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Chiral separation of β-methyl-amino acids by ligand exchange using capillary electrophoresis and HPLC

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Dedicated to Professor Dr Gottfried Blaschke on the occasion of his 65th birthday.

Abstract

This paper deals with the chiral separation of optical isomers of β-methyl-amino acids by CE and HPLC using the principle of ligand-exchange. Capillary zone electrophoresis was carried out using Cu(II) complexes of L-4-hydroxyproline (L-4-Hypro), *N*-(2-hydroxypropyl)-L-4-hydroxyproline (HP-L-4-Hypro) and *N*-(2-hydroxyoctyl)-L-4-hydroxyproline (HO-L-4-Hypro) as chiral selectors, added to the electrolyte. The HPLC separations were performed on a chiral stationary ligand-exchange chromatography phase containing L-4-Hypro chemically bonded to silica gel. With both techniques nearly all compounds investigated are baseline resolved using different background electrolytes and mobile phases, respectively.

Keywords: β-Methyl-amino acids; Capillary zone electrophoresis; Enantiomer separation; High performance liquid chromatography; Ligand-exchange

1. Introduction

The topographical features of a peptide and their relationships to biological activity comprise one of the most important fields of peptide research. The topographical features involve the three-dimensional arrangements of the side-chain groups in a peptide, which determine its surface architecture. Since side-chain groups in peptides are generally quite flexible in the dihedral angle χ_1 to give *gauche*(−), *trans* and *gauche*(+) conformations, it is clear that the specific arrangement of these side-chain groups relative to one another can dramatically alter the three-dimensional architecture of peptide and such changes will directly affect the biological activity. Several types of unusual amino acids have recently been designed in order to constrain the side-chain functional groups of natural amino acids. In particular, stereospecific β-methyl substitution is widely used to constrain or bias populations of χ_1 rotamers of an aromatic side-chain. β-Methyl substitution tends to eliminate at least one of the three χ_1 rotamers, depending on the substituent configuration at positions α and β [1].

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The unusual β-methylated amino acids have been produced synthetically and control of their enantiopurity requires analytical methods. A recent review gives an overview of chromatographic methods for the chiral separation of β-alkyl-amino acids [2]. Some data are available on the application of gas chromatography [3,4] and thin-layer chromatography [5] in the separation of β-methyl amino acids.

Indirect chiral separation of unusual β-alkyl-amino acids by HPLC was previously reported using chiral derivatization reagents such as 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate [3,4,6–9] and (1*S*,2*S*)-1,3-diacetoxy-1-(4-nitrophenyl)-2-propyl-isothiocyanate [10]. Direct separation was carried out on teicoplanin [4,9,11,12], ristocetin A [13] and crown ether [3,9] phases.

A powerful technique for the chiral separation of amino acids was shown to be ligand-exchange chromatography (LEC) introduced by Davankov et al. [14]. This technique was employed for compounds coordinating with transition metal ions forming complexes of different stabilities [15]. While Davankov [14] separated amino acid enantiomers using polymer resins, which contained L-amino acid residues as metal complexes, Gübitz et al. [16–18] adapted this principle to HPLC using chemically bonded silica gel phases consisting of amino acids bonded to silica gel. Subsequently, numerous papers dealing with different applications of chiral LEC appeared [15,19].

The principle of ligand-exchange (LE) has been adapted to CE by Zare's group using L-histidine [20] or aspartam/Cu(II) [21] complexes as electrolyte additives for the chiral separation of Dns-amino acids. Direct separation of underivatized amino acids using L-Pro or L-4-hydroxyproline (L-4-Hypro) as chiral selectors was reported by Schmid and Gübitz [22]. *N*-alkyl-hydroxyproline derivatives were found to show improved enantioselectivity for amino acids compared to L-4-Hypro [23,24]. These selectors were also applied successfully to the chiral separation of underivatized aliphatic and aromatic amino acids [24], dipeptides [23], sympathomimetics [25], hydroxy acids and β-blockers [26]. An overview of different applications of ligand-exchange capillary electrophoresis (LECE) has been given by Gübitz et al. [27].

In this article the chiral separation of enantiomers of β-methyl-amino acids (Fig. 1) comparing LECE and LEC is described. While for CE Cu(II) complexes of L-4-Hypro, *N*-(2-hydroxypropyl)-L-4-hydroxyproline (HP-L-4-Hypro) and *N*-(2-hydroxyoctyl)-L-4-hydroxyproline (HO-L-4-Hypro) were used, the HPLC separations were carried out on a L-4-Hypro stationary phase.

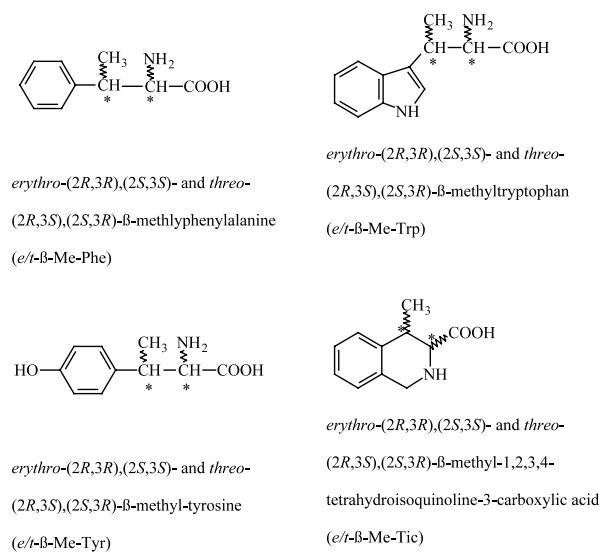

Fig. 1. Structure of the compounds investigated.

2. Experimental

2.1. Chemicals

All chemicals were of analytical grade. L-4-Hypro, copper(II) sulfate and ammonia were purchased from Fluka (Buchs, Switzerland). Phosphoric acid, sodium hydroxide, hydrochloric acid, methanol (HPLC grade), potassium dihydrogen phosphate, LiChrosorb 100, 5 μm were from Merck (Darmstadt, Germany). Dimethlysulfoxide (DMSO) was from Sigma–Aldrich (St. Louis, MO, USA). The water was double-distilled and deionized. The β-methyl-amino acids were synthesized as described previously [2]. Samples

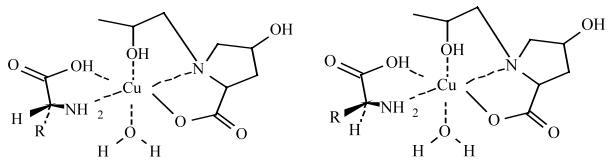

Fig. 2. Structure of the mixed ternary complexes of HP-L-4-Hypro with D and L amino acids.

and elecrolytes were dissolved in double-distilled water (1 mg/ml) and filtered through a 0.45 μm pore size filter (Schleich/Schuell, Dassel, Germany). HP-L-4-Hypro, HO-L-4-Hypro were synthesized following a procedure described previously [24]. The background electrolyte (BGE) contained L-4-Hypro, HP-L-4-Hypro or HO-L-4-Hypro and copper(II) sulfate in 5 mM phosphoric acid. If necessary, pH was adjusted by 5% ammonia to pH 4.3. Before and after use, the capillaries were flushed with 0.25 M sodium hydroxide and water.

Preparation of the chiral stationary phase (CSP) was performed as described in [18]. The mobile phase for HPLC (potassium dihydrogen phosphate and copper(II) sulfate pH 4.5) was filtered through a cellulose nitrate filter (Sartorius AG, Goettingen, Germany) with pore size of 0.2 μm and degassed with helium for 20 min.

2.2. Instrumentation

CE was performed by a fully automated ^{3D}CE (Hewlett–Packard, Palo Alto, CA, USA) equipped with a diode array detector. Fused silica capillary (40 cm, 31.5 cm effective length, 50 μm I.D.) was purchased from Microquarz (Munich, Germany). Detection was accomplished via measurement of the UV absorption at 208 nm. The capillary was thermostated at 25 °C. Samples were injected hydrodynamically (30 mbar∗3 s) and during measurement 10 kV were applied.

HPLC was carried out with a HP 1090 Liquid Chromatograph (Hewlett–Packard, Palo Alto, CA, USA) equipped with a diode array detector (detection at 223 nm), size of the column was 15 cm × 0.46 cm I.D., temperature was 50 °C. Samples were injected automatically by an autosampler (15 μl) and flow rate was 2 ml/min.

3. Results and discussion

The separation mechanism of ligand-exchange has been described previously [15]. Briefly, the separation is based on the formation of ternary mixed metal complexes between the selector and the D-and L-analytes (Fig. 2). The different mixed complexes formed with each of the enantiomers possess different complex stability constants and different elution times, allowing the chiral separation.

3.1. Capillary zone electrophoresis

First L-4-Hypro was used as chiral selector added to the BGE. The ratio between selector and Cu(II) was always 2:1. The pH-optimum for the separation of β-methyl-amino acids was found to be 4.3. Under the conditions applied, out of eight compounds four were baseline resolved and two partially, as can be seen from Table 1. Migration order was checked by spiking with the authentic enantiomers and was found to be (2*R*,3*R*) before (2*S*,3*S*) for the *erythro* and (2*R*,3*S*) before (2*S*,3*R*) for the *threo* epimers. The stereoisomers in a mixture of the *erythro* and *threo*-Me-amino acids could be partially resolved; data are shown in Table 2.

A significant improvement in resolution was obtained with HP-L-4-Hypro as chiral selector and a decrease in migration time was observed. Compared to L-4-Hypro a lower selector concentration was found to be sufficient to achieve baseline separation for all enantiomers except β-Me-Tic. Additionally, in the case of a mixture of *erythro* and *threo*-β-Me-Trp all four stereoisomers were baseline resolved (Fig. 3) and migration order was found to be *erythro*-(2*R*,3*R*), *threo*-(2*R*,3*S*), *threo*-(2*S*,3*R*) and *erythro*-(2*S*,3*S*). The stereoisomers of the other compounds were partially (β-MePhe) or not resolved (β-MeTyr) (Table 2). A further decrease in migration time was obtained using HO-L-4-Hypro as chiral selector but the resolution decreased compared to the application of HP-L-4-Hypro as chiral selector. Under these conditions all enantiomers except β-Me-Tic were baseline separated (Table 1). The stereoisomers in mixtures of *erythro* and *threo*-β-

Table 1
Separation data for β-methyl-amino acid enantiomers by ligand-exchange-CE using different conditions

Compound	t_1 (2*R*, 3*R*)	t_2 (2*S*, 3*S*)	α	R_s	BGE[a]
erythro-β-MePhe	16.26	16.92	1.041	0.992	A
	16.45	19.68	1.197	5.800	B
	9.84	10.76	1.094	3.070	C
erythro-β-MeTrp	19.93	21.07	1.057	1.200	A
	14.77	18.18	1.230	7.110	B
	11.44	13.00	1.136	4.110	C
erythro-β-MeTyr	19.99	21.86	1.094	1.316	A
	13.89	16.90	1.216	5.556	B
	12.24	13.95	1.140	4.010	C
erythro-β-MeTic	20.69	–	1	0	A
	14.01	–	1	0	B
	12.66	–	1	0	C
	(2*R*, 3*S*)	(2*S*, 3*R*)			
threo-β-MePhe	17.75	18.05	1.017	0.652	A
	16.73	18.55	1.109	2.395	B
	10.77	11.62	1.079	1.867	C
threo-β-MeTrp	19.77	20.42	1.033	1.140	A
	15.19	17.24	1.135	5.010	B
	12.23	13.53	1.106	2.661	C
threo-β-MeTyr	19.93	20.89	1.048	1.200	A
	13.90	15.36	1.106	2.560	B
	12.99	14.68	1.130	2.945	C
threo-β-MeTic	21.24	–	1	0	A
	13.98	14.61	1.045	1.385	B
	11.73	–	1	0	C

[a] BGE—A: 80 mM L-4-Hypro, 40 mM Cu(II), in 5 mM phosphoric acid solution adjusted to pH 4.3 with ammonia. B: 20 mM HP-L-4-Hypro, 10 mM Cu(II), in 5 mM phosphoric acid solution adjusted to pH 4.3 with ammonia. C: 20 mM HO-L-4-Hypro, 10 mM Cu(II), in 5 mM phosphoric acid solution adjusted to pH 4.3 with ammonia.

Table 2
Separation data for the four stereoisomers of β-methyl-amino acids by ligand-exchange-CE

Compound	α_1	α_2	α_3	R_{s1}	R_{s2}	R_{s3}	Selector[a]
β-MePhe	1.041	1.049	1.017	1.826	1.497	0.822	A
	1.017	1.109	1.061	0.452	2.395	1.420	B
	1.016	1.062	1.045	0.728	1.880	1.320	C
β-MeTrp	1.008	1.025	1.032	0.371	1.090	1.432	A
	1.028	1.135	1.055	0.768	4.111	2.419	B
	1	1.098	1.036	0	2.779	1.306	C
β-MeTyr	1.003	1.045	1.006	0.136	2.068	2.026	A
	1	1.106	1.100	0	2.200	2.841	B
	1.014	1.120	1.034	0.702	3.568	1.026	C

Separation factor α and resolution R_s are given.

[a] Selector—A: 80 mM L-4-Hypro, 40 mM Cu(II), in 5 mM phosphoric acid solution adjusted to pH 4.3 with ammonia. B: 20 mM HP-L-4-Hypro, 10 mM Cu(II), in 5 mM phosphoric acid solution adjusted to pH 4.3 with ammonia. C: 20 mM HO-L-4-Hypro, 10 mM Cu(II), in 5 mM phosphoric acid solution adjusted to pH 4.3 with ammonia.

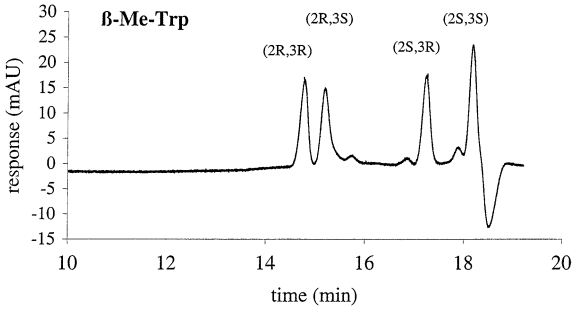

Fig. 3. Electropherogram of the chiral separation of the stereoisomers of *erythro*-(2*R*,3*R*),(2*S*,3*S*)- and *threo*-(2*R*,3*S*),(2*S*,3*R*)-β-Me-Trp. Conditions: 20 mM HP-L-4-Hypro, 10 mM Cu(II), in 5 mM phosphoric acid solution adjusted to pH 4.3 by 5% ammonia.

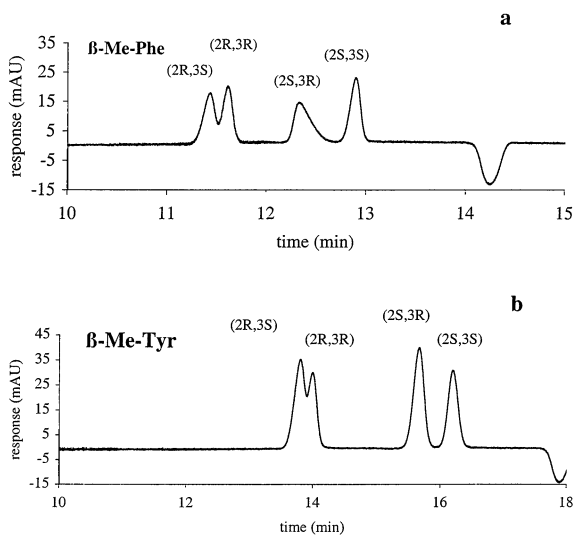

Fig. 4. Electropherogram of the chiral separation of the stereoisomers of (a) *erythro*-(2*R*,3*R*),(2*S*,3*S*) and *threo*-(2*R*,3*S*),(2*S*,3*R*)-β-Me-Phe, (b) *erythro*-(2*R*,3*R*),(2*S*,3*S*) and *threo*-(2*R*,3*S*),(2*S*,3*R*)-β-Me-Tyr. Conditions: 20 mM HO-L-4-Hypro, 10 mM Cu(II), in 5 mM phosphoric acid solution adjusted to pH 4.3 by 5% ammonia.

Me-Phe and *erythro* and *threo*-β-Me-Tyr, respectively (Fig. 4) were partially resolved and in this case migration order was found to be *threo*-(2*R*,3*S*), *erythro*-(2*R*,3*R*), *threo*-(2*S*,3*R*) and *erythro*-(2*S*,3*S*), respectively (Table 2). The higher resolution obtained using *N*-alkyl-hydroxyproline derivatives as chiral selectors might be a proof that the OH-group in the side chain of the selector participates in complex formation and is an important factor for chiral recognition.

Fig. 5 shows an example for the possibility of applying this method for enantiopurity control in synthesis of β-methyl-amino acids using HP-L-4-Hypro as chiral selector. Under the conditions applied an amount of only 0.3% of (2*R*,3*R*) *erythro*-β-Me-Phe is still detectable in a sample of the (2*S*,3*S*) enantiomer.

3.2. High performance liquid chromatography

A chiral ligand-exchange chromatography phase containing L-4-Hypro as chiral selector described previously for the chiral separation of amino acids was used [18]. As mobile phase 0.2 M KH_2PO_4, pH 4.5 and 0.1 mM Cu(II) was used with a flow rate of 2 ml/min. Under these conditions baseline separations for all amino acid enantiomers, except *erythro*-β-Me-Tic and *threo*-β-Me-Tic (data not shown) were obtained (Table 3). Additionally, in mixtures of *erythro* and *threo*-β-Me-Phe, *erythro* and *threo*-β-Me-Tyr and *erythro* and *threo*-β-Me-Trp, respectively, the four stereoisomers were partially resolved, as shown in Table 4 (in the case of β-Me-Tyr the *threo*-(*2R*,3*S*) and *erythro*-(2*R*,3*R*) stereoisomers were not resolved). Using 0.05 M KH_2PO_4 in the mobile phase, *threo*-β-Me-Tic showed baseline resolution and *erythro*-β-Me-Tic was partially resolved (Fig. 6, Table 3). The elution order was found to be the same as in CE. The stereoisomers elute in the order *threo*-

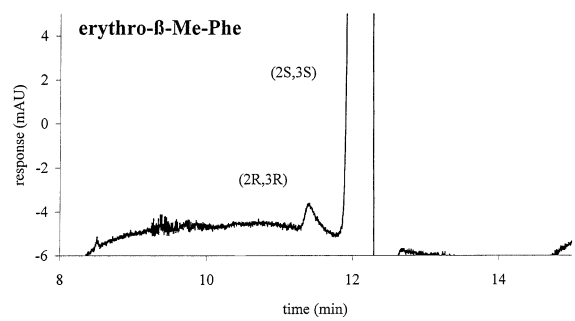

Fig. 5. Electropherogram of the chiral separation of the enantiomers of *erythro*-(2*R*,3*R*),(2*S*,3*S*)-β-Me-Phe, containing only 0.3% (2*R*,3*R*). Conditions: 20 mM HP-L-4-Hypro, 10 mM Cu(II), in 5 mM phosphoric acid solution adjusted to pH 4.3 by 5% ammonia.

Table 3
Separation data for β-methyl-amino acid enantiomers by ligand-exchange-HPLC using different mobile phases

Compound	k'_1 (2*R*, 3*R*)	k'_2 (2*S*, 3*S*)	α	R_s	m.p.[a]
erythro-β-MePhe	1.82	5.42	2.98	2.91	A
erythro-β-MeTrp	3.02	7.52	2.49	2.19	A
erythro-β-MeTyr	1.13	6.84	6.00	3.84	A
erythro-β-MeTic	2.77	3.37	1.21	0.58	B
	(2*R*, 3*S*)	(2*S*, 3*R*)			
threo-β-MePhe	1.63	3.92	2.41	2.27	A
threo-β-MeTrp	2.00	6.26	3.10	2.62	A
threo-β-MeTyr	1.14	4.50	3.94	2.50	A
threo-β-MeTic	1.50	2.99	1.99	1.61	B

[a] Mobile phase—A: 0.2 M phosphate solution pH 4.5, 0.1 mM Cu(II). B: 0.05 M phosphate solution pH 4.5, 0.1 mM Cu(II).

Table 4
Separation data for the 4 stereoisomers of β-methyl-amino acids by ligand-exchange-HPLC

Compound	α_1	α_2	α_3	R_{s1}	R_{s2}	R_{s3}
β-MePhe	1.127	2.139	1.429	0.469	1.910	1.017
β-MeTrp	1.047	2.067	1.202	0.081	1.874	0.581
β-MeTyr	1	3.893	1.547	0	3.282	1.483

Separation factor α and resolution R_s are given. Mobile phase: 0.2 M phosphate solution, pH 4.5, 0.1 mM Cu(II).

(2*R*,3*S*), *erythro*-(2*R*,3*R*), *threo*-(2*S*,3*R*) and *erythro*-(2*S*,3*S*).

4. Conclusions

It has been shown that the principle of ligand-exchange offers an effective possibility for enantiomer separation of β-methyl-amino acids. This principle was applied both to CE and HPLC. With HPLC using an L-4-Hypro CSP, all amino acid enantiomers, including β-Me-Tic, were separated. However, only partial resolution was obtained for the four stereoisomers (two pairs of enantiomers). The use of L-4-Hypro as chiral selector was shown to be more efficient in HPLC compared to CE.

In the CE-investigations, L-4-Hypro, HP-L-4-Hypro and HO-L-4-Hypro were compared as chiral selectors. HP-L-4-Hypro was found to be superior to L-4-Hypro and HO-L-4-Hypro. Baseline separations were achieved for the enantiomers of all the amino acids investigated except β-Me-Tic. With HP-L-4-Hypro and HO-L-4-Hypro the 4 stereoiso-

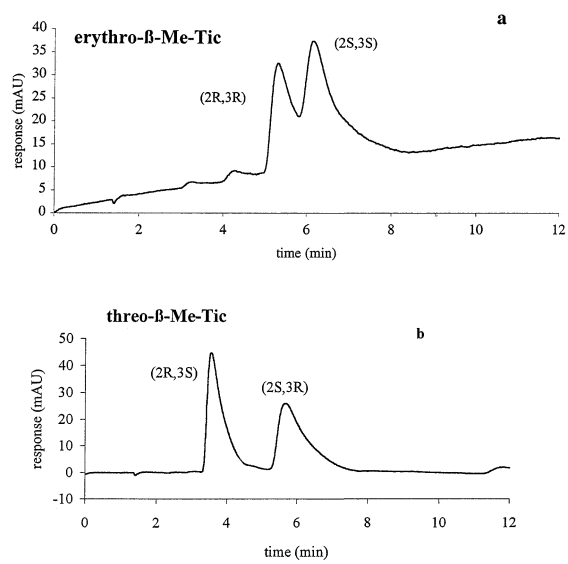

Fig. 6. Chromatogram of the chiral separation of (a) *erythro*-(2*R*,3*R*),(2*S*,3*S*)-β-Me-Tic, (b) *threo*-(2*R*,3*S*),(2*S*,3*R*)-β-Me-Tic. Conditions: 0.05 M phosphate solution, pH 4.5, 0.1 mM Cu(II), flow rate = 2 ml/min.

mers of β-Me-Phe, β-Me-Trp and β-Me-Tyr could be resolved.

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