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Chromatographic behaviour of opioid peptides containing β-methylphenylalanine isomers

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Abstract

A reversed-phase high-performance liquid chromatographic (RP-HPLC) method was developed to obtain pure *erythro*[2*S*3*S*, 2*R*3*R*]- and *threo*[2*S*3*R*, 2*R*3*S*]-β-methylphenylalanine. These amino acids were incorporated into an enkephalin, H-Tyr-D-Ala-Gly-β-MePhe-Val-Val-Gly-NH_2, and into a deltorphin C, H-Tyr-D-Ala-β-MePhe-Asp-Val-Val-Gly-NH_2, analogue, which yielded four diastereoisomers of the peptides. The diastereoisomers were separated on different columns and with different eluent systems. The sequence of elution of the peptide diastereoisomers was determined after hydrolysis of the peptides. For identification of the β-methylphenylalanine enantiomers, enzymatic degradation and an RP-HPLC method were used, with application of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide as derivatizing reagent.

1. Introduction

In the synthesis of receptor selective peptides, unusual amino acids are often used. These unnatural amino acids can either be prepared in racemic form, which is usually the fastest method, or by asymmetric synthesis. Even when using the latter strategy, the chiral purity of the amino acid is often not complete, and small amounts of the stereoisomers can be present.

After incorporation into the peptides, the resulting diastereoisomers may have similar or different physico-chemical or biological properties. The crude peptides are fragmentally purified by reversed-phase high-performance liquid chromatographic (RP-HPLC) methods. In many cases, these purifications are difficult, chromatography of the diastereoisomeric peptides leading to overlapping peaks. Nevertheless, because of the large differences in biological potencies and agonist or antagonist activities of peptide stereoisomers, it is very important to ensure complete separation on both analytical and preparative scales. Another problem is the determination of the configurations of the unusual amino acids in the diastereoisomeric peptides following purification. We described earlier a method for the determination of the configurations of certain unnatural aromatic amino acids in peptide diastereoisomers [1].

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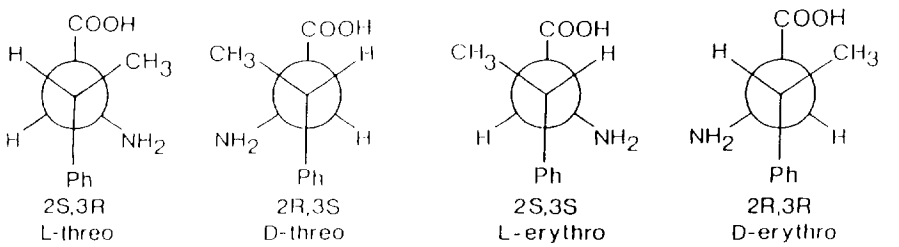

Fig. 1. Structures of four optical isomers of β-methylphenylalanine.

This paper describes a new RP-HPLC method for the preparation of pure *erythro*[(2*S*,3*S*) and (2*R*,3*R*)] and pure *threo*[(2*S*,3*R*) and 2*R*,3*S*)]-β-methylphenlyalanine (β-MePhe). β-MePhe contains two chiral centres, and four stereoisomers are possible (Fig. 1); when incorporated into a peptide, these give rise to different diastereoisomers. We synthesized all four isomers of [β-MePhe[3]]deltorphin C (H-Tyr-D-Ala-β-MePhe-Asp-Val-Val-Gly-NH_2) and of the [β-MePhe[4]] enkephalin analogue (H-Tyr-D-Ala-Gly-β-MePhe-Val-Val-Gly-NH_2) and investigated the effects of the side-chain conformations of the four β-MePhe isomers on the opioid receptor affinity and selectivity [2]. A method was developed for separation of the four diastereoisomers of β-MePhe-containing peptides and for establishment of the configuration of the β-MePhe in these diastereoisomers.

2. Experimental

2.1. Chemicals and reagents

β-MePhe was synthesized by a slight modification of the method of Kataoka et al. [3]. Pure *erythro*[(2*S*,3*S*)- and (2*R*,3*R*)]-β-MePhe and *threo*[(2*S*,3*R*) and (2*R*,3*S*)]-β-MePhe were obtained by chromatographic separation of crystallized β-MePhe. Standard (2*S*,3*S*)-, (2*R*,3*R*)-, (2*S*,3*R*)- and (2*R*,3*S*)-β-MePhe for chromatographic identification were prepared by the method of Kataoka et al. [3]. Their purity and structures were confirmed by fast atom bombardment (FAB) MS and by NMR spectroscopy. The chemical shifts and coupling constants are in agreement with the results of Tsuchihashi et al. [4] and Hruby et al. [5].

Deltorphin C and enkephalin analogues were prepared by solid phase peptide synthesis, using Boc chemistry, starting from 4-methylbenzhydrylamine resin [2,6]. Two syntheses were carried out for both peptides, with Boc-D,L-*erythro*-β-MePhe or Boc-D,L-*threo*-β-MePhe. Both syntheses gave crude diastereomeric peptides.

The mixtures of diastereomeric peptides were separated by means of HPLC, with one of the chromatographic systems described below, and the pure peptides were isolated as white powders. The configuration of β-MePhe in each peptide was determined after hydrolysis to the amino acids, by application of L-amino acid oxidase [7] in combination with the chromatographic methods described previously [8].

L-Amino acid oxidase and 1-fluoro-2,4-dinitrophenyl-5-L-alanin amide (FDAA) were purchased from Sigma (St. Louis, MO, USA).

4-Methylbenzhydrylamine resin was purchased from Bachem Feinchemikalien (Bubsendorf, Switzerland). Amino acids, HPLC-grade solvents (methanol and acetonitrile) and other reagents of analytical-reagent grade were obtained from Merck (Darmstadt, Germany).

Buffers were prepared with doubly distilled water and further purified by pumping through a Type HV 0.45-μm filter (Millipore, Molsheim, France).

2.2. Apparatus

HPLC measurements were performed with three chromatographic systems: (A) the Waters system consisted of an M-600 low-pressure gra-

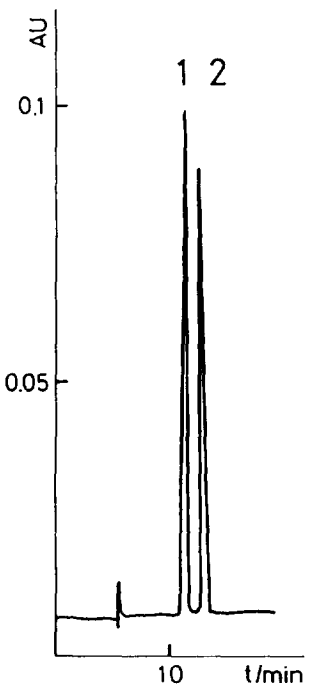

Fig. 2. Chromatogram of D,L-*threo*- and D.L-*erythro*-β-methylphenylalanine. Column, Vydac 218TP54 C_{18}; flow-rate, 1.5 ml/min; mobile phase, 0.2% acetonitrile in water; detection at 210 nm. Peaks, 1 = D,L-*threo*-; 2 = D.L-*erythro*-β-MePhe.

dient pump, an M-996 photodiode-array detector and a Millenium 2010 Chromatography Manager data system (Waters Chromatography, Division of Millipore, Milford, MA, USA); (B) the HPLC system consisted of an L-6000 liquid chromatographic pump (Merck–Hitachi, Tokyo, Japan), a UV 308 spectrophotometric detector (Labor MIM, Budapest, Hungary) and an HP 3395 integrator (Hewlett-Packard, Waldbronn, Germany); and (C) the Gilson Autoprep system contained Gilson Model 302 and 303 pumps, a Gilson Model 115 detector and a Gilson Model 712 system controller (Gilson Medical Electronics, Villiers le Bel, France).

The columns used for analytical separations were as follows: (I) Nucleosil 10 C_{18} (250 × 4.6 mm I.D.), 10-μm particle size (Macherey–Nagel, Düren, Germany); (II) Nova-Pak C_{18} (150 × 3.9 mm I.D.), 4-μm particle size (Waters Chromatography); (III) LiChrospher RP-18 C_{18} (125 × 4 mm I.D.), 5-μm particle size (Merck); and (IV) Vydac 218TP54 C_{18} (250 × 4.6 mm I.D.), 5-μm particle size (Separations Group, Hesperia, CA, USA). For semi-preparative separation, Vydac 218TP1010 C_{18} (250 × 10 mm I.D.), 10-μm particle size, and Vydac 218TP101522 C_{18} (250 × 22 mm I.D.), 10–15-μm particle size, columns (Separations Group) were used.

Quantitative amino acid analysis was performed on an HP 1090 Amino Quant amino acid analyser (Hewlett-Packard). The column used was Hypersyl ODS C_{18} (200 × 2 mm I.D.), 5-μm particle size (Shandon Scientific, Runcorn, Cheshire, UK).

^{1}H NMR spectra were measured on a Bruker (Zug, Switzerland) AM 400 spectrometer.

FAB mass spectra were recorded on a MS 902S double-focussing spectrometer (AEI Scientific Apparatus Division, Manchester, UK) with xenon at 8 kV as bombarding gas.

2.3. Peptide hydrolysis

The deltorphin C and enkephalin analogues containing different stereoisomers of β-MePhe in positions 3 and 4 were hydrolysed under argon pressure in 6 *M* HCl in PTFE bombs in a microwave oven [9]. The solvent was removed by flushing with argon. The dry samples were used for different types of derivatization.

2.4. Enzymatic assay of β-MePhe

A 1-mg amount of *erythro*-D,L- or *threo*-D,L-β-MePhe was dissolved in 0.1 *M* Tris buffer (pH 7.2) in a test-tube and 10 μl of L-amino acid oxidase were added. The test-tube was filled with oxygen, tightly capped and incubated for 24 h at 37°C. The reaction product was used for derivatization reactions.

2.5. Derivatization of β-MePhe

A 0.5–1-mg amount of *erythro*-D,L- or *threo*-D,L-β-MePhe was derivatized with FDAA by the method of Marfey [10].

3. Results and discussion

It was reported previously [8] that D,L-*erythro*- and D,L-*threo*-β-MePhe are not separated on an RP-HPLC column. Only the derivatized *erythro* and *threo* forms could be separated in a conventional buffer–organic modifier system. We now find that with pure water as eluent, the *erythro* and *threo* forms can be separated very well. A better peak shape (more symmetrical peaks) can be achieved if the mobile phase contains 0.1–0.5% of acetonitrile as organic modifier in water (Fig. 2). This finding leads to the separation of D,L-*erythro*- and D,L-*threo*-β-MePhe within a short time on a preparative scale, instead of the very time-consuming crystallization procedure. These pure amino acids were incorporated into [β-MePhe^4]enkephalin and [β-MePhe^3]deltorphin C. The crude peptides were purified by gel-filtration chromatography (Sephadex G-10) with 30% aqueous acetic acid as eluent. Final separation of the diastereoisomers and purification of the lyophilized solid were achieved by semi-preparative RP-HPLC based on one of the analytical methods mentioned below. The analytical separations were carried out on four different reversed-phase columns, in two different buffer systems: a 0.1% aqueous solution of trifluoroacetic acid (TFA) or a 0.01 *M* aqueous

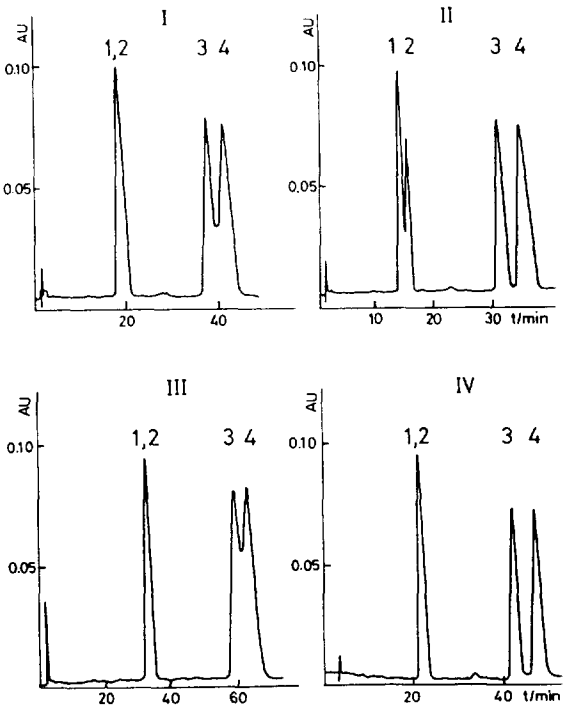

Fig. 3. Chromatograms of four diastereoisomers of [β-MePhe^3]deltorphin C. Column, (I) Nucelosil 10 C_{18}, (II) Nova Pak C_{18}, (III) LiChrospher RP C_{18} and (IV) Vydac 218TP54 C_{18}; flow-rate, 0.8 ml/min; mobile phase, 0.1% TFA–acetonitrile (80:20); detection at 210 nm. Peaks, 1 = D-*threo*; 2 = D-*erythro*; 3 = L-*threo*; 4 = L-*erythro*.

Table 1
Retention factors (k') and resolutions (R_s) of four diastereoisomers of [β-MePhe^3]deltorphin on different columns

Column	Eluent composition (TFA–CH_3CN)	k'				R_s		
		D-*threo*	D-*erythro*	L-*threo*	L-*erythro*	D–D	D–L	L–L
I	80:20	5.15	7.31	19.08	21.65	3.11	9.56	1.39
II	80:20	4.21	6.14	16.29	18.07	3.00	9.26	1.13
III	80:20	4.28	6.14	16.29	18.08	3.06	9.16	1.18
IV	80:20	3.72	5.04	12.78	14.64	3.20	12.67	2.05

Column: (I) Nucleosil 10 C_{18}; (II) Nova Pak C_{18}; (III) LiChrospher RP C_{18}; (IV) Vydac 218TP54 C_{18}. TFA = 0.1% aqueous solution of trifluoroacetic acid; Flow-rate, 0.8 ml/min; detection at 210 nm. $R_{s,D-D}$ represents the separation of D-*threo*- and D-*erythro*-[β-MePhe^3]deltorphin C isomers; $R_{s,D-L}$ represents the separation of D-*erythro*- and L-*threo*-[β-MePhe^3]deltorphin C isomers; $R_{s,L-L}$ represents the separation of L-*threo*- and L-*erythro*-[β-MePhe^3]deltorphin C isomers.

solution of potassium dihydrogenphosphate (pH 3). The organic modifier was acetonitrile or methanol.

3.1. Separation of diasteroisomers of [β-MePhe^3]deltorphin C

The chromatographic behaviour of the four pure diastereoisomers of [β-MePhe^3]deltorphin C was studied by utilizing four different columns with different mobile phases. The results are given in Table 1 and Fig. 3.

On variation of the organic content of the mobile phase in the range 80–20% at 80% organic modifier content, only two peaks were observed and, on decreasing of the acetonitrile content to 20%, the four diastereoisomers could be separated well. With respect to the four columns used, there was no difference in separation capability, but the same separation on columns II and III could be achieved within a shorter time. With respect to the effects of the buffers and organic modifiers, the 0.1% TFA–acetonitrile system seemed most useful; application of phosphate buffer or methanol did not improve the separation. After peaks 1, 2, 3 and 4 had been collected, lyophilized and hydrolysed, the composition of the amino acids in the samples was determined by amino acid analysis. The configuration of the β-MePhe in the four peptides was determined by enzymatic assay with L-amino acid oxidase. For this reason, D,L-*erythro*-, D,L-*threo*-β-MePhe and the four hydrolysed peptides were digested with L-amino acid oxidase. The reaction products were derivatized with FDAA and analysed by RP-HPLC [8].

This procedure revealed that the first peak in Fig. 3 corresponds to [*threo*-D-MePhe^3]deltorphin C, the second to [*erythro*-D-β-MePhe^3]deltorphin C, the third to [*threo*-L-β-MePhe^3]deltorphin C and the fourth to [*erythro*-L-β-MePhe^3]deltorphin C. From the chromatograms, it is clear that the peptide diastereoisomers containing the D-form of β-MePhe can be separated very well from the L-forms, but that the separation of the *erythro* and *threo* forms is more critical.

3.2. Separation of diastereoisomers of the [β-MePhe^4]enkephalin analogue

The optimum mobile phase compositions for the separation of the four isomers of [β-

Table 2
Retention factors (k') and resolutions (R_s) of four diastereoisomers of [β-MePhe^4]enkephalin with different eluent systems and columns

Column	Eluent composition	k'				R_s		
		L-*threo*	L-*erythro*	D-*threo*	D-*erythro*	L–L	L–D	D–D
	TFA–CH_3CN							
I	80:20	6.70	6.83	18.62	19.62	<0.4	10.02	0.53
II	80:20	4.79	4.79	15.21	16.00	–	8.59	<0.40
III	80:20	9.64	10.07	19.00	20.00	0.55	9.62	0.93
IV	85:15	8.30	8.74	22.20	22.70	0.98	17.95	<0.40
	TFA–CH_3OH							
I	60:40	10.04	10.25	21.36	23.42	<0.4	6.23	0.87
II	60:40	8.72	9.58	20.08	22.50	1.10	10.24	1.12
III	65:35	13.09	13.09	24.46	26.27	–	5.68	0.59
IV	65:35	9.95	9.95	19.95	22.43	–	8.69	1.55

Column: (I) Nucleosil 10 C_{18}; (II) Nova Pak C_{18}; (III) LiChrospher RP C_{18}; (IV) Vydac 218TP54 C_{18}. TFA = 0.1% aqueous solution of trifluoroacetic acid; Flow-rate, 0.8 ml/min; detection at 210 nm. $R_{s,L-L}$ represents the separation of L-*threo*- and L-*erythro*-[β-MePhe^4]enkephalin isomers; $R_{s,L-D}$ represents the separation of L-*erythro*- and D-*threo*-[β-MePhe^4]enkephalin isomers; $R_{s,D-D}$ represents the separation of D-*threo*- and D-*erythro*-[β-MePhe^4]enkephalin isomers.

MePhe[4]]enkephalin are given in Table 2 and Fig. 4. The complete separation of all four isomers in one chromatogram poses considerable difficulties. The procedure for the identification of the isomers is the same as described earlier for [β-MePhe[3]]deltorphin C. The elution sequence for the four diastereomers was L-*threo*, L-*erythro*, D-*threo*, D-*erythro*, i.e. opposite to that observed for [β-MePhe[3]]deltorphin C. The 0.1% TFA–acetonitrile system is suitable for separation of the L- and D-forms, while the separation of the L-*threo* from L-*erythro* and D-*threo* from D-*erythro* forms is completely or partially unsuccessful. Column III seems suitable for the separation of the D-*threo* and D-*erythro* forms while column IV gives the best separation of the L-*threo* and L-*erythro* forms. A better separation can be achieved if the mobile phase contains methanol instead of acetonitrile. On column II, all four isomers are detectable although the resolution of the L-*threo* from the L-*erythro* form is not complete. On changing the buffer system from TFA to phosphate, the results for the separation were worse for any mobile phase composition, except 0.01 *M* phosphate buffer (pH 3)–methanol (60:40), in which the D-*threo* and D-*erythro* forms could be separated with $R_s > 1.3$ within 20 min. It should be mentioned that many variations of gradient elution were tested, but without any considerable improvement in resolution.

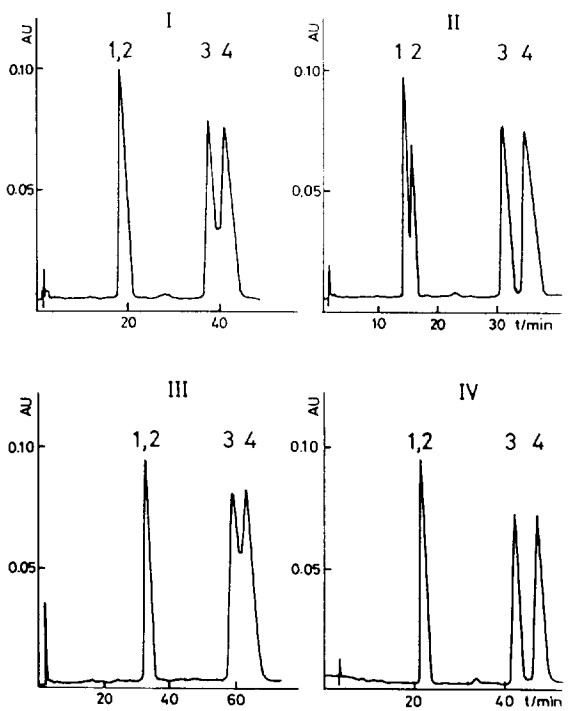

Fig. 4. Chromatogram of four diastereoisomers of [β-MePhe[4]]enkephalin. Column and mobile phase. (I) Nucleosil 10 C_{18}, 0.1% TFA–methanol (60:40), (II) Nova Pak C_{18}, 0.1 TFA–methanol (60:40), (III) LiChrospher RP C_{18}, 0.1% TFA–methanol (65:35) and (IV) Vydac 218TP54 C_{18}, 0.1% TFA–methanol (65:35); flow-rate, 0.8 ml/min; detection at 210 nm. Peaks, 1 = L-*threo*; 2 = L-*erythro*; 3 = D-*threo*; 4 = D-*erythro*.

An important feature of the interaction of peptides and receptors is the three-dimensional structure of the peptide ligand [11]. The structure depends on the amino acid composition, their sequence, their configuration, their side-chain conformation in the peptide and the backbone conformation of the peptide. In the opioid peptides, the distance between tyrosine and phenylalanine is an important factor. The incorporation of one of the four isomers of β-MePhe instead of phenylalanine may result in optimization of this distance, thereby affecting the biological properties, and also the chromatographic behaviour. In the [β-MePhe[4]]enkephalin analogues, the tyrosine and β-MePhe are in the 1,4-positions and the elution sequence is L before D. A similar elution sequence can be observed for H-Tyr-D-Pen-Gly-β-MePhe-D-Pen-OH (Pen = penicillamine) in the TFA–acetonitrile system [1,5]. In [β-MePhe[3]]deltorphin C, the 1,3-positions of tyrosine and β-MePhe leads to a different elution sequences; the first pair of peaks correspond to the D-forms and the L-forms are eluted as the third and fourth peaks. A general prediction of the elution sequence is therefore not possible.

4. Conclusions

Our results demonstrate that the RP-HPLC separation of L- from D-β-MePhe-containing peptides is especially good. However, separation of the *threo* from the *erythro* isomers (epimers at the β-carbon) may be difficult. Therefore, a

synthetic strategy which uses racemic *threo-* or *erythro-β*-MePhe for incorporation in peptides is acceptable in view of the excellent separation of the L- and D-stereoisomers. However, particular attention should be paid to the diastereomeric purity of the *threo* or *erythro* amino acids, as a small amount of the β-carbon epimers (e.g., L-*threo* in L-*erythro*) in the final peptide may be difficult to remove.

Acknowledgement

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