Journal of Chromatography, 366 (1986) 283–292
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 788

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF ISOPEPTIDES*

GYULA SZÓKÁN* and GABRIELLA KELEMEN
Institute of Organic Chemistry, Eötvös University, Muzeum krt. 4/B, H-1088 Budapest (Hungary)
and
ANGELA TÖRÖK
Department of Medical Chemistry, University Medical School, Dóm tér 8, H-6720 Szeged (Hungary)
(First received March 13th, 1986; revised manuscript received May 12th, 1986)

SUMMARY

High-performance liquid chromatographic (HPLC) methods are described for (1) the structural analysis of clavicepamines, their analogues and branched-chain polypeptides, (2) the analysis in synthetic stages of isopeptides (analysis of half-protected derivatives and purity control of active esters) and (3) the differentiation between α- and iso-peptides (such as α- and γ-glutamyl peptides). Pre-column derivatization was used to label the free amino groups for the structural investigation. Dansylated and hydrolysed isopeptides were analysed by HPLC methods based on isocratic separation of α-, ε- and bis-Dns-lysines using Hypersil, ODS-Hypersil and Partisil PAC columns. For the analysis of peptide active esters, an RP-HPLC method was developed, with methanol–acetonitrile–water mobile phases containing an acidic buffer. Peptides containing α-glutamyl residues were analysed for γ-isomer content in the same system.

INTRODUCTION

Isopeptides have the bonding amide group at the ω-position and not between the α-amino and α-carboxyl amino acids forming the peptide. They can exist in the case of multi-functional amino acids such as Lys, Orn, Arg, Glu and Asp. Naturally occurring isopeptides of Lys are rare, being found in some peptide antibiotics, in bacterial cell walls and in cross-linkage structures of several proteins[1]. Recently, clavicepamines containing lysine isopeptides as fundamental structural units and ε-polylysine were isolated from natural sources[1]. The well known synthetic branched polypeptide antigens and immunoadjuvants contain some isopeptide structures[2]. The metabolism of peptides, synthetic peptide couplings and their side reactions also lead to isopeptide formation[3].

* This paper was presented at the *5th International Symposium on HPLC of Proteins, Peptides and Polynucleotides, Toronto, November 4–6, 1985*. The majority of the papers presented at this Symposium have been published in *J. Chromatogr.*, Vol. 359 (1986).

0021-9673/86/$03.50

Numerous recent studies have documented the advantages of HPLC in peptide analyses[4,5]. In this paper we demonstrate the potential usefulness of RP-HPLC methods for the structural analysis of isopeptides, the differentiation of α- and isopeptide bonds, the exact determination of a peptide network, monitoring of synthetic stages, the analysis of free, protected and half-protected fragments and metabolites and the identification of side products.

EXPERIMENTAL

Materials

The peptides investigated were synthesized by the Polypeptide Research Group of the Hungarian Academy of Sciences, Budapest, the Institute of Organic Chemistry, Eötös University, Budapest, and the Department of Medical Chemistry, University Medical School of Szeged, Szeged, Hungary. The abbreviations used follow the rules of the IUPAC–IUB Commission on Biochemical Nomenclature[6]. Abbreviations (except for the three-letter codes of the common amino acids) are as follows:

Dns-Cl	dansylchloride	5-dimethylaminonaphthalene-1-sulphonylchloride
Z	$C_6H_5CH_2O-\underset{\displaystyle O}{\overset{\displaystyle \|}{C}}=O$	benzyloxycarbonyl
Bzl	$C_6H_5CH_2-$	benzyl
ONp	$p\text{-}NO_2-C_6H_5-O-$	*p*-nitrophenyl-ester
BOC	$(CH_3)_3C-O-\underset{\displaystyle O}{\overset{\displaystyle \|}{C}}-$	*tert.*-butoxycarbonyl

The preferred nomenclature for isopeptides follows the recommendation of Plessing *et al.*[7]:

α–HNCHCO–	Dns–Lys–OH	α-dansyllysine
$(CH_2)_4$	H–Lys–OH	Lys(Dns) ε-dansyllysine
εNH–	Dns	
α–Lys–	Dns–Lys–OH	Dns–Lys(Dns) bis-dansyllysine
ε	Dns	

AA, amino acid; AA-Lys, α-peptide; Lys(AA), ε-peptide; AA-Lys(AA), α,ε-peptide.

The peptides investigated are listed in Tables I-IV.

The peptides were characterized chromatographically by peak numbers, retention times, capacity factors and quantitative analysis. Derivatization with dansyl chloride (Dns-Cl) Pierce, Rockford, IL, U.S.A.) was carried out according to the method of Hartley[8]. Solutions of standard dansyl amino acids (Pierce) and of hydrolysates were prepared in methanol prior to liquid chromatography. α-Dansyllysine standard was prepared from ε-Z-Lys and ε-dansyllysine was prepared from α-Z-Lys, followed by hydrolysis in our laboratory.

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 1011 loop injector; Rheodyne, Berkeley, CA, U.S.A.). The column effluents were monitored at 215, 254 or 280 nm, depending on the derivatizing reagent, protecting groups and amino acid residues. The packing materials were Hypersil, ODS-Hypersil (Shandon Southern Products, Runcorn, U.K.) and Partisil 10-PAC (Whatman, Clifton, NJ, U.S.A.).

Columns (250 × 4 and 125 × 4 mm I.D.) were made of internally polished stainless steel. The columns were packed either by the balanced density method (Hypersil) or by the slurry method (ODS-Hypersil); the Partisil 10-PAC column was ready-packed by the manufacturer.

All solvents were of analytical-reagent grade. The mobile phases are listed in Tables II–IV. The chromatograph was operated isocratically at ambient temperature and the mobile phase flow-rates were between 0.6 and 1.2 cm^3/min. Peaks were recorded on a Type OH-814/1 chart recorder (Radelkis, Budapest, Hungary). "t_0" was measured as the solvent peak.

Thin-layer chromatography

TLC was carried out on Merck silica gel plates (Kieselgel 60, Art 5553, 80 × 40 mm and 20 × 8 cm), using the developing solvents listed in Figs. 1 and 2. The spots were rendered visible under UV light and by treatment with iodine vapour or, after chlorination, by use of potassium iodide–*o*-toluidine spray reagent.

Identification of peptide bonds

To determine the types of peptide bonds in the recently isolated clavicepamines, a strategy involving dansylation (with Dns-Cl), hydrolysis (in 6 *N* HCl) and HPLC separation of Dns-amino acids was applied.

HPLC was used to identify and quantitate Dns-amino acids after pre-column derivatization[9,10] with Dns-Cl at the free amino groups of the N-terminal and in the side chain of peptides and hydrolysis in 6 *N* HCL, as described[11]. Separation of α-Dns-Lys peptides and of α-, ε- and bis-Dns-lysines was achieved isocratically on an ODS-Hypersil column with methanol–phosphate buffer as the eluent, on a Partisil 10-PAC column with acetonitrile–water containing 1% acetic acid as the eluent and on a Hypersil column with ethyl acetate–isopropanol–concentrated ammonia solution as the eluents, based on preliminary thin-layer chromatographic (TLC) results[12].

RESULTS AND DISCUSSION

Optimization and the use of different systems minimized the disturbing effect of Dns-OH and Dns-NH_2 side products in quantitative measurements. In system A Dns-OH is eluted at the front, in system B it is very far from the others and in system C (silica column) it elutes together with DnsNH_2 at the beginning of the chromatographic pattern. The combination of these three systems provides the opportunity for quantitative measurements of all Dns-amino acids without using gradient elution.

The chromatographic patterns (Fig. 1) of the ε-Lys peptides revealed sharp

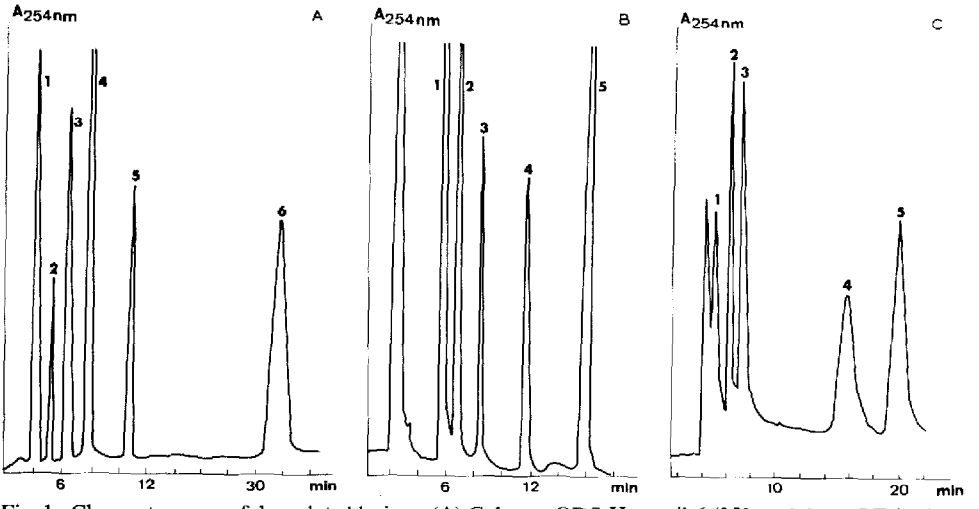


Fig. 1. Chromatograms of dansylated lysines. (A) Column, ODS-Hypersil-6 (250 × 4.6 mm I.D.); eluent, methanol–0.03 *M* phosphate buffer (pH 7.3) (55:45, v/v); flow-rate, 0.6 cm^3/min; detection, 254 nm. 1 = Dns-OH; 2 = Dns-Ala; 3 = Dns-Lys; 4 = Lys-(Dns); 5 = Dns-Phe; 6 = Dns-Lys-(Dns). (B) Column, Partisil 10-PAC (250 × 4.6 mm I.D.); eluent, acetonitrile–water–acetic acid (35:65:1, v/v); flow-rate, 1 cm^3/min; detection, 254 nm. 1 = Lys(Dns); 2 = Dns-Lys(Dns); 3 = Dns-Lys; 4 = Dns-Ala; 5 = Dns-OH. (C) Column, Hypersil-6 (250 × 4 mm I.D.); eluent, ethyl acetate–isopropanol–25% ammonia solution (50:40:15, v/v); flow-rate, 0.9 cm^3/min; detection, 254 nm. 1 = Dns-NH_2; 2 = Dns-Lys(Dns); 3 = Dns-OH; 4 = Lys-(Dns); 5 = Dns-Lys.

peaks corresponding to α- and bis-Dns-Lys, whereas α-peptides showed ε- and bis-Dns-Lys peaks and poly-(ε-Lys) gave α-Dns-Lys. From these patterns, the type of peptide bond could be deduced (see Table I). The different types of lysine bonds were also evaluated quantitatively[11] by a method used previously to determine the primary structure of di-, oligo- and poly-isopeptides and to analyse the surface topography of polylysine-based branched-chain polypeptides[11].

TABLE I

IDENTIFICATION OF PEPTIDE BONDS ON THE BASIS OF HPLC PATTERNS OF DANSYLATED AND HYDROLYSED COMPOUNDS

Peptide	*Peaks*			*Peptide bond (α or ε)*
	Dns-Lys	*Lys(Dns)*	*Dns-Lys(Dns)*	
Lys-Lys	–	+	+	α
Lys(Lys)	+	–	+	ε
Lys(Phe)	+	–	– Dns-Phe	ε
Phe-Lys	–	+	– Dns-Phe	α
Lys(Gly)	+	–	– Dns-Gly	ε
Gly-Lys	–	+	– Dns-Gly	α
poly(α-Lys)	–	+	(+)	α
poly(ε-Lys)	+	–	(+)	ε
poly[Lys(Ala)m]	(+)	+	(+) Dns-Ala	α, ε
Clavicepamines	+		+	ε

Clavicepamines showed only α-Dns-Lys as the major peak, suggesting that Lys ε-amino groups are fully blocked in the peptide bond and that Lys isopeptides are fundamental structural units of clavicepamines (see Table I and ref. 1). Thus, it appears that a similar technique can be used in the structural investigation of other isopeptides, such as ornithine, any α,ω-diaminoalkanoic acid and arginine.

Chromatography of isopeptide intermediates

To confirm the isopeptide structures, ε-oligo- and poly-lysines were synthesized in our laboratory[1] and the synthetic steps were analysed by HPLC. The retention data of the half-protected isopeptides are summarized in Table II.

The retention times of protected amino acids were greater than those of the corresponding Lys or Orn peptide (*e.g.*, compare IV and VII with III and VIII in Table II), the magnitude of the increase depending on the number of protecting

TABLE II

RETENTION DATA OF THE HALF-PROTECTED ISOPEPTIDES AND SOME AMINO ACID DERIVATIVES

Column, ODS-Hypersil-6 (125 × 4 mm I.D.); eluent, methanol–0.01 *M* sodium acetate buffer (pH 4.0) (50:50, v/v); flow-rate, 1.1 cm³/min; detection, 254 mm.

	Compound	t_R *(min)*	*k'*
I	Z—Phe—Orn	8.9	3.7
II	Z—D—Phe—D—Orn	8.9	3.7
III	Z—D—Phe—Orn	8.4	3.5
IV	Z—Phe—OH	10.0	4.0
V	Z—Glu—OBzl + Lys	13.2	6.3
VI	Z—Glu—OBzl + Orn	14.2	6.1
VII	Z—Glu—OBzl	22.0	9.0
VIII	Z—Glu(OBzl)—Lys	20.2	10.2
IX	Z—Glu(OBzl)	20.2	8.2
X	Z—Orn(Z)—Lys	16.3	7.2
XI	Z—Orn(Z)—Orn	15.9	7.4
XII	Z—Orn(Z)	17.3	6.9
XIII	Z—Lys(Z)—Orn	21.9	9.7
XIV	Z—Lys(Z)	25.7	10.3
XV	Z—Arg(Z_2)—Lys	13.1	6.7
XVI	Z—Arg	2.3	0.2
XVII	Z—Arg(Z_2)	15.2	6.6

groups (see Fig. 2, XVI and XVII). The peptide analogues had similar t_R values (*e.g.*, V and VI, X and XI). The retention times of α-peptides are different from those of the same ω-peptides (*e.g.*, V and VIII), indicating their non-identical properties.

The enantiomeric peptides have the same t_R values (*e.g.*, I and II). It was possible to separate the diastereomeric peptides (*e.g.*, II and III) so that racemization was detectable. Fig. 3 shows that preparative purification can be controlled by this system and that the rate of isopeptide couplings can be measured. Further, impurities can be detected in the end-products (see Fig. 3).

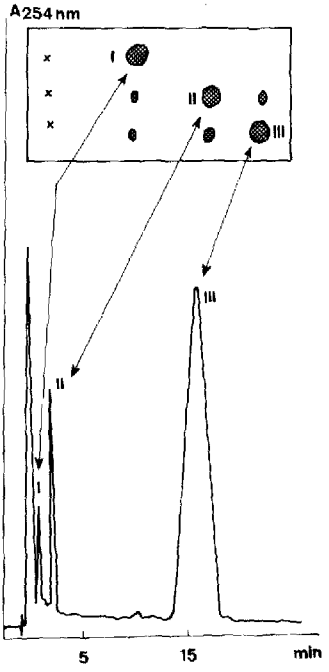


Fig. 2. Analysis of Z-protected Arg derivatives. Comparison of HPLC peaks to TLC spots. HPLC: column, ODS-Hypersil-6 (125 × 4 mm I.D.); eluent, methanol–0.01 *M* ammonium acetate buffer (pH 4) (50:50, v/v); flow-rate, 1.1 cm³/min. TLC: silicagel 60 (Merck), *n*-butyl alcohol–acetic acid–water (4:1:1, v/v). *I* = Z-Arg-OH (XVI); *II* = Z-Arg(Z)-OH; *III* = Z-(Arg(Z_2)-OH) (XVII).

The identification and quantitation of peptide fragments and starting amino acid derivatives assumes importance in the light of their potentially confounding role in further peptide synthetic steps and in peptide end-product analyses. During RP-LC the protecting groups used (*e.g.*, Z as amino and benzyl ester as carboxyl-protecting groups) generally increase the retention times owing to their higher hydrophobicity (XVI and XVII). On the other hand, these groups give easily detected chromophores, allowing more precise and more sensitive identification of any amino acid derivatives.

In the case of Arg peptides (XV), the number of Z groups (1, 2 or 3) at the Arg residues increases the retention times on the RP column. The TLC data and the HPLC pattern are related in Fig. 2, so that synthetic stages can be monitored by both means.

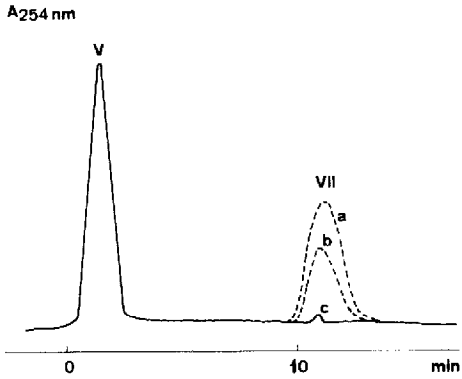


Fig. 3. Analysis of end-product and purification steps in the reaction Z-Gly-OBzl + Lys → Z-Glu-OBzl-Lys. (a) Pattern of crude product; (b) after first crystallization; (c) after tetrahydrofuran extraction. For chromatographic parameters, see Table II.

Fig. 3 gives another example of practical utilization. The HPLC pattern suggested the possibly that the crude half-protected synthetic Lys-isopeptides could be purified. On the basis of retention times, the residue of the starting materials [Z_x-Arg, Z-Glu-(OBzl)] could be extracted by tetrahydrofuran from the end-product, which has a more polar character because of the presence of free Lys amino and carboxyl groups.

The ω-polypeptides of diamino monocarboxylic acids were synthesized via polycondensation[1]. The key intermediate active esters, which are very sensitive to

TABLE III

RETENTION TIMES OF ACTIVE ESTERS OF LYSINE ISOPEPTIDES

Columns, ODS-Hypersil-6 (125 × 4 mm I.D.); eluent, methanol–0.02 *M* sodium acetate buffer (pH 4.0)–acetonitrile (40:30:30, v/v); flow-rate, 1 cm^3/min; detection, 254 nm.

Isopeptide p-nitrophenyl ester	t_R *(min)*	k'
Z — Lys — ONp BOC	3.8	0.9
Z — Lys — ONp Z — Lys BOC	8.8	2.2
Z — Lys — ONp Z — Lys Z — Lys BOC	18.2	8.1

TABLE IV

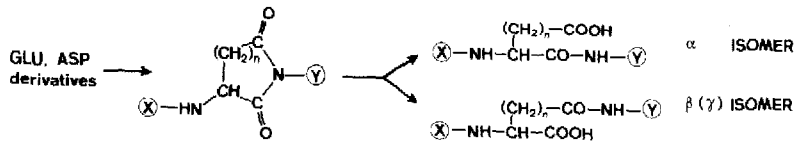
CHROMATOGRAPHIC DATA FOR ISOMERIC DERIVATIVES

Derivative	*Packing*	*Eluent (v/v)*	t_R *(min)*		k'		R_s
			α	β (γ)	α	β (γ)	
Z-Glu (OBzl)	ODS-Hypersil***	Methanol–0.01 *M* sodium acetate buffer (pH 4) (50:50)	22.0	20.0	3.0	8.3	1.80
Z-Glu (OBzl)Lys	ODS-Hypersil	As above	20.2	13.2	10.2	6.3	3.20
Z-Asp-Phe-NH_2*	RP-18 (Knauer)	Methanol–water–acetic acid (60:40:2)	8.0	9.0	2.0	3.5	1.25
BOC-Glu (EDAZ)**	ODS-Hypersil	Methanol–0.01 *M* sodium acetate buffer (pH 4) (70:30)	3.3	2.4	2.3	1.4	1.76

* Personal communication from B. Penke (Szeged). Column dimensions, 250 × 4 mm I.D.

** EDA-Z = $-CH_2CH_2-NH-Z$.

*** ODS-Hypersil columns dimensions, 125 × 4 mm I.D.



Scheme 1. Isopeptides and transpeptidation.

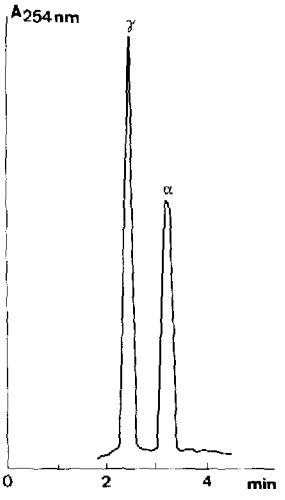


Fig. 4. Chromatographic pattern of aminoethylglutamine–isoglutamine transpeptidation. Column, ODS-Hypersil-6 (125 × 4 mm I.D.; eluent, methanol–0.01 *M* sodium acetate buffer (pH 4) (70:30, v/v); flow-rate, 1.2 cm^3/min; detection, 254 nm.

any nucleophilic attack, were analysed by HPLC using the ternary eluent system with an acidic buffer throughout (pH 4.0–4.5). Representative chromatographic data for lysine isopeptide-*p*-nitrophenyl esters are shown in Table III.

Transpeptidation

The difference in retention times between α- and ω-isomers made it possible to detect some side reactions in peptide synthesis. The α → γ transpeptidation that occurs in glutamyl peptides and the α → β shift in aspartyl peptides (see Scheme 1) results in isopeptide formation[3]. According to the data in Table II, several critical peptide separations were achieved by RP-HPLC, chiefly with glutamyl and aspartyl dipeptides, in which 0.1–0.2% of isopeptide content was determined in the α-isomer. Thus rates of transpeptidation were easily monitored. Chromatographic data are summarized in Table IV. Aminoethylglutamine and isoglutamine derivatives (Table IV) also gave baseline separations (see Fig. 4). With N-protected α- and γ-glutamic acid benzyl esters (VII and IX), the impurities could be determined after preparation in each other (see Fig. 5).

The reported data demonstrate the usefulness of RP-HPLC for monitoring different stages in the synthesis of isopeptides, and suggest that applications of HPLC systems extend well beyond the purity control of peptides.

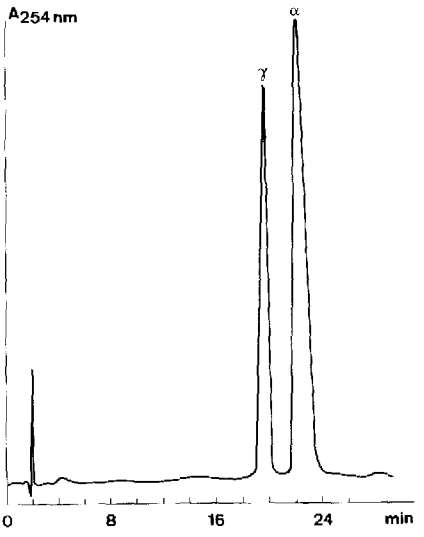


Fig. 5. Separation of N-protected α- and γ-glutamic acid benzyl esters. For chropmatographic data, see Table II.

ACKNOWLEDGEMENTS

The authors thank Dr. B. Penke and Dr. E. Tyihák for reagents and discussions and M. Hoffman for editorial assistance. Sincere thanks are expressed to M. Almás for her excellent technical assistance with the HPLC measurements and with the synthetic work.

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