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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETECTION OF SIDE REACTIONS IN PEPTIDE SYNTHESIS*

GYULA SZÓKÁN*
Institute of Organic Chemistry, Eötvös L. University, Múzeum krt. 4/B, H-1088 Budapest (Hungary)
and
ANGELA TÖRÖK and BOTOND PENKE
Department of Medical Chemistry, University Medical School, Dóm tér 8, H-6720 Szeged (Hungary)
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SUMMARY

High-performance liquid chromatographic systems with chemically bonded packing materials were elaborated for monitoring synthetic stages during peptide coupling, purity control of peptides produced by solid-phase methods and detection of impurities formed as a consequence of side reactions such as oxidation, desulphation, racemization, transpeptidation, alkylation and decomposition. The technique was applied to the analysis of peptide hormones, neuropeptides, cyclopeptides, isopeptides and branched polypeptides based on poly-lysine. The mobile phases were methanol–acetonitrile–water mixtures containing phosphate, acetate, carbonate buffers or trifluoroacetic acid (pH 2–9). After optimization, baseline separations could be achieved for some critical peptide separations.

INTRODUCTION

During organic synthesis, at least some unreacted starting materials must be separated from the reaction product in the majority of steps. Often a process yields two or more products and, in the case of peptide synthesis, the complexity of the intermediates and the similarity of the by-products to the desired material sometimes makes their separation difficult. The influence of impurities on the biological activities of the peptides underscores the importance of sensitive methods for the detection of these impurities in synthetic peptide end-products.

High-performance liquid chromatography (HPLC) is very useful in the analysis of amino acids, peptides, proteins, their metabolites and derivatives. Its superior analytical performance with reversed-phase, chemically bonded packings has been demonstrated in peptide analysis[1–5].

* Presented in part at the *5th International Symposium on HPLC of Proteins, Peptides and Polynucleotides, Toronto, 1985*. Abbreviations used are as recommended by the IUPAC-IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.*, 247 (1972) 977–983.

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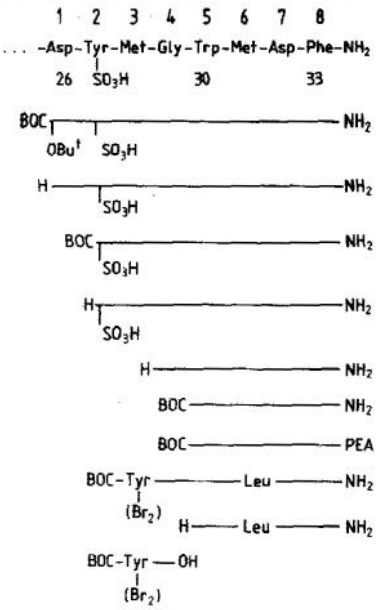
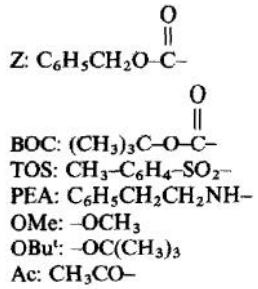
TABLE I

MATERIALS

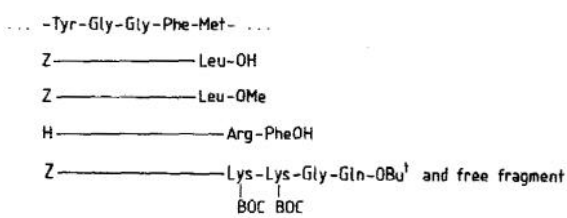
CCK, CCK-OP (octapeptide) and its derivatives



*Abbreviations**



β-LPH (β-lipotropin) fragments, derivatives



α-MSH and its fragments

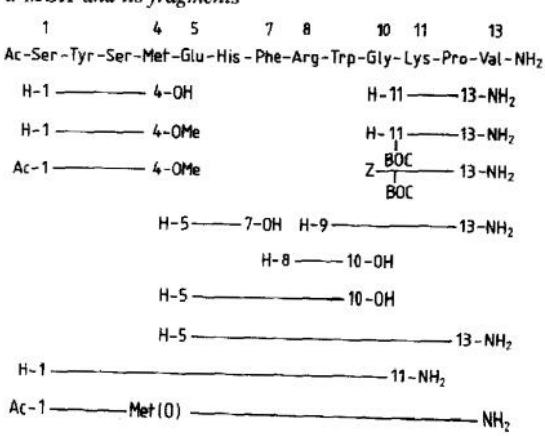
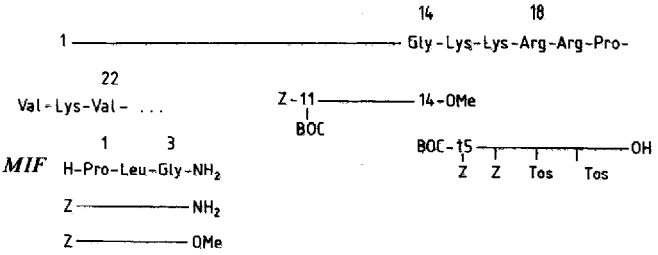


TABLE I (*continued*)

CCK, CCK-OP (octapeptide) and its derivatives	*Abbreviations**

ACTH fragments



Branched polypeptides
Poly[Lys(Ala)$_m$]$_n$
Z-DL-Ala-DL-Ala-DL-Ala-Leu-OPcp

DSIP analogue
H-Trp-AcA-Asp-Ala-Ser-Gly-Glu-OH

Glutamyl peptides
Z-pGlu-Val-OMe
Glu(OMe)-Val(OMe)
pGlu-Val-OMe
Glu(OH)-Val-OMe

ε-Lysine peptides
BOC-ε-tetra-α-Z-lysine methyl ester

Cyclopeptides
Pro-(Gly)$_n$-Pro
| |
Pro-(Gly)$_n$-Pro
n = 1, 2, 3

* See second part of footnote on page 267.

In this report, we demonstrate the usefulness of HPLC methods for monitoring steps in the synthesis of some peptide hormones and neuropeptides, such as α-melanocyte-stimulating hormone (α-MSH), cholecystokinin (CCK) and adrenocorticotropin (ACTH), melanotropin-inhibiting factor (MIF), enkephalins and their analogues, fragments and derivatives, isopeptides of Lys, cyclopeptides and branched polypeptides based on poly-Lys. We have analyzed the side reactions (oxidation, desulphation, racemization, incomplete deprotection, decomposition, transpeptidation, ring closure, alkylation), summarized by Bodánszky and Kisfaludy[6–8], which commonly occur in peptide synthesis.

EXPERIMENTAL

Materials

The peptides investigated (Table I) were synthesized by the Research Group

for Peptide Chemistry of the Hungarian Academy of Sciences, Budapest; the Institute of Organic Chemistry, Eötvös L. University, Budapest and the Department of Medical Chemistry, University Medical School of Szeged.

In mixtures, peptides were characterized chromatographically by the number of peaks, retention times, capacity factors and quantitative contents data.

High-performance liquid chromatography

Separations were performed on a laboratory-assembled instrument consisting of a reciprocating piston pump (Type 1515; Orlita, Giessen, F.R.G.), a variable-wavelength UV monitor fitted to an 8-μl flow-cell (Model 212; Cecil, Cambridge, U.K.) and a sample injector (Model 7011 loop injector; Rheodyne, Berkeley, CA, U.S.A.). Column effluents were monitored at 215, 254 and 280 nm, depending on the protecting groups and amino acid residues. The packing materials were usually Hypersil ODS, Hypersil SAS, Hypersil (Shandon Southern Products, Runcorn, U.K.) or Chromsil (Laboratory MIM, Esztergom, Hungary). All solvents used were of the highest reagent grade. The mobile phases are listed in Tables III and IV. The chromatograph was operated isocratically at ambient temperature and the mobile phase flow-rates were between 0.6 and 2.0 cm^3/min. Peaks were recorded on a Type OH-814/1 chart recorder (Radelkis, Hungary).

RESULTS

Purity of starting materials

Protecting groups generally increase retention times on reversed-phase (RP) columns (see Fig. 1) because of their higher hydrophobicity, *e.g.*, Z, BOC, Tos as amine protecting groups and alkyl- (Me, Et, Bu^t) and benzyl ester as carboxyl protecting groups. However, most of these groups also give favourable chromophores for detection (250–280 nm). For example, the esters of MSH and ACTH fragments are well separated from the unesterified ones (Tables II, III). In the case of monoamino-dicarboxylic acids, the α esters are sometimes contaminated with ω esters. The N-benzyloxycarbonylglutamic acid α-benzyl ester was found by RP-HPLC[10] to be contaminated by the corresponding γ isomer. The method is also applicable to the detection of α-ester contaminants in the γ isomer. Protection of primary amino groups and of the guanidino moiety of arginine by the group Z can also occur. The presence of Z groups increases the retention time, t_R, on RP columns. Since t_R is dependent on the number of Z groups, the contents of compounds with up to three such groups could be determined with quantitative content data[10].

Coupling control

After coupling, the fully protected end-product generally has an increased retention time as compared with the starting N- and C-terminal fragments. Fig. 2 illustrates this for gastrin fragments, where the peaks correspond to the starting dipeptide, BOC-Tyr(Br_2)-Gly-OH, and tetrapeptide, H-Trp-Leu-Asp-Phe-NH_2, and the end-product hexapeptide, BOC-Tyr(Br_2)-Gly-Trp-Leu-Asp-Phe-NH_2.

Hydrolysis

HPLC monitoring of the hydrolysis of α-MSH, ACTH, enkephalin and MIF

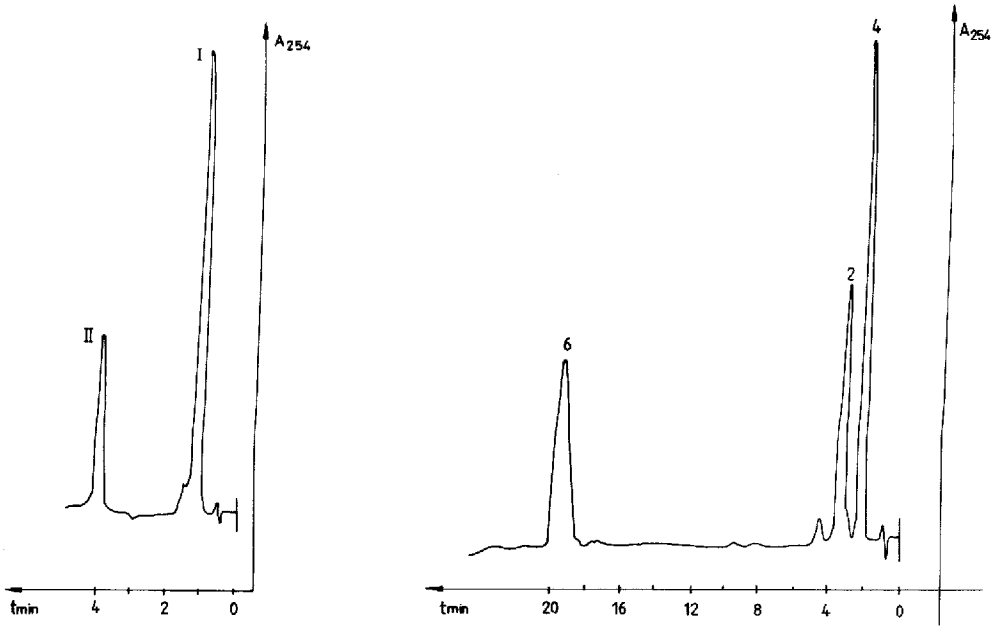


Fig. 1. Separation of protected and free enkephalin derivatives. I = Free peptide, H-Tyr-Gly-Gly-Phe-Met-Lys-Lys-Gly-Gln-OH; II = protected peptide, Z-Tyr-Gly-Gly-Phe-Met-Lys(BOC)-Lys(BOC)-Gly-Gln-OBut. Packing: Hypersil ODS-6 (125 mm × 4 mm). Eluent: methanol–0.01 *M* phosphate buffer (pH 2.1)–acetonitrile (3:3:4, v/v/v); flow-rate 1.5 cm³/min. Detection: 254 nm.

Fig. 2. HPLC analysis of peptide coupling in the case of BOC-Tyr(Br_2)-Gly-OH (2) + H-Trp-Leu-Asp-Phe-NH_2 (4) → BOC-Tyr(Br_2)-Gly-Trp-Leu-Asp-Phe-NH_2 (6). Packing: Hypersil ODS-6 (125 mm × 4 mm). Eluent: methanol–0.01 *M* phosphate buffer (pH 2)–acetonitrile (3:6:4, v/v/v); flow-rate 1.3 cm³/min. Detection: 254 nm.

TABLE II

RETENTION DATA FOR α-MSH DERIVATIVES ON HYPERSIL ODS

Column: 125 mm × 4 mm. Flow-rates: 1.4 cm³/min (gradient) and 1.2 cm³/min (isocratic). TFA = Trifluoroacetic acid.

Peptide	t_R *(min)*	*k'*	*Eluent*
α-MSH	19.8	15.0	Acetonitrile–water–TFA gradient
α-MSH-Met(O)⁴	15.6	11.0	
Z-11———13-NH_2 | BOC	3.1	2.2	Methanol–water–acetonitrile (30:30:40)
H-1-4-OH	3.4	2.5	Methanol–0.01 *M* phosphate (pH 2.1) (30:60)
H-1-4-OMe	4.3	3.5	
Ac-1-4-OMe	5.9	4.8	
H-11-13-NH_2	1.8	0.1	Acetonitrile–0.01 *M* phosphate (pH 2.1) (30:90)
H-5-7-OH	2.7	1.3	
H-8-10-OH	3.9	1.6	
H-1-4-OH	5.0	2.0	

TABLE III

RETENTION DATA FOR SOME NEUROPEPTIDES AND THEIR DERIVATIVES ON HYPERSIL ODS

Column: 125 mm × 4 mm.

Peptides	t_R *(min)*	k'	*Eluents*
MIF			
Pro-Leu-Gly-NH_2	1.4	0.75	Methanol–water–acetonitrile
Z-Pro-Leu-Gly-NH_2	3.0	2.7	(30:30:40)*
Z-Pro-Leu-Gly-OMe	5.3	5.6	
Enkephalins			
Z-Tyr-Gly-Gly-Phe-Leu-OMe	3.2	2.2	Acetonitrile–methanol–water**
Z-Tyr-Gly-Gly-Phe-Leu-OH	2.35	1.35	(20:50:30)
Met^5-enkephalin-Arg^6-Phe^7***	7.2	3.5	Methanol–0.01 *M* phosphate (pH 7.5) (55:45)*
Z-enkephalin-Lys^6-Lys^7-Gly^8-Gln^9-OBu^t (BOC BOC)	3.9 (1.1 for free peptide)	2.85	Methanol–0.01 *M* phosphate (pH 2.1)–acetonitrile (30:30:40)§
ACTH			
BOC-11——14-OMe (Z)	3.30	2.30	Methanol–water–acetonitrile* (30:30:40)
BOC-11——14-OH (Z)	1.90	0.90	
BOC-15————19-OH (Z Z Tos Tos)	2.80	1.80	

* Flow-rate: 1.1 cm^3/min.
** Flow-rate: 1.4 cm^3/min.
*** Column: 250 mm × 4 mm. Flow-rate: 1.1 cm^3/min.
§ Flow-rate: 1.5 cm^3/ min.

derivatives indicates that in all cases, the acid, *i.e.*, the peptide with free C-terminal, is eluted before the corresponding esters since it is more polar than the methyl esters or amides (Tables II, III). The separation also indicated the presence of an unidentified side-product, formed during hydrolysis in the case of protected Z-Leu-enkephalin methyl ester and probably arising from the decomposition of a Z group.

Products from solid-phase synthesis

A peptide synthesized by solid-phase methods[11] can contain a variety of impurities as a consequence of the synthesis strategy and of incomplete reaction steps. Failed, deleted, blocked, partially deprotected and racemized sequences are examples of such impurities. Thus, the analysis of crude products requires highly efficient RP packings. For example, an analogue of delta sleep-inducing peptide (DSIP) was synthesized by solid-phase methods, and the crude product was purified by moderate-pressure liquid chromatography (MPLC) (Fig. 3A). The fraction of interest, tested by amino acid analysis, showed more peaks in analytical HPLC (Fig. 3B). The ana-

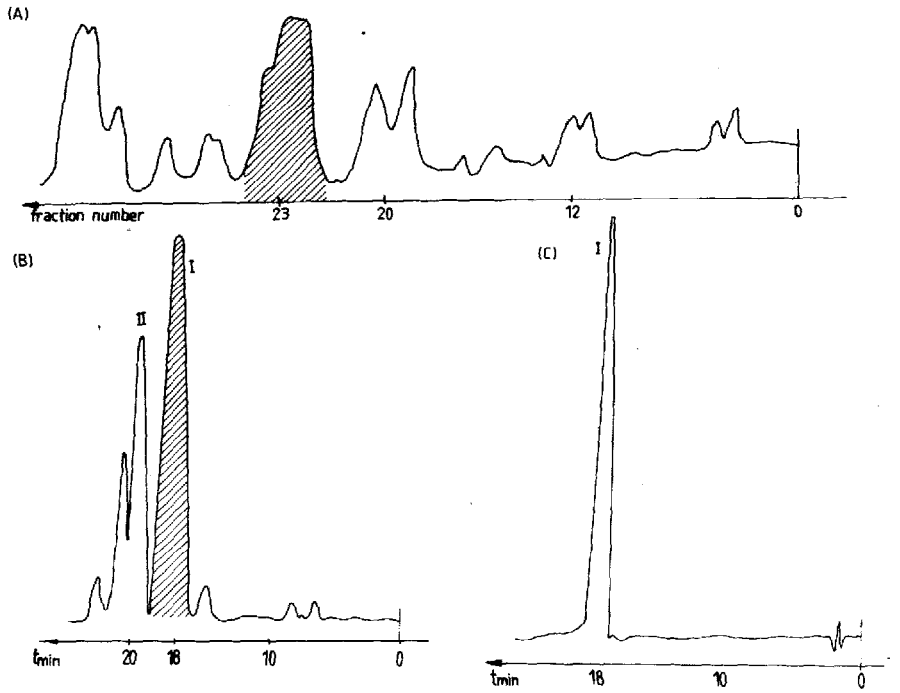


Fig. 3. Chromatograms of the synthetic DSIP analogue: H-Trp-AcA-Asp-Ala-Ser-Gly-Glu-OH. (A) MPLC pattern of 150 mg crude product synthesized by the solid-phase method. Packing: LiChrosorb RP-18 (25–40 μm, 600 mm × 8 mm (Merck). Eluents: A, 0.1 *M* ammonium acetate buffer (pH 6.5); B, 60% acetonitrile in solvent A. Gradient: 2% B per minute; flow-rate 3.2 cm^3/min. Fraction volume: 3 ml. Detection: 254 nm. (B) Semipreparative HPLC pattern of 200 μg from fraction 23. Packing: Hypersil ODS-6 (250 mm × 4 mm). Eluent: acetonitrile–0.01 *M* ammonium acetate buffer (pH 4) (17:83, v/v); flow-rate 1.2 cm^3/min. Detection: 280 nm. Repeating of 200 μl loading; collection of fraction corresponding to peak I. (C) Analytical HPLC control of pure fraction I under the same chromatographic conditions as in (B). Retention time: 18.2 min.

lytical separation was scaled up to semi-preparative conditions and using the same HPLC conditions the sample injection at maximum loading capacity was repeated (Fig. 3C). Pure peptide was produced and collected in quantities sufficient for biological assay and final structure identification.

Fig. 4 shows the HPLC monitoring of the purification of a synthetic protected tetrapeptide, confirming the efficiency of the HPLC separation.

Alkylation

In the deprotection of protected BOC and OBu^t peptides, the tryptophan residue can suffer *tert.*-butylation at the indole ring (Scheme A). We have previously developed a separation system on Hypersil SAS for analysis of Trp derivatives containing up to three *tert.*-butyl groups in the indole ring[12]. The compounds are eluted in the order of the number of *tert.*-butyl groups in the molecule, consistent with the hydrophobic nature of the compound (Fig. 5). The ACTH fragments containing Trp residues showed the same behaviour[12].

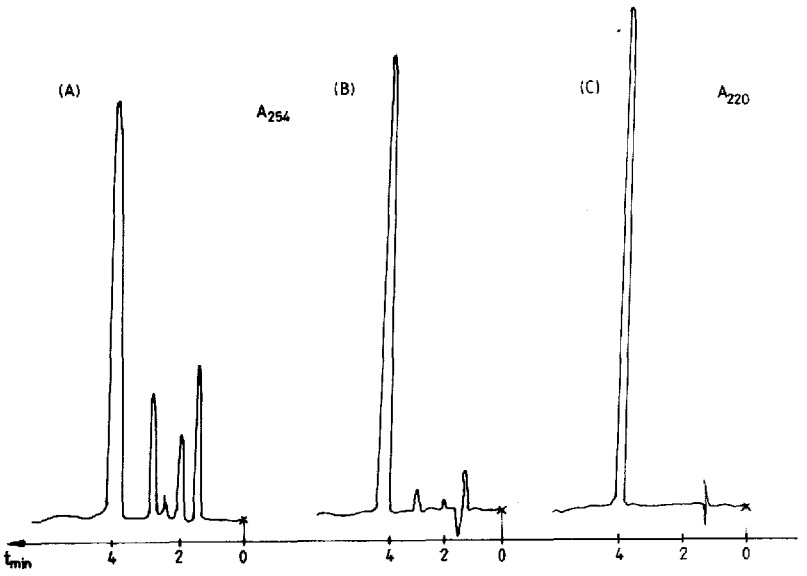


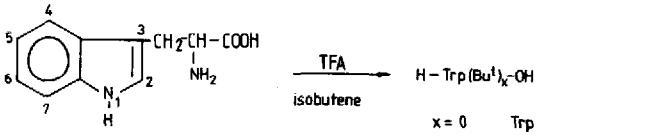
Fig. 4. Analysis of synthetic protected ε-lysine tetrapeptide: BOC-ε-tetra-α-Z-lysine methyl ester. Packing: Hypersil ODS-6 (125 mm × 4 mm). Eluent: methanol–0.01 *M* sodium acetate buffer (pH 4) (80:20, v/v); flow-rate 1.3 cm³/min. Detection: 254, 220 nm. Chromatographic patterns of (A) the crude product, (B) semipurified material and (C) pure compound after recrystallization.

Oxidation: Met → Met(O)

The methionine residues in peptides are very susceptible to oxidation, forming methionine sulphoxide or sulphone. The end-product, α-MSH, was well separated from the sulphoxide impurity at the Met⁴ residue. Fig. 6 shows a chromatogram of the crude synthetic α-MSH. The efficiency of the separation was increased by gradient elution. The sulphoxide derivative has a lower retention time on the RP column than that of the pure hormone because of the slightly higher polarity of the derivative.

Desulphation: Tyr(SO₃H) → Tyr

The synthesis of cholecystokinin peptides involves two problems: methionine oxidation, as mentioned above, and sulphation–desulphation at the tyrosine residue. Thus HPLC analysis of CCK peptides requires very high efficiency because of the presence of sensitive residues (sulphated Tyr and Met). By means of our isocratic system, both fragments and side-products were separated. Table IV lists the retention



x = 0	Trp
x = 1	2-Bu^t-Trp
x = 2	2,5-di-Bu^t-Trp
x = 3	2,5,7-tri-Bu^t-Trp

Scheme A. *tert.*-Butylation of tryptophan.

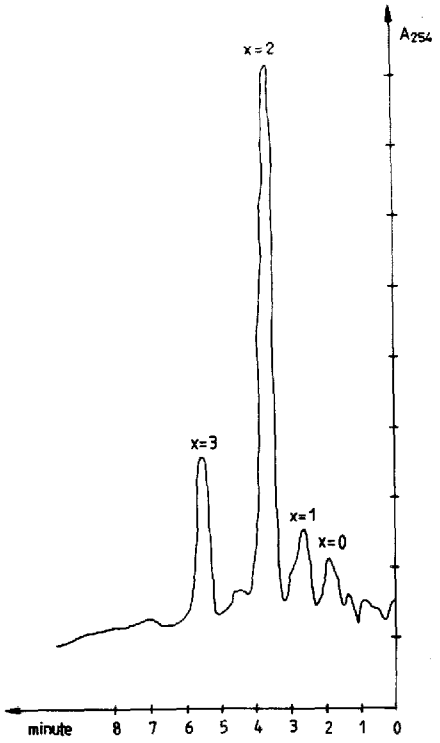


Fig. 5. Separation of *tert.*-butyl derivatives of tryptophan containing up to three *tert.*-butyl groups (as stated and shown in Scheme A). Packing: Hypersil SAS-5 (125 mm × 4 mm). Eluent: methanol–water–acetic acid (84:14:2, v/v/v); flow-rate 1.1 cm^3/min. Detection: 254 nm.

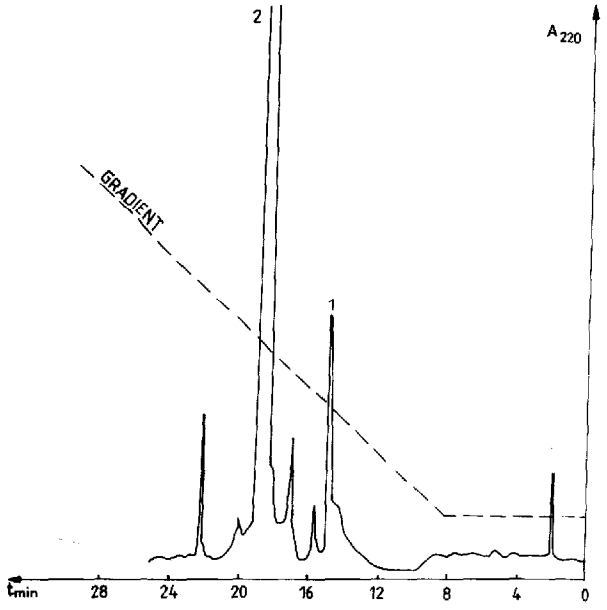


Fig. 6. Purity control of 10 μg synthetic α-MSH. Packing: Hypersil ODS-6 (125 mm × 4 mm). Eluents: A, acetonitrile–water–trifluoroacetic acid (20:80:0.1); (B), acetonitrile–water–trifluoroacetic acid (60:40:0.1, v/v/v). Gradient: 1% B per minute; flow-rate 1.4 cm^3/min. Detection: 220 nm. Peaks: 1 = ^{4}Met(0) α-MSH; 2 = α-MSH.

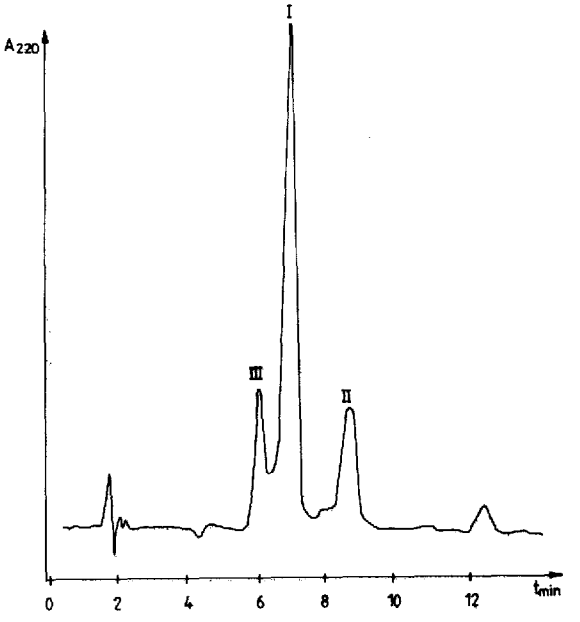


Fig. 7. Chromatogram of synthetic CCK octapeptide. Packing: μBondapak C_{18} (300 mm × 3.9 mm, Waters). Eluent: acetonitrile–0.01 *M* ammonium acetate buffer (pH 4.2) (25:75, v/v); flow-rate 2 cm^3/min. Detection: 220 nm. Peak I corresponds to CCK-OP. The peaks at 6.1 and 8.7 min correspond to the impurities Met(O)-CCK-OP(III) and des(SO H)CCK-OP(II)

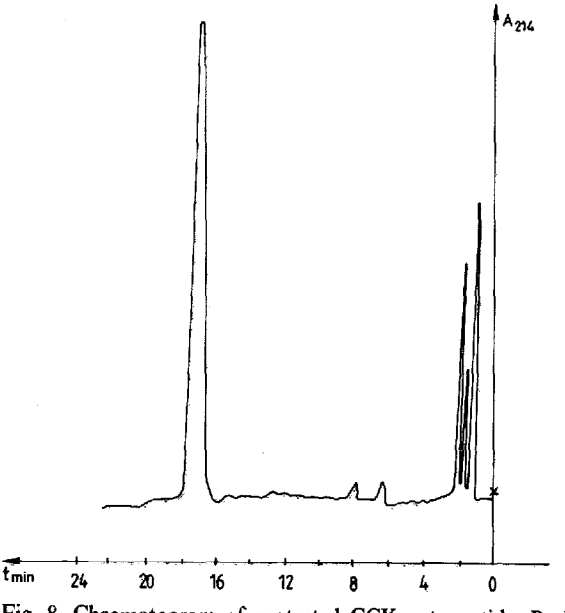


Fig. 8. Chromatogram of protected CCK octapeptide. Packing: Hypersil ODS-6 (250 mm × 4 mm). Eluent: acetonitrile–0.01 *M* sodium acetate buffer (pH 4) (15:85, v/v); flow-rate 1.3 cm^3/min. Detection: 214 nm. The peak at 16.7 min corresponds to BOC-Asp(OBu^t)-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2, the other peaks are impurities.

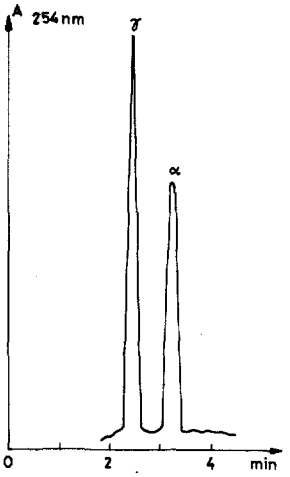


Fig. 9. Chromatographic pattern of aminoethylglutamine–isoglutamine transpeptidation (see Scheme B). Packing: Hypersil ODS-6 (125 mm × 4 mm). Eluent: methanol–0.01 *M* sodium acetate buffer (pH 4) (70:30, v/v); flow-rate 1.2 cm^3/min. Detection: 254 nm.

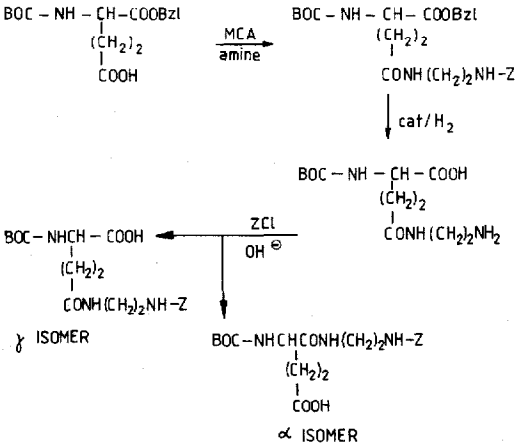
TABLE IV

RETENTION DATA OF CCK PEPTIDES ON C_{18} COLUMNS

SE = Sulphate ester.

Peptide	t_R *(min)*	k'	*Eluent*
*Hypersil ODS (250 mm × 4 mm)**			
BOC-CCK8-SE	16.7	15.6	Acetonitrile–0.01 *M* sodium
BOC-CCK7-SE	2.8	1.8	acetate (pH 4) (15:85)
CCK8-SE	12.1	5.66	Acetonitrile–0.01 *M*
CCK7-SE	16.2	8.0	triethylammonium
CCK6	14.2	6.88	phosphate (pH 6.5)
*μBondapak (300 mm × 3.9 mm)***			(25:75)
CCK8-SE	13.2	5.0	
Met(O)-CCK8-SE	11.8	4.3	
CCK8-SE	6.85	3.03	Acetonitrile–0.01 *M*
CCK8	8.7	3.53	ammonium acetate
Met(O)-CCK8-SE	6.1	2.60	(pH 4.2) (25:75)
*Hypersil ODS (125 mm × 4 mm)****			
BOC-CCK4	6.6	5.60	Acetonitrile–0.01 *M* phosphate
BOC-CCK4-PEA	11.1	10.10	(pH 7.5) (30:70)
BOC-Tyr(Br_2)^1-Leu^4-CCK6	18.8	17.8	Methanol–0.01 *M* phosphate
BOC-Tyr(Br_2)-Gly-OH	2.8	1.8	(pH 2.1)–acetonitrile
Leu^2-CCK4	1.8	0.8	(30:60:40)

* Flow-rate: 1.1 cm^3/min.
** Flow-rate: 1.3 cm^3/min.
*** Flow-rate: 2.0 cm^3/min.



Scheme B. Transpeptidation in the synthesis of the glutamine derivative.

times of some free and protected CCK peptides on Hypersil ODS. Figs. 7 and 8 show the analytical results.

The CCK octapeptides are eluted in the following order: oxidized, sulphated and desulphated compounds (Fig. 7), consistent with their hydrophobicities. The BOC- and OBu^t-protected derivatives present as impurities showed the expected increase in retention times (Fig. 8).

Transpeptidation

The difference in retention times between α and ω isomers makes it possible to detect transpeptidation side reactions in peptide synthesis[10]. The $\alpha \rightarrow \gamma$ transpeptidation occurring in glutamyl peptides and the $\alpha \rightarrow \beta$ shift in aspartyl peptides result in isopeptide formation.

Török *et al.*[13] synthesized biologically active peptides containing basic non-proteinogenic amino acids derived from glutamic acid. They used *tert.*-butyloxycarbonyl α- and γ-benzyl-L-glutamate as starting materials, from which the amide analogues were synthesized by mixed carbonic acid anhydride (MCA) methods with monobenzyloxycarbonylethylenediamine (Scheme B). The BOC-protected derivatives of the two amide analogues were obtained by catalytic hydrogenolysis. In the last step, the protected α analogue was produced in larger quantity by acylation with isobutyl chloroformate, while in the case of the γ analogue, aminoethylglutamine and isoglutamine derivatives were obtained because of transpeptidation. These gave baseline separations as shown in Fig. 9.

The chromatographic data for such critical peptide separations were summarized in our previous paper[10], chiefly for glutamyl and aspartyl dipeptides. In our experience, 5–20% of the transpeptidation rates was found based on baseline RP-HPLC separations. In general, 0.1–0.2% of the related isopeptide could be determined in the α isomer by our method.

Racemization

Racemization results in diastereomeric peptide formation and these peptides

TABLE V

RETENTION TIMES OF Pro-Gly CYCLOPEPTIDES

Column: Hypersil ODS, 125 mm × 4 mm. Detection: 215 nm. Eluent: methanol–water (15:60); flow-rate: 1.3 cm^3/min.

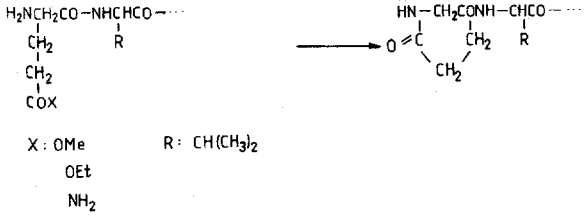
Cyclopeptide	t_R *(min)*	k'
Hexa (n = 1)	4.2	4.2
Hepta	4.4	4.5
Octa (n = 2)	5.0	5.2
Deca (n = 3)	4.6	4.7

can be separated under normal chromatographic conditions. Thus racemization is easily detectable by HPLC[14]. In our Hypersil ODS system, the stereoisomeric Z-Phe-Orn dipeptides (LL, DD or DL compounds) could be separated directly[10]. Alternatively, racemization can be determined by measuring the D-amino acid content, or D/L amino acid ratios after peptide hydrolysis by Marfey's method[9] using 1-fluoro-2,4-dinitrophenyl-5-L-alanine-amide as derivatizing agent. Isocratic methods to separate these amino acid derivatives were developed in our laboratory on Hypersil ODS columns. For example, the Z-protected DL-Ala-DL-Ala-DL-Ala-Leu-active ester (pentachlorophenyl ester), synthesized by a stepwise method with hydrolysis of the methyl ester, showed 18% racemization. The determination was based on the isocratic separation of the D- and L-Leu and D- and L-Ala derivatives with methanol–acetonitrile–0.02 M sodium acetate (pH 4) (35:10:60, v/v) as buffer, flow-rate 0.8 cm^3/min.

Cyclization, N-terminal ring closure

As the linear peptide chain and the cyclic peptide skeleton show different chromatographic behaviours, the side-products can be separated from the synthetic cyclopeptides. The ring number is not a major determinant of retention times on RP columns. The retention data for Pro-Gly cyclopeptides, summarized in Table V, indicate only very small differences in t_R. It is well known that peptide chains with glutamyl residues at the N-terminus preferentially form pyroglutamyl residues as a consequence of ring closure (Scheme C).

For example, the production of Glu(OMe)-ValOMe gives free acid and pyroglutamyl derivatives. The major product and these derivatives could be differentiated by HPLC on a Chromspher silica column (Table VI).



Scheme C. Pyroglutamyl-ring formation.

TABLE VI

RETENTION TIMES OF PYROGLUTAMYL DERIVATIVES

Column: Chromspher Sil, 6 μm, 250 mm × 4 mm. Eluent: methanol–acetonitrile–0.01 *M* sodium acetate/acetic acid (pH 4) (7:7:6, v/v/v); flow-rate 0.8 cm^3/min. Detection: 220 nm.

Peptide	t_R *(min)*	*k'*
Z-pGlu-Val-OMe	3.6	1.0
pGlu-Val-OMe	3.9	1.1
Glu(OH)-Val-OMe	4.0	1.2
Glu(OMe)-Val-OMe	7.4	3.1

The reported data demonstrate the usefulness of HPLC for monitoring a variety of side reactions in peptide synthesis.

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REFERENCES

1 I. Molnár and Cs. Horváth, *J. Chromatogr.*, 142 (1977) 623.
2 W. S. Hancock, C. A. Bishop, R. L. Prestige, D. R. K. Harding and M. T. W. Hearn, *J. Chromatogr.*, 153 (1978) 391.
3 J. E. Rivier, *J. Liq. Chromatogr.*, 1 (1978) 343.
4 E. C. Nice and M. J. O'Hare, *J. Chromatogr.*, 162 (1979) 401.
5 W. S. Hancock, *CRC Handbook of HPLC for the Separation of Amino Acids, Peptides and Proteins*, Vols. I, II, CRC Press Boca Raton, FL, 1984.
6 M. Bodanszky, in E. Gross and J. Meienhofer (Editors), *The Peptides*, Vol. 5, Academic Press, New York, 1983, p. 112.
7 L. Kisfaludy, in I. Z. Siemion and G. Kupryszewsky (Editors), *Peptides 1978*, Wroclaw Univ. Press, Wroclaw, 1979, pp. 25–40.
8 L. Kisfaludy, in A. Eberle, R. Geiger and T. Wieland (Editors), *Perspectives in Peptide Chemistry*, Karger, Basle, 1981, pp. 58–66.
9 D. Marfey, *Carlsberg Res. Commun.*, 49 (1984) 591–596.
10 Gy. Szókán, G. Kelemen and A. Török, *J. Chromatogr.*, 366 (1986) 283.
11 G. Bárány and R. B. Merrifield, in E. Gross and J. Meienhofer (Editors), *The Peptides*, Vol. 2, Academic Press, New York, 1978, p. 3.
12 J. H. Knox and Gy. Szókán, *J. Chromatogr.*, 171 (1979) 439.
13 A. Török, K. Kovács and Gy. Szókán, *Advances in Liquid Chromatography*, Akademiai Kiado, Budapest, 1986, in press.
14 D. S. Kemp, in E. Gross and J. Meienhofer (Editors), *The Peptides*, Vol. 1, Academic Press, New York, 1977.