application of preparative reversed-phase high-performance liquid chromatography for the purification of a synthetic underivatised peptide

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During the past few years, reversed-phase high-performance liquid chromatography (HPLC) has emerged as a powerful technique for analysing the purity of underivatised peptides[1–8]. In recent theoretical and experimental studies[9–13], a number of different approaches have been explored by which secondary solution equilibria, *e.g.* pH and ion-pairing, of underivatised peptides can be manipulated to influence selectivity and resolution of these substances on microparticulate reversed-phase supports. In particular, the addition of hydrophilic and hydrophobic counterionic species to the mobile phase at low pH has proved very useful for the analysis of underivatised peptides of widely different intrinsic polarities.

Currently a wide variety of peptides are being synthesised for use in biological investigations[14]. A major difficulty with these syntheses has been the production of high purity products in gram quantities. In the past, liquid chromatographic techniques such as ion-exchange and gel filtration have been used for purification of the products of solid phase or solution phase peptide syntheses. These techniques tend to be slow, exhibit low separation efficiencies and can result in low recoveries. Fully protected peptides have been successfully purified[15] using preparative normal-phase HPLC on porous silica gel microparticles with sample sizes 5 g and larger. However, with these packing materials unprotected peptides show poor resolution, long retention times and generally low recoveries.

It is the purpose of this report to show that it is also possible to purify gram quantities of underivatised peptides by reversed-phase HPLC using polyethylene car-

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tridges, containing 10–20 μm octadecylsilica particles, which can be radially compressed. A model tetrapeptide, L-leucyl–glycyl–glycyl–glycine, (L-Leu(Gly)$_3$), extensively used for intestinal transport studies[16], was prepared by standard solution phase methods and purified to homogeneity by this preparative HPLC approach. The time for the chromatographic separation for up to 10 g of the tetrapeptide was under 30 min.

EXPERIMENTAL

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 a U6K universal liquid chromotograph injector, coupled to an M450 variable wavelength UV Spectrophotometer (Waters Assoc.) and an Omniscribe two-channel chart recorder (Houston Instruments, Austin, Texas, U.S.A.). A μBondapak C_{18} column (10 μm, 30 cm $\times$ 4 mm I.D.) purchased from Waters Assoc. was 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 and recorder. An M450 variable wavelength detector (Waters Assoc.) was connected in series with the refractive index detector, and coupled to an Omniscribe two-channel recorder (Houston Instruments). A Waters Assoc. Prep PAK-500-C_{18} cartridge (75 μm mean particle size 30 cm $\times$ 5.7 cm) was used for the purification. Sample injections were made using a Gastight 1010W 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, 0.45 μm 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 (I.C.I., Wellington New Zealand) and distilled before use. Trifluoroacetic acid (TFA) (Halocarbon Products, Hackensack, N.J., U.S.A.) was also distilled before use. The tetrapeptide, L-Leu(Gly)$_3$, was prepared by a standard solution phase synthetic method, the details of which will be reported elsewhere. A commercial sample of L-Leu(Gly)$_3$ was purchased from Vega Biochemicals (Tucson, Ariz., U.S.A.). Glycine and L-leucine were obtained from BDH (Poole, England).

Methods

Analytical HPLC was carried out at a flow-rate of 1.5 ml/min using 0.05% TFA in water, pH 2.3, as mobile phase.

All chromatography was carried out at room temperature (*ca.* 22°). Peptides were dissolved in the mobile phase at a concentration of 5 mg/ml and 10–25 μl volumes injected onto the columns. Similarly, 25 μl aliquots from the preparative HPLC runs were analysed under identical elution conditions with and without the commercial standard present.

For the preparative separations, a flow-rate of 100 ml/min was maintained (back pressure 100 p.s.i.). The mobile phase, water–methanol–TFA (95:5:0.05) was degassed completely by vacuum aspiration or by a helium gas purge. The crude sample was loaded in amounts between 1 and 10 g in 10 ml of the eluting solvent. Immediately after collection each fraction was neutralised to pH 7 with ammonium hydroxide, concentrated on a rotary evaporator and lyophilised.

RESULTS AND DISCUSSION

The analytical HPLC profile of the crude product from the solution synthesis of L-Leu(Gly)$_3$ is shown in Fig. 1a. As has been noted previously[4,17] with other peptides, the peak shape obtained for the crude L-Leu(Gly)$_3$ as well as the commercial standard (Fig. 1b) with mobile phases containing TFA is poor compared to that obtained with orthophosphate systems[1,11] at a similar pH. However, TFA was chosen as the hydrophilic ion-pairing reagent on account of its apparent volatility as the ammonium salt, a property which should allow it to be removed at the final step of the purification, by lyophilisation. The resolution of the peaks is not optimal but represents a maximal loading before resolution is lost completely. Lowering the sample size dramatically improved the resolution but would give no indication of a maximum loading weight of the product for use on the preparative system.

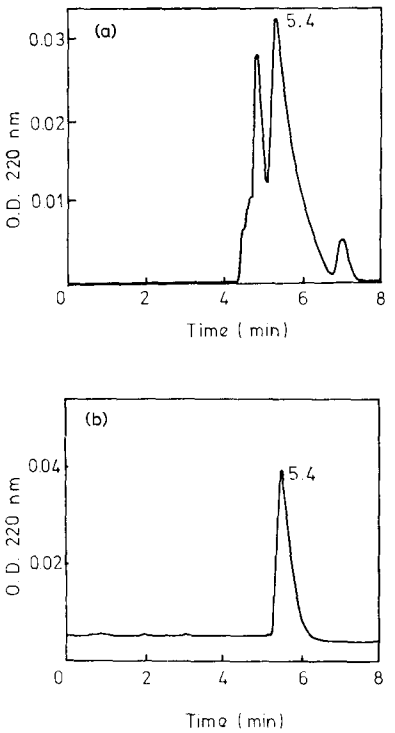

Fig. 1. The elution profile of the crude synthetic L-Leu(Gly)$_3$ (a) and the commercially obtained standard L-Leu(Gly)$_3$ (b). Chromatographic conditions: Column, μBondapak C_{18}; mobile phase, water–0.05% TFA, pH 2.3; flow-rate, 1.5 ml/min.

Fig. 2 shows the preparative HPLC chromatogram for the separation of 1 g of the crude tetrapeptide product. Following analytical HPLC of the collected fractions (Fig. 3) a close correlation between the profiles obtained under analytical and preparative conditions was apparent. The degree of separation achieved during the preparative purification is easily discerned. The major component evident in the HPLC analysis of the fraction 5 had a retention time identical to authentic L-Leu(Gly)$_3$. To improve resolution, the leading edge of the preparative fraction 5 was recycled under the same elution conditions and fractions 6, 7, 8 and 9 (Fig. 2) were collected. Only 2 l of solvent were required for this preparative run which was completed in 27 min including the recycle. In view of the excellent separation of L-Leu(Gly)$_3$ from other contaminants at the one gram scale, the sample load was subsequently increased to 10 g of crude product without loss of resolution. The purified tetrapeptide, recovered from the major recycled peak (Fig. 2, fraction 8), had a coincident retention time under analytical HPLC conditions to that of L-Leu(Gly)$_3$ standard. (*cf.* Fig. 3, fractions 5 and 8 with Fig. 1b).

A known amount of the purified product was loaded onto the preparative system in the absence of any ion-pairing reagent and collected, lyophilised and reweighed. Recovery was greater than 95%. The product obtained after lyophilisation of fraction 8 exhibited an unretained peak corresponding to a small amount of ammonium TFA which resisted repeated lyophilisation. The product could, however, be easily desalted by rechromatography on the preparative system equilibrated in

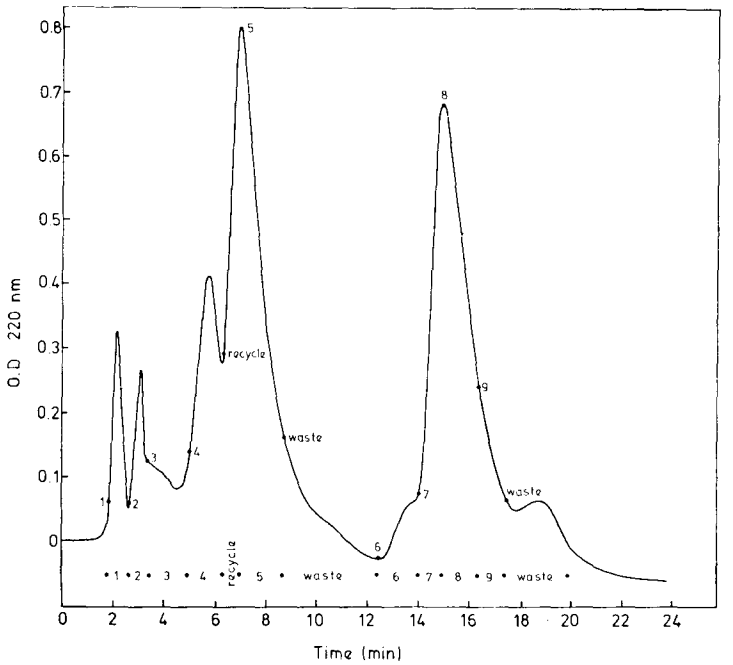

Fig. 2. The elution profile of the preparative purification of 1 g crude L-Leu(Gly)$_3$. Chromatographic conditions: Column, Prep PAK-500/C_{18} cartridge; mobile phase, water–methanol–TFA (95:5:0.05), pH 2.3; flow-rate, 100 ml/min.

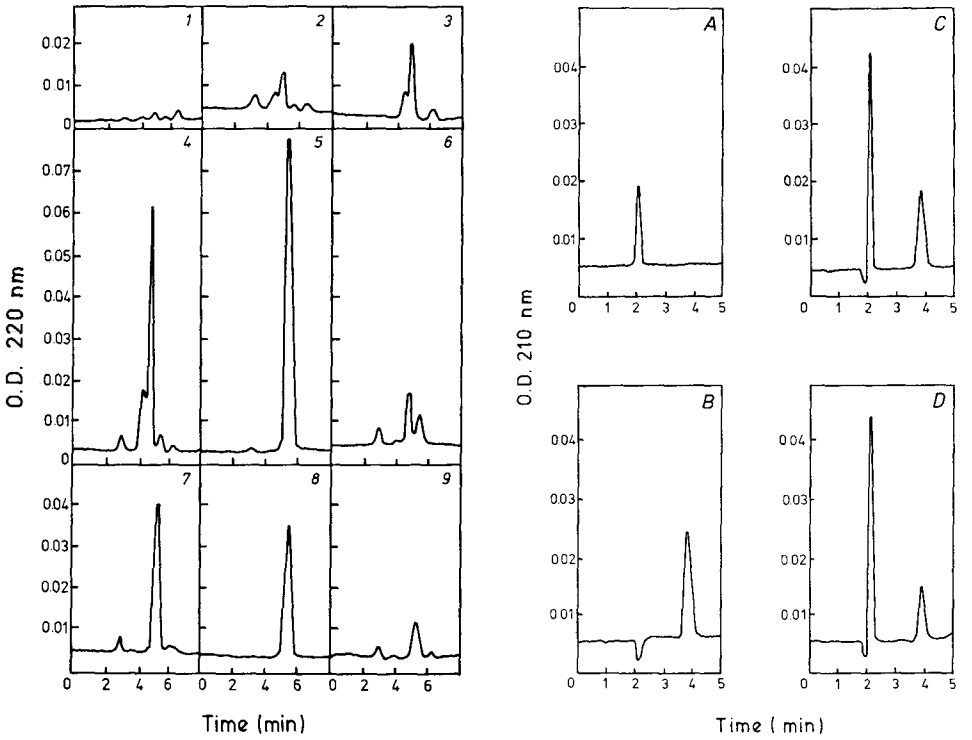

Fig. 3. The analytical HPLC profiles of the collected fractions (1–9) from the preparative separation of the crude L-Leu(Gly)$_3$. Chromatographic conditions as in Fig. 1.

Fig. 4. The elution profiles of the amino acid analysis by HPLC. (A) Glycine, (B) L-leucine, (C) the hydrolysate of the L-Leu(Gly)$_3$ obtained by preparative HPLC, (D) the hydrolysate of the commercial standard. Chromatographic conditions as in Fig. 1.

water–methanol (9:1) without the TFA present. Under these elution conditions the L-Leu(Gly)$_3$ was obtained, following lyophilisation, free of solvent contaminants.

Further positive identification of the purified product was achieved by N.M.R. and mass spectrometry. Analytical reversed-phase HPLC using several mobile phases containing ion-pairing reagents of different polarities[9,12,13] failed to reveal any peptide impurities in the recovered product. A sample of the purified peptide was hydrolysed and subjected to amino acid analysis by both conventional autoanalyser techniques and reversed-phase HPLC. The latter technique, as has been reported previously[18], is particularly useful to distinguish constituent underivatised amino acids differing in hydrophobicity. As can be seen from Fig. 4 both the commercial standard and the purified synthetic peptide show, following hydrolysis, exact correlation with the standard constituent amino acids, glycine and leucine.

Based on the above observations with the model peptide, we consider that the rapid and extremely efficient purifications of underivatised peptides in quantities up to at least 10 g should now be possible by means of preparative reversed-phase HPLC using inexpensive solvents, in reasonable volumes and with excellent recoveries of the desired product. In a subsequent paper the purification by this means of a range of biologically active synthetic underivatised peptides will be described.

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