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Note

Application of chemically bonded packing materials to high-performance liquid chromatography of peptides and amino-acid derivatives

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High-performance liquid chromatography (HPLC) has been used on an analytical scale to assay the purity of peptide hormones, antibiotics, peptide fragments and to analyse reaction mixtures involving peptides[1–10]. Recent work by Molnár and Horváth[11] and by Kraak *et al.*[12] has demonstrated the superior analytical performance which can be achieved by using reversed-phase chemically bonded packing materials. HPLC has also been used on a preparative scale for the purification of synthetic peptides[13,14].

In this note we demonstrate the potential of HPLC for monitoring stages in peptide synthesis and its application to the analysis of protected and unprotected peptide hormone fragments (fragments of adrenocorticotropin), enkephalin analogues, by-products of peptide synthesis containing tryptophan residues, and basic derivatives of glutamic acid oligomers. The column packing materials used were an octadecyl silica capped with trimethylsilyl groups (ODS–TMS silica) and a short chain reversed-phase silica (SAS silica). The characteristics of these materials and some of their applications have been described by Knox and Pryde[15].

EXPERIMENTAL

Equipment

The chromatographs used were a modified DuPont Model 820 instrument, and a laboratory assembled instrument of which the principal components were either an air driven pump (Haskel, Burbank, Calif., U.S.A.) or a reciprocating piston pump (Orlita type 1515, Giessen, G.F.R.) and a variable-wavelength photometer fitted to an 8-μl flow cell (Cecil Model 212, Cambridge, Great Britain). Columns 125 mm long and 5 mm in bore were of internally polished stainless steel. Injection, by 10-μl microsyringe, was made through a septum centrally into a bed of glass beads placed on top of the column packing.

Packing Materials

The ODS–TMS and SAS silicas were in-house preparations with a particle

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size of around 6 μm. The materials are similar to ODS and SAS Hypersils marketed by Shandon Southern Products, Runcorn, Great Britain.

About 2 g of ODS–TMS or SAS silica was made into a slurry with *ca.* 10 cm^3 of slurrying liquid (80% methanol, 20% of 0.1% (w/v) aqueous sodium acetate solution). The slurry was stirred vigorously, and agitated in an ultrasonic bath for 5 min. A volume of 280 cm^3 of slurry liquid was placed in the pump reservoir, and system was pressurised to 200 bar. The column was filled with the slurry liquid and fitted to the bottom of a precolumn which was then filled with the slurry. After connection to the pump 200 m^3 of the slurry liquid was passed at a constant pressure of about 200 bar. After shutting off the flow, the pressure was allowed to fall to zero before disconnecting the column.

*Biochemicals**

The following peptides, amino acids and modified peptides were examined.

Protected peptides

(1) Z-Tyr-Gly-Gly-Phe-Leu-OMe (thin-layer chromatographically (TLC)-pure and hydrolysis products)

(2) Z-Tyr-Gly-Gly-Phe-Leu-OH (standards, Peptide Research Group, Hungarian Academy of Sciences, Budapest, Hungary.)

(3) BOC-Lys-Pro-Val-Gly-OMe (TLC-pure and hydrolysis products)

(4) BOC-Lys-Pro-Val-Gly-OH

(5) Z-[Glu(DIME)]$_n$-OBzl $n = 1,2$; DIME = $-HNCH_2CH_2N(CH_3)_2$ (prepared by Gy. Szókán).

Modified free peptides. (from M. Low, Gedeon Richter, Budapest, Hungary).

(1) H-Arg-Trp-Gly-OH

(2) H-Arg-Trp(Bu^t)-Gly-OH.

Amino acids

(1) Tryptophan derivatives Trp(Bu^t)$_n$ where $n = 0, 1, 2, 3$.

RESULTS AND DISCUSSION

Any routine analysis of peptides must provide: (a) adequate speed of analysis for use in control of synthesis (b) adequate resolution to give complete separation of mixtures of protected and free peptides, starting materials, and by-products of coupling reactions.

The choice of an elution system was made on the basis of sample solubility. Chromatography was carried out using 125 × 5 mm columns packed with ODS–TMS and SAS silicas. Typically 2–20 μg of peptides were chromatographed. Chromatography required a maximum of 15 min. Impurities could easily be detected at

* Abbreviations used in this paper follow the rules of the IUPAC-IUB Commission on Biochemical Nomenclature. See *J. Biol. Chem.*, 247 (1972) 977–983.

levels as low as 1 %. Detection of Tyr, Trp, Phe derivatives was at 280 or 254 nm and in other cases at 224 nm. Eluents were methanol–water, methanol–water–acetic acid, acetonitrile–water and acetonitrile–methanol–water mixtures. For chromatography of free peptides and amino acids the eluent was buffered at pH 4 to 4.5 with 0.01 *M* acetic acid–sodium acetate mixtures. The pressure was varied between 30 and 125 bar; flow-rates were between 1 and 2 cm^3/min.

Figs. 1 and 2 show chromatograms of so-called "TLC-pure" samples of the protected peptides Z-Tyr-Gly-Gly-Phe-Leu-OMe and BOC-Lys-Pro-Val-Gly-OMe using

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ODS–TMS silica and a detection wavelength of 224 nm. They show several impurities. The second of these protected peptides is clearly the more pure.

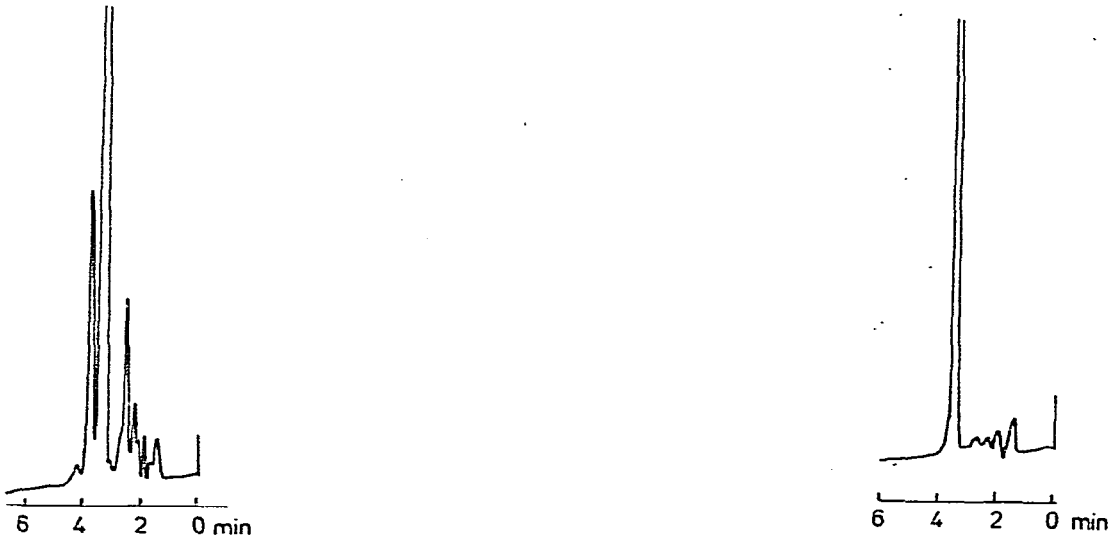

Fig. 1. Chromatogram of "TLC-pure" Z-Tyr-Gly-Phe-Leu-OMe. Packing: ODS–TMS silica; eluent: methanol–water–acetonitrile (3:2:4, v/v/v); sample: 9 μg; flow-rate: 1.2 cm^3/min; detection: 224 nm; 0.22 a.u.f.s.

Fig. 2. Chromatogram of "TLC-Pure" BOC-Lys-Pro-Val-Gly-OMe. Conditions as for Fig. 1 except sensitivity 0.05 a.u.f.s.

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Fig. 3 shows chromatograms, under slightly different elution conditions, of Z-Tyr-Gly-Gly-Phe-Leu-OMe (P) and the products of its hydrolysis by 2 *M* KOH in ethanol at 0° for 2 h. The formation of at least three products is seen, the major product being the free acid Z-Tyr-Gly-Gly-Phe-Leu-OH (A).

Fig. 4 shows analogous chromatograms under the same conditions for BOC-Lys-Pro-Val-Gly-OMe (P) and its hydrolysis products, the acid again being clearly separated (A). In both cases the acid being more polar than the methyl ester elutes before the ester.

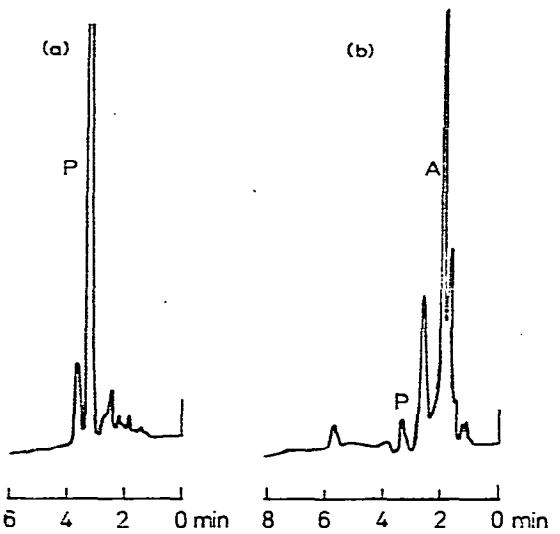

Fig. 3. Analysis of Z-Tyr-Gly-Gly-Phe-Leu-OMe. (a) "TLC–pure" sample; (b) sample hydrolysed for 2 h at 0° with 2 *M* KOH. Packing: ODS–TMS silica; eluents: methanol–water–acetonitrile; (a) 3:2:4, (b) 5:3:2 (v/v/v); sample: (a) 4 μg; flow-rate: 1.2 cm^3/min; detection: 254 nm; (a) 0.02 a.u.f.s., (b) 0.05 a.u.f.s.

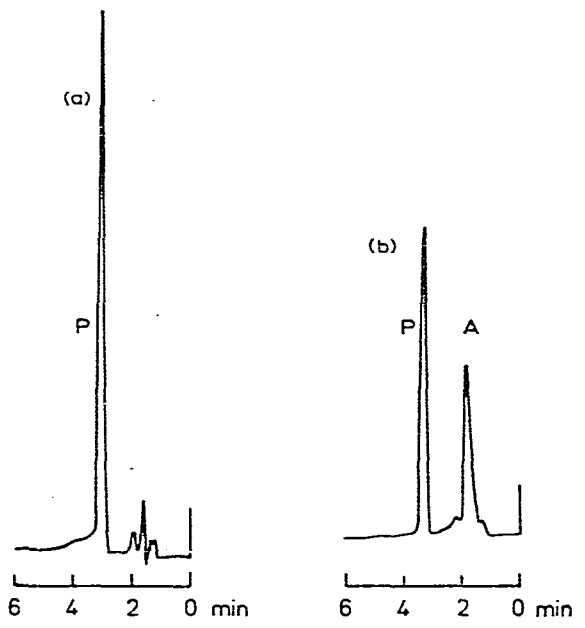

Fig. 4. Analysis of BOC-Lys-Pro-Val-Gly-OMe. (a) "TLC-pure" sample, (b) sample hydrolysed for 30 min. Packing: ODS–TMS silica, eluent: methanol–water–acetonitrile (3:3:4, v/v/v); sample: 10 μg; flow-rate: 1.0 cm^3/min; detection: 254 nm; 0.05 a.u.f.s.

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Figs. 1–4 show the potential for use of HPLC in quality control for the various various steps in peptide synthesis.

Fig. 5 shows the analysis of an unprotected tripeptide and its *tert.*-butyl derivative. In general peptides having free amino and acid groups give poorly shaped peaks even with buffered eluents.

Fig. 6 demonstrates that oligomers, which have very similar structure, can be very well separated, while Fig. 7 shows that SAS-silica is especially useful for the resolution of tryptophan derivatives containing 0, 1, 2, 3 *tert.*-butyl groups at the indole-ring .The compounds are eluted in the order of the number of *tert.*-butyl groups in the molecule in agreement with expectation for reversed-phase behaviour.

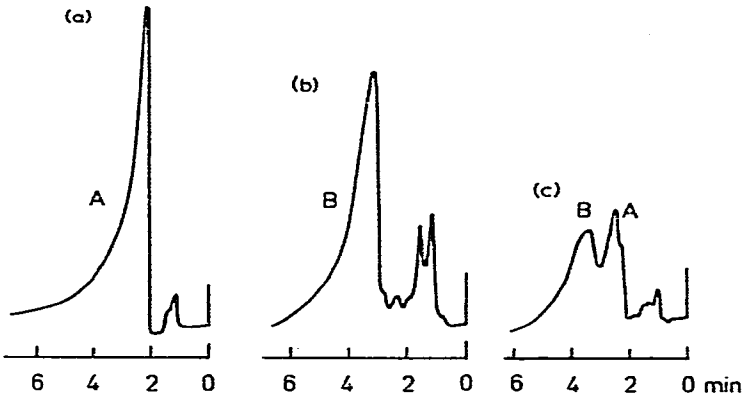

Fig. 5. Chromatogram of unprotected peptides. (a) Arg-Trp-Gly (A); (b) Arg-Trp(Bu^t)-Gly, (c) mixture of A and B. Packing: ODS–TMS silica; eluent: methanol–water–acetonitrile (7:3:5 v/v/v); sample: 6 μg; flow-rate: 1.4 cm^3/min; detection: 254 nm; 0.02 a.u.f.s.

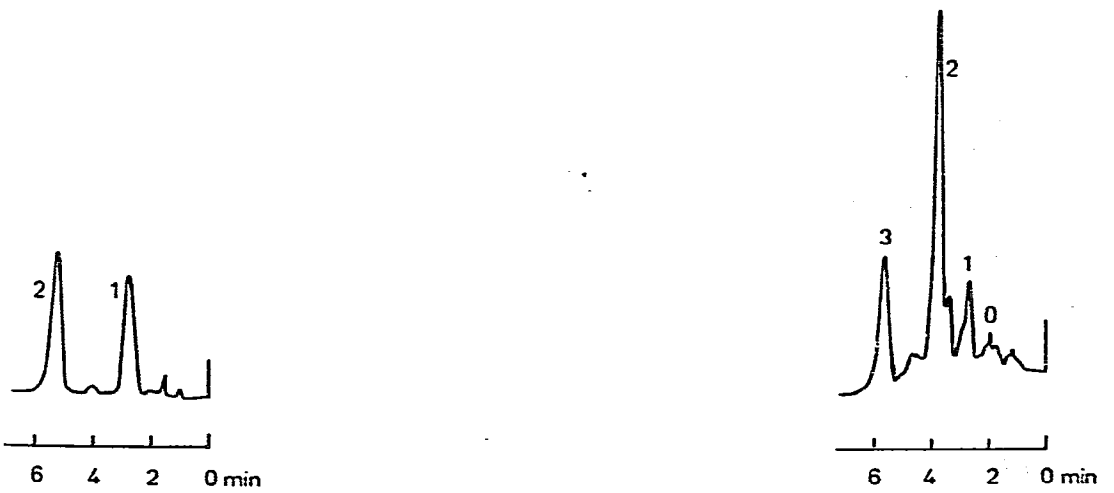

Fig. 6. Separation of oligomers Z-Glu(DIME)-OBzl (1) and Z-[Glu(DIME)]$_2$-OBzl (2). Packing: ODS–TMS silica, eluent: methanol–water–acetonitrile (5:4:11, v/v/v); sample: 5 μg of each oligomer; flow-rate: 1.3 cm^3/min; detection: 254 nm; 0.05 a.u.f.s.

Fig. 7. Separation of *tert.*-butyl derivatives of tryptophan containing 0–3 *tert.*-butyl groups (as stated in figure). Packing: SAS silica; eluent: methanol–water–acetic acid (84:14:2, v/v/v); sample: 10 μg; flow-rate: 1.1 cm^3/min; detection: 254 nm; 0.05 a.u.f.s.

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