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Monitoring of optical isomers of β-methylphenylalanine in opioid peptides

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ABSTRACT

D,L-*erythro*- and D,L-*threo*-β-methylphenylalanines were synthesized and incorporated into the delta opioid receptor-selective deltorphin C peptide by solid-phase peptide synthesis. The enantiomeric composition of β-methylphenylalanine and the diastereomeric composition of the peptide were determined. To distinguish exactly between the two enantiomeric pairs of β-methylphenylalanine, different liquid, gas and thin-layer chromatographic methods are suggested, while the four diastereomers of [β-MePhe^3]deltorphin C can be identified and separated conveniently by high-performance liquid chromatography.

INTRODUCTION

Diastereomers of peptides with biological activity often exhibit antagonistic or agonistic properties which differ greatly from each other. Diastereomeric peptides, isomers in which one or more asymmetric centres have opposite configurations, may have similar or different physico-chemical or biological properties.

In the synthesis of receptor-selective peptides, unusual racemic amino acids are often used. It is therefore very important to develop effective chromatographic methods for the separation of the mixture of diastereomeric peptides and for the characterization and identification of the optical isomers of the amino acids in the pure peptide.

Several papers and reviews covering the development of enantioselective separations have been published. High-performance liquid chromatographic (HPLC) methods can be divided into three main groups: direct separation on chiral columns [1–3], separation on achiral columns with chiral mobile phase additives [3–5] and separation of the diastereoisomers formed by precolumn derivatization with chiral reagents [3,6–17].

The gas chromatographic (GC) separation of amino acid enantiomers is based on precolumn derivatization and analysis on a chiral capillary column [18–21]. The thin-layer chromatography (TLC) of enantiomeric compounds has been possible since Chiralplate (a plate containing a chiral selector) became available [22–27].

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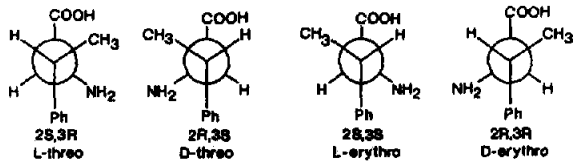

Fig. 1. Structures of four optical isomers of β-methylphenylalanine.

This paper describes the separation of the β-methylphenylalanine (β-MePhe) isomers by different chromatographic methods. β-MePhe has two chiral centres, and four stereoisomers are possible (Fig. 1). To separate these four isomers, different chromatographic methods were applied. HPLC was carried out with pre-column derivatization with acetic anhydride, *o*-phthaldialdehyde (OPA)–mercaptopropionic acid and 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (FDAA; Marfey reagent). GC was performed with the N-trifluoroacetylated isobutyl ester derivative and MS detection. For TLC analysis, the free amino acid was used.

The β-MePhe isomers were built into a peptide, [β-MePhe^3]deltorphin C (Tyr-D-Ala–β-MePhe–Asp–Val–Val–Gly–NH_2) and the diastereomers were separated by HPLC. Methods were developed to check the optical purity of the peptide by HPLC and TLC. The former is also suitable for preparative purification.

EXPERIMENTAL

Chemicals and reagents

β-MePhe was prepared by a slightly modified method of Kataoka *et al.* [28]. Pure *erythro*-(2*S*,3*S*)- and -(2*R*,3*R*)-β-MePhe · HCl were easily obtained from N-acetyl-β-MePhe by hydrolysis with 3 *M* HCl and crystallization from water. *threo*-(2*S*,3*R*)- and -(2*R*,3*S*)-β-MePhe · HCl were obtained from the mother liquor by repeated crystallization. Structures were confirmed by NMR spectroscopy; the chemical shifts and coupling constants are in agreement with the results of Tsuchihashi *et al.* [29] and Hruby *et al.* [30]. (2*S*,3*S*)-, (2*R*,3*R*)-, (2*S*,3*R*)- and (2*R*,3*S*)-β-MePhe · HCl were prepared by Kataoka *et al.*'s method [28] and used as standards.

Deltorphin C analogues were synthesized by solid-phase peptide synthesis by using Boc chemistry, starting from 4-methylbenzhydrylamine resin [30]. Two syntheses were carried out, using Boc-D,L-*erythro*-β-MePhe or Boc-D,L-*threo*-β-MePhe. Both syntheses gave crude diastereomeric peptides. The mixture of diastereomeric peptides were separated by HPLC using a Vydac semi-preparative column and an acetonitrile–aqueous trifluoroacetic acid (TFA) mobile phase system. Each crude peptide gave two main peaks, corresponding to the two diastereomeric peptides, and were isolated as white powders. The peptides containing optically pure β-MePhe were identified by an enzymatic method [31] using L-amino acid oxidase or other methods described below.

FDAA was purchased from Sigma (St. Louis, MO, USA), 4-methylbenzhydrylamine resin from Bachem Feinchemikalien (Bubendorf, Switzerland), trifluoroacetic anhydride (99+%) from Janssen Chimica (Beerse, Belgium) and HPLC-grade solvents (methanol and acetonitrile) and other reagents of analytical-reagent grade from Merck (Darmstadt, Germany).

Buffers were prepared with doubly distilled water and further purified by pumping through a 10-μm filter. The pH was adjusted with phosphoric acid (phosphate buffer), acetic acid (acetate buffer) or sodium hydroxide.

TLC was performed on commercially available precoated DC Fertigplatten Kieselgel F_{254} plates (Merck) and on a Chiralplate (Macherey–Nagel, Düren, Germany), which is coated with a reversed-phase silica gel and impregnated with a chiral selector (2*S*,4*R*,2′*RS*)-N-(2′-hydroxydodecyl)-4-hydroxyproline and copper(II) ions. Spots were revealed by spraying with 0.1% ninhydrin reagent.

Apparatus

The LC system consisted of a liquid chromatographic pump (Merck–Hitachi, Tokyo, Japan), a Model 308 variable-wavelength UV spectrophotometric detector (Labor MIM, Budapest, Hungary) and an HP 3395 integrator (Hewlett-Packard, Waldbronn, Germany). The columns used were Vydac 218TP104 C_{18} (250 × 4.6 mm I.D.), 10-μm particle size, a semi-preparative Vydac 218TP1010 C_{18} (250 × 10 mm I.D.), 10-

μm particle size (Separations Group, Hesperia, CA, USA), and Nucleosil $10C_{18}$ (250 × 4.6 mm I.D.), 10-μm particle size (Macherey–Nagel).

GC was performed on a Model 3200 GC–MS system (Finnigan, San Jose, CA, USA) connected to an on-line Finnigan Incos data system, which controlled repetitive data scanning of the mass spectrometer and acquired and processed all the data obtained. The samples were analysed on a Chirasil-L-Val III (Alltech, Deerfield, IL, USA) capillary column (50 m × 0.25 mm I.D.). The operating conditions were injection port temperature 250°C, carrier gas helium [0.5 atm inlet pressure (1 atm = 101 325 Pa)] and splitting ratio 1:30. The column temperature was varied in the range 65–220°C, depending on the nature of the amino acids.

Quantitative amino acid analysis was performed on an HP1090 Amino Quant amino acid analyser (Hewlett-Packard). The column used was Hypersil ODS C_{18} (200 × 2 mm I.D.), 5-μm particle size (Shandon Scientific, Runcorn, Cheshire, UK).

^{1}H NMR was performed on a Bruker (Zug, Switzerland) AM 400 spectrometer.

Peptide hydrolysis

The deltorphin C analogues containing different stereoisomers of β-MePhe in position 3 were hydrolysed in 6 *M* HCl at 110°C for 24 h in a Pierce Reacti-vial under argon pressure. The solvent was removed by flushing with argon. The dry sample was then derivatized for GC and LC or used for amino acid analysis.

Derivatization of the amino acid for HPLC

As D,L-*erythro*- and D,L-*threo*-β-MePhe are not separated on an RP-HPLC column, a suitable derivatization procedure is required. In the formation of N-acetyl-β-MePhe, conventional derivatization with acetic anhydride in acidic media leads to racemization. However, on derivatization in an alkaline medium racemization is not observed. The following procedure was used: 2 mg of β-MePhe were cooled in an ice-bath, 350 μl of 1 *M* NaOH were added, the mixture was stirred for a few seconds until the salt had dissolved, 20 μl of acetic anhydride were added and the solution was kept at 0°C for 40 min.

The OPA–mercaptopropionic acid derivatives of D,L-*erythro*- and D,L-*threo*-β-MePhe were formed in the automated Amino Quant system by method of Schuster [32].

FDAA was used for the formation of the four diastereomers with β-MePhe as described by Marfey [12].

Derivatization for GC–MS

The N-trifluoroacetylated isobutyl ester of the β-MePhe (TAB-β-MePhe) was prepared by the reaction with isobutanol in 3 *M* HCl following reaction with trifluoroacetic anhydride [33].

RESULTS AND DISCUSSION

Separation of β-MePhe isomers

In the course of the synthesis of the β-MePhe enantiomers, the first main stage is crystallization of the D,L-*erythro* and D,L-*threo* forms. Separation of the *erythro* and *threo* forms on an RP-HPLC column is not possible. To follow the crystallization, the N-acetyl form of β-MePhe was prepared and analysed. In some instances, resolution could be observed for the non-derivatized D,L-*threo*- and D,L-*erythro*-β-MePhe at higher pH (>6), but the separation was not reproducible. This separation was observed mostly during analysis with new columns, probably owing to the higher efficiency of such columns.

Fig. 2 shows the k' values for the N-acetyl-D,L-*threo* and N-acetyl-D,L-*erythro* forms of β-MePhe at different eluent compositions and pH values. The *threo* form elutes first. Separation of the two forms starts if the methanol concentration is below 20–40%, depending on the pH. The resolution (R_s) also increases with decreasing methanol concentration and with decreasing pH. Higher pH does not increase the separation of the N-acetyl forms, as was observed in some instances with non-derivatized β-MePhe. At low pH, the N-acetylamino acid is more protonated and has a more hydrophobic character and a higher k'. On changing the mobile phase composition, with acetonitrile being used instead of methanol and TFA or sodium acetate instead of

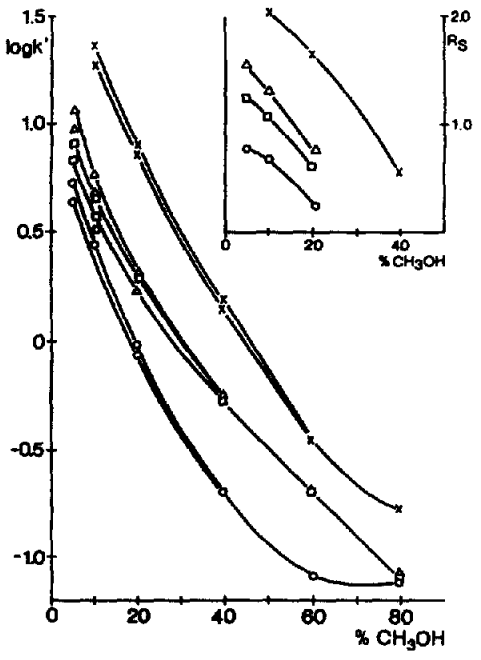

Fig. 2. Plots of capacity factor (k') and resolution (R_s) of D,L-*threo*- and D,L-*erythro*-N-acetyl-β-MePhe *vs.* percentage of methanol in eluent (*threo* elutes first). Column, Nucleosil $10C_{18}$ (250 × 4.6 mm I.D.); buffer, 0.01 *M* KH_2PO_4; detection, UV at 220 nm. pH: × = 2.2; △ = 4.2; □ = 6.5; ○ = 8.

phosphate buffer, the character of the curves was unchanged.

In amino acid analysis, after OPA derivatization, on a Hypersil ODS C_{18} column with acetonitrile–sodium acetate (pH 7.2) as the mobile phase [32], D,L-*erythro*-β-MePhe elutes first and D,L-*threo*-β-MePhe second. The k' value for the D,L-*erythro* form is 9.76 and that for the D,L-*threo* form is 10.55; $R_s = 1.70$.

With FDAA as derivatizing agent, four diastereomers were formed from the four stereoisomers of β-MePhe. The chromatographic behaviour of these diastereomers is not known; FDAA is generally used for the derivatization of amino acids containing one chiral centre. The results in Tables I and II reveal that predominantly the L-*erythro*,*threo*- and D-*erythro*,*threo*-β-MePhe derivatives are resolved. The effects of pH on the capacity factor (k'), selectivity factor (α) and resolution (R_s) were investigated (Table I). In a narrow range of pH around 2, the resolution is good for the L- and D-forms, but the high k' is disadvantageous. At pH 4, α and R_s have lower values. pH 3 seems suitable and in addition the *erythro* and *threo* forms are partially resolved.

The effect of the eluent composition is shown in Table II. A decrease of the organic content of the mobile phase increases the value of k' and improves the separation of the L- and D- forms. At a given mobile phase composition (organic content-to-buffer ratio = 30:70) the k' values depend on the nature of the buffer. The extremely high values of k' in the TFA system are disadvantageous and are probably connected with the pH being lower than 3. Of the three buffers, sodium acetate seems the most suitable

TABLE I

DEPENDENCE OF CAPACITY FACTOR (k'), SELECTIVITY FACTOR (α) AND RESOLUTION (R_s) OF β-METHYLPHENYLALANINE FDAA DERIVATIVES ON pH

Column, Nucleosil $10C_{18}$ (250 × 4.6 mm I.D.); eluent, CH_3CN–0.01 *M* KH_2PO_4 (30:70); detection, UV at 340 nm. α_{L-D} and $R_{s,L-D}$ represent separation of L- and D-forms.

pH of eluent	k'				α_{L-D}	$R_{s,L-D}$
	L-		D-			
	erythro	*threo*	*erythro*	*threo*		
2.0	11.66		18.66		1.60	5.52
3.0	4.32	4.65	8.38	8.98	1.80	5.60
4.0	3.95		5.00		1.27	1.91

TABLE II

DEPENDENCE OF CAPACITY FACTOR, SELECTIVITY FACTOR AND RESOLUTION OF β-METHYLPHENYLALANINE FDAA DERIVATIVES ON ELUENT COMPOSITION

Column, Nucleosil $10C_{18}$ (250 × 4.6 mm I.D.); detection, UV at 340 nm. Buffers: KH_2PO_4 = 0.01 *M* KH_2PO_4 (pH 3); TFA = 0.1% aqueous solution of trifluoroacetic acid; NaOAc = 0.01 *M* sodium acetate (pH 3). α_{L-L} and $R_{s,L-L}$ represent separation of L-*erythro*- and L-*threo*-β-MePhe derivatives; α_{L-D} and $R_{s,L-D}$ represent separtion of L-*threo*- and D-*erythro*-β-MePhe derivatives; α_{D-D} and $R_{s,D-D}$ represent separation of D-*erythro*- and D-*threo*-β-MePhe derivatives.

Composition of eluent	*k'*				α_{L-L}	α_{L-D}	α_{D-D}	$R_{s,L-L}$	$R_{s,L-D}$	$R_{s,D-D}$
	L-		D-							
	erythro	*threo*	*erythro*	*threo*						
CH_3OH–CH_3CN–KH_2PO_4										
30 : 70	4.32	4.65	8.38	8.97	1.08	1.80	1.07	0.63	5.60	0.63
40 : 7 : 53	5.25	5.95	13.33	14.00	1.13	2.24	1.05	1.09	10.54	0.65
CH_3OH–CH_3CN–TFA										
30 : 70	20.00	21.00	27.33	29.00	1.05	1.19	1.06	0.58	2.56	0.54
CH_3OH–CH_3CN–NaOAc										
30 : 70	5.41	5.91	10.80	11.22	1.09	1.82	1.04	0.98	6.38	0.44
10 : 20 : 70	8.83	9.83	24.33	24.33	1.11	2.47	1.00	1.11	9.45	–

as the chromatograms obtained have the best peak shape. The eluents containing two organic modifiers give better α and R_s values. For the separation of L-*erythro,threo* and D-*erythro,threo* derivatives, many systems are suitable. Separation of the *erythro* and *threo* forms is a more difficult problem. For the resolution of L-*erythro* and L-*threo* derivatives, we found some systems having an R_s greater than 1 (Table II and Fig. 3), but the D-*erythro* and D-*threo* derivatives overlapped.

In addition to HPLC, TLC and GC were also applied for resolution of the β-MePhe stereoisomers. The four stereoisomes of β-MePhe were analysed among the other amino acids originating from the hydrolysis of [β-MePhe^3]deltorphin C. The R_F values are given in Table III. On the plate, the elution sequence for the β-MePhe enantiomers was L-*threo,erythro-* and D-*threo,erythro*-β-MePhe. The L-*erythro* and L-*threo* forms gave one spot on the plate, whereas D-*threo-* and D-*erythro*-β-MePhe were resolved well.

For GC, the amino acid was derivatized into the N-trifluoroacetylated isobutyl ester form. With a GC–MS system for analysis, an electron impact (EI) ionization mass spectrum was obtained to confirm the expected structure of the derivative. Fig. 4 shows part of the chromatogram, registered at m/z 218.0 ± 0.5, for the separation of four TAB-β-MePhe derivatives. The elution sequence on a Chirasil-L-Val III column was D-*erythro*, L-*erythro-* D-*threo* and L-*threo* derivatives. The D-*threo* and L-*threo* derivatives could be resolved very well, whereas the D-*erythro* and L-*erythro* forms overlapped.

TABLE III

R_F VALUES OF FOUR ISOMERS OF β-METHYL PHENYLALANINE AND OTHER AMINO ACIDS FROM HYDROLYSED [β-MePhe^3]DELTORPHIN C, COMPARED WITH STANDARD AMINO ACIDS

Chiralplate with acetonitrile–methanol–water (4:1:1, v/v/v).

Amino acid	R_F	
	Hydrolysate	Standard
Tyr	0.63	0.62
L-*erythro*,L-*threo*-β-MePhe	0.58	0.58
Val	0.51	0.51
D-*threo*-β-MePhe	0.48	0.48
Asp	0.44	0.43
D-*erythro*-β-MePhe	0.39	0.40
D-Ala	0.37	0.38
Gly	0.32	0.32

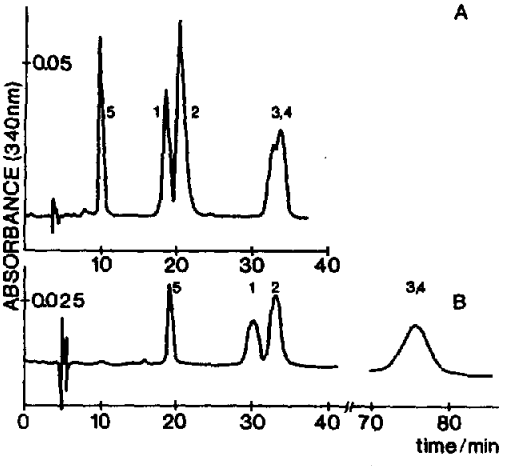

Fig. 3. Chromatograms of β-MePhe FDAA derivatives. Column, Nucleosil $10C_{18}$ (250 × 4.6 mm I.D.); eluent, (A) methanol–acetonitrile–0.01 *M* KH_2PO_4 (pH 3) (40:7:53) and (B) methanol–acetonitrile–0.01 *M* sodium acetate (pH 3) (10:20:70); detection, UV at 340 nm. Peaks: 1 = L-*erythro*; 2 = L-*threo*; 3 = D-*erythro*; 4 = D-*threo*; 5 = unreacted FDAA.

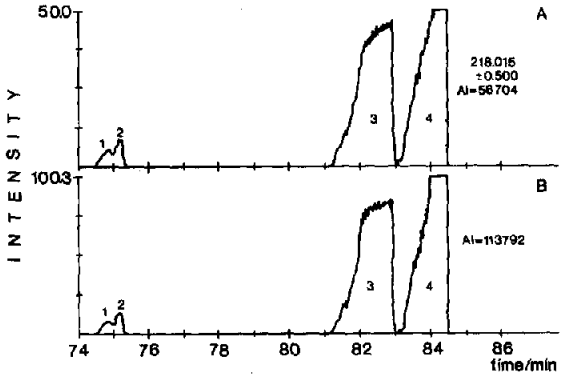

Fig. 4. GC–MS of TAB-β-MePhe derivatives. Column, Chirasil-L-Val III (50 × 0.25 mm I.D.); oven temperature, programmed from 65 to 220°C at 0.5°C/min. (A) Ion chromatogram of mass 218 $[M - CF_3 - CO - NH_2]^+$; (B) total ion chromatogram: 2.5% D,L-*erythro*-β-MePhe, 97.5% D,L-*threo*-β-MePhe. Peaks: 1 = D-*erythro*; 2 = L-*erythro*; 3 = D-*threo*; 4 = L-*threo*.

Separation of diastereomers of [β-MePhe]deltorphin C

The enantiomers of β-MePhe were incorporated into the peptide [β-MePhe^3]deltorphin C. Two separate syntheses were performed with D,L-*erythro*- and D,L-*threo*-β-MePhe. The crude peptide was purified by HPLC on a Vydac column with acetonitrile–0.1% TFA as the mobile phase. A suitable mobile phase system contained about 20% of organic modifier. Each chromatogram had two main peaks. These peaks were identified after separation of the peptide on a semi-preparative Vydac column following hydrolysis or enzymatic digestion. The hydrolysate containing the amino acids was analysed by one of the chromatographic methods mentioned above. In both instances, the first peak on the Vydac column corresponded to the D-*erythro* or D-*threo* isomers of deltorphin C and the second peak to the L-*erythro* or L-*threo* isomers.

HPLC of the [β-MePhe^3]deltorphin C diastereomers with the acetonitrile–TFA system led to the total separation of the four isomers (Fig. 5). On the Vydac column, the sequence of elution was D-*threo*, D-*erythro*, L-*threo* and L-*erythro*. With a 20% organic modifier content, the separation was very satisfactory and the α and R_s values were good. This result indicates that the four diastereomers can be separated and purified on the preparative column.

Analysis of the diastereomers of [β-MePhe^3]deltorphin C was also performed by

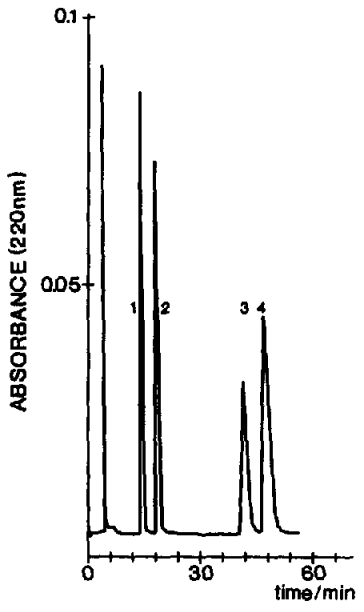

Fig. 5. Chromatogram of [β-MePhe^3]deltorphin C diastereomers Column, Vydac 218TP104 C_{18} (250 × 4.6 mm I.D.); eluent, acetonitrile–0.1% TFA (20:80); detection, UV at 220 nm. Peaks: 1 = D-*threo*; 2 = D-*erythro*; 3 = L-*threo*; 4 = L-*erythro*.

TLC. The results are given in Table IV. With a convention Kieselgel F_{254} plate, the L-*threo* diastereomer can be separated partially from the other three (Table IV, column A). On the Chiralplate, three diastereomers can be resolved (column A'): L-*threo*, D,L-*erythro* (in one spot) and D-*threo*. With both plates these separations

TABLE IV

R_F VALUES FOR THE FOUR ISOMERS OF [β-MePhe^3]DELTORPHIN C

A = Kieselgel with acetonitrile–methanol–water (4:1:1, v/v/v); A' = Chiralplate with acetonitrile–methanol–water (4:1:1, v/v/v); B = Kieselgel with *n*-butanol–acetic acid–water (4:1:1, v/v/v); C = Kieselgel with *n*-butanol–pyridine–acetic acid–water (13:12:3:10, v/v).

Peptide	R_F			
	A	A'	B	C
L-*threo*-[β-MePhe^3]deltorphin C	0.47	0.38	0.49	0.65
L-*erythro*-[β-MePhe^3]deltorphin C	0.43	0.33	0.48	0.64
D-*erythro*-[β-MePhe^3]deltorphin C	0.43	0.33	0.48	0.64
D-*threo*-[β-MePhe^3]deltorphin C	0.42	0.26	0.49	0.64

TABLE V

SEPARATION OF β-MePhe ISOMERS, DERIVATIVES AND ITS PEPTIDES BY DIFFERENT CHROMATOGRAPHIC METHODS

||| = $R_s > 2$ or difference between two R_F values $\Delta R_F > 0.1$. || = $2 > R_s > 1$ or difference between two R_F values $0.1 > \Delta R_F > 0.05$. | = $R_s < 1$ or difference between two R_F values $\Delta R_F < 0.05$.

Method	Analysed component	Elution sequence of β-MePhe isomers and derivatives
		1 2 3 4
HPLC	N-Ac-β-MePhe	D,L-*threo* ||| D,L-*erythro*
	OPA-β-MePhe	D,L-*erythro* ||| D,L-*threo*
	β-MePhe + FDAA	L-*erythro* || L-*threo* ||| D-*erythro*	D-*threo*
GC	TAB-β-MePhe	D-*erythro* || L-*erythro* ||| D-*threo* || L-*threo*
Chiral TLC	β-MePhe	L-*erythro*,*threo* ||| D-*threo* || D-*erythro*
		Elution sequence of [β-MePhe^3]deltorphin C isomers
		1 2 3 4
HPLC		D-*threo* ||| D-*erythro* ||| L-*threo* ||| L-*erythro*
TLC	Kieselgel	L-*threo*	D-*threo*, D,L-*erythro*
	Chiralplate	L-*threo*	D,L-*erythro* || D-*threo*

can be achieved with the acetonitrile–methanol–water (4:1:1, v/v/v) system. With a more polar system (columns B and C), the R_F values are higher and no separation can be observed.

CONCLUSIONS

The described procedures can be applied for the separation and identification of the β-MePhe enantiomers and peptides containing them. A comparison of the different methods is given in Table V. The exact enantiomer content of β-MePhe can be determined with a combination of the procedures, and the four diastereomers of [β-MePhe^3]deltorphin C can be identified conveniently by HPLC.

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REFERENCES

1 W.H. Pirkle and J. Finn, in J. Morrison (Editor), *Asymmetric Synthesis, Vol. I, Analytical Methods*, Academic Press, New York, 1983, Ch. 6.
2 W.F. Lindner and C. Petterson, in I. Wainer (Editor), *Liquid Chromatography in Pharmaceutical Development: an Introduction*, Aster, Springfield, VA, 1985, Part 1.
3. D.W. Armstrong and S.M. Han, *CRC Crit. Rev. Anal. Chem.*, 19 (1988) 175.
4 V.A. Davankov, A.A. Kurganov and A.S. Bochkov, *Adv. Chromatogr.*, 22 (1983) 71.
5 C. Petterson, *Trends Anal. Chem.*, 7 (1988) 209.
6 W.F. Lindner, in L. Crane and B. Zief (Editors), *Chromatographic Chiral Separations*, Marcel Dekker, New York, 1987, p. 91.
7 W.F. Lindner, in J.F. Lawrence and R.W. Frei (Editors), *Chemical Derivatization in Analytical Chemistry*, Vol. 2, Plenum Press, New York, 1982, p. 145.
8 T. Nambara, in W.S. Hancock (Editor), *CRC Handbook of HPLC for the Separation of Amino Acids, Peptides and Proteins*, Vol. I, CRC Press, Boca Raton, FL, 1984, p. 383.
9 N. Nimura, H. Ogura and T. Kinoshita, *J. Chromatogr.*, 202 (1980) 375.
10 T. Kinoshita, Y. Kasahara and N. Nimura, *J. Chromatogr.*, 210 (1981) 77.
11 N. Nimura, A. Toyama and T. Kinoshita, *J. Chromatogr.*, 316 (1984) 547.

12 P. Marfey, *Carlsberg Res. Commun.*, 49 (1984) 591.
13 S. Einarsson, B. Josefsson, P. Möller and D. Sanchez, *Anal. Chem.*, 59 (1987) 1191.
14 H. Brückner and C. Gah, *J. Chromatogr.*, 555 (1991) 81.
15 H. Brückner, R. Wittner and H. Godel, *Chromatographia*, 32 (1991) 383.
16 H. Brückner and B. Strecker, *Chromatographia*, 33 (1992) 586.
17 S. Einarsson and G. Hansson, in C.T. Mant and R.S. Hodges (Editors), *High Performance Liquid Chromatography of Peptides and Proteins*, CRC Press, Boca Raton, FL, 1991, p. 369.
18 C.W. Gehrke, F.E. Kaiser, K.C.T. Kuo and R.W. Zumwalt, in R.W. Zumwalt, K.C.T. Kuo and C.W. Gehrke (Editors), *Amino Acid Analysis by Gas Chromatography*, Vol. I, CRC Press, Boca Raton, FL, 1987, Ch. 2.
19 E. Bayer, G. Nicholson and H. Frank, in R.W. Zumwalt, K.C.T. Kuo and C.W. Gehrke (Editors), *Amino Acid Analysis by Gas Chromatography*, Vol. II, CRC Press, Boca Raton, FL, 1987, Ch. 2.
20 H. Frank, in R.W. Zumwalt, K.C.T. Kuo and C.W. Gehrke (Editors), *Amino Acid Analysis by Gas Chromatography*, Vol. II, CRC Press, Boca Raton, Fl, 1987, Ch. 3.
21 I. Abe, S. Kuramoto, in R.W. Zumwalt, K.C.T. Kuo and C.W. Gehrke (Editors), *Amino Acid Analysis by Gas Chromatography*, Vol. III, CRC Press, Boca Raton, FL, 1987, Ch. 2.
22 K. Günther, J. Martens and M. Schickenadz, *Angew. Chem.*, 96 (1984) 514.
23 K. Günther, J. Martens and M. Schickenadz, *Naturwissenschaften*, 72 (1985) 149.
24 K. Günther, *J. Chromatogr.*, 448 (1988) 11.
25 *Chemolog Hi-Lites*, 13, No. 1 (1989) 10.
26 J. Martens and R. Bhushan, *Int. J. Pept. Protein Res.*, 34 (1989) 433.
27 G. Tóth, M. Lebl and V.J. Hruby, *J. Chromatogr.*, 504 (1990) 450.
28 YI. Kataoka, Y. Seto, M. Yamamoto, T. Yamada, S. Kuwata and H. Watanabe, *Bull. Chem. Soc. Jpn.*, 49 (1976) 1081.
29 G. Tsuchihashi, S. Mitamura and K. Ogura, *Bull. Chem. Soc. Jpn.*, 52 (1979) 2167.
30 V.J. Hruby, G. Tóth, C.A. Gehrig, L.-F. Kao, R. Knapp, G.K. Lui, H.I. Yamamura, T.H. Kramer, P. Davis and T.F. Burks, *J. Med. Chem.*, 34 (1991) 1823.
31 J.P. Greenstein and M. Winitz, *The Chemistry of Amino Acids*, Wiley, New York, 1961, p. 2065.
32 R. Schuster, *J. Chromatogr.*, 431 (1988) 271.
33 A. Péter, G. Laus, D. Tourwé, E. Gerlo and G. Van Binst, *Pept. Res.*, 6 (1993) 48.