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Enantioseparation of β-methyl-substituted amino acids with cyclodextrins by capillary zone electrophoresis☆

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ABSTRACT

Direct capillary zone electrophoretic methods were developed for the separation of the enantiomers of unnatural β-methyl-amino acids such as *erythro*- and *threo*-β-methylphenylalanine, β-methyltyrosine, β-methyltryptophan and β-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid. Capillary zone electrophoresis was carried out using sulfopropylated-α-CD (SP2-α-CD), sulfopropylated-β-CD (SP2-β-CD) both with a degree of substitution of 2 moles/mole cyclodextrin, and sulfopropylated-β-CD (SP4-β-CD) with a degree of substitution of 4 moles/mole β-cyclodextrin. The effects of selector and buffer concentrations, electrolyte pH and applied voltage were studied on the separation efficiency. Varying the electrophoretic conditions with application of 20 kV, hydrodynamic injection, unmodified silica capillary, three different buffers (borate, phosphate and acetate) and modified cyclodextrins as chiral selectors all compounds investigated are nearly baseline resolved. The elution sequence was determined in most cases.

1. Introduction

The incorporation of conformationally constrained α-amino acids into peptides allows the study of structure–activity relationships and the synthesis of peptide analogs with improved pharmacological properties [1–4]. Special mention must be made of the constrained analogs of aromatic amino acids, the three-dimensional arrangement of the side-chain moiety of aromatic amino acid residue is crucial in eliciting the desired response.

The side-chain groups in peptides are generally quite flexible in the dihedral angle χ_1 to give *gauche* (−), *trans* and *gauche* (+) conformations, and 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 effect the biological activity. The side-chain conformation can be constrained by introducing an alkyl-group at the β-position of an α-amino acid residue without significantly perturbing the backbone conformation. 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 [5].

The unusual β-methyl-substituted amino acids have two chiral centers and two pairs of enantiomers are possible (Fig. 1). They have been produced synthetically and control of their enantiopurity requires analytical methods. In the course of peptide synthesis some racemization may occur which also requires precise analytical procedures for the identification and determination of all four stereoisomers. Chiral separations of unusual β-methyl-substituted amino acid enantiomers mainly involve high-performance liquid chromatographic (HPLC) methods. Indirect HPLC separation was carried out by application of different chiral derivatizing agents [6–13]. Direct HPLC separations were performed on teicoplanin [11–15], ristocetin A [16], crown ether [10,12], ligand exchange- [17,18], amylose- [18,19] and crown ether-based [20] chiral stationary phases (CSPs). Some data are available on the application of gas-chromatography [10,11] and thin-layer chromatography [9,21] on the separation of β-methyl-amino acids. Recently amino acid analysis by CE have been reviewed by Rizzi [22] and Chankvetadze [23], but very few papers dealing with capillary electrophoresis (CE) of β-methyl-amino acids have appeared. Gübitz and coworkers [17] applied ligand-exchange CE, Érchegyi et al. [24] separated *erythro* and *threo* stereoisomers of β-methyl-3-(2-naphthylalanine) by CE and very recently Jiang et al. [25] resolved some racemic *erythro*- and *threo*-β-methyl-amino acid enantiomers. Survey of literature data revealed that the baseline separation of all four stereoisomers in one run is rarely attainable.

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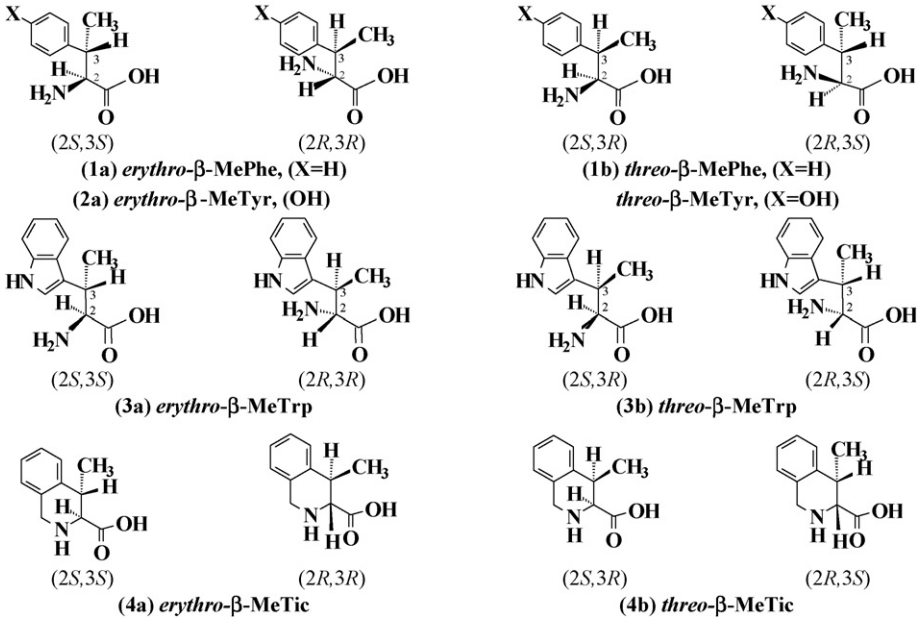

Fig. 1. Structure of analytes.

The present paper describes direct capillary zone electrophoretic method for the enantioseparation of racemic β-methyl-amino acids with application of different sulfated cyclodextrins (CDs) and CD sulfates (CDSs). To achieve total separation of four stereoisomers in one electrophoretic run the separation was optimized by variation of the electrophoretic conditions. The effects of different parameters on the selectivity, such as the nature and pH of the buffer, the mobile phase composition, concentration of chiral additives, applied voltage were examined and are discussed. The elution sequence was determined in all cases.

2. Experimental

2.1. Chemicals and reagents

Racemic *erythro*-(2*S*,3*S* and 2*R*,3*R*)-β-methylphenylalanine (*erythro*-β-MePhe) (**1a**) and *threo*-(2*S*,3*R* and 2*R*,3*S*)-β-methylphenylalanine (*threo*-β-MePhe) (**1b**); *erythro*-(2*S*,3*S* and 2*R*,3*R*)-β-methyltyrosine (*erythro*-β-MeTyr) (**2a**) and *threo*-(2*S*,3*R* and 2*R*,3*S*)-β-methyltyrosine (*threo*-β-MeTyr) (**2b**); *erythro*-(2*S*,3*S* and 2*R*,3*R*)-β-methyltryptophan (*erythro*-β-MeTrp) (**3a**) and *threo*-(2*S*,3*R* and 2*R*,3*S*)-β-methyltryptophan (*threo*-β-MeTrp) (**3b**); *erythro*-(2*S*,3*S* and 2*R*,3*R*)-4-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (*erythro*-β-MeTic) (**4a**) and *threo*-(2*S*,3*R* and 2*R*,3*S*)-4-methyl-1,2,3,4-tetrahy-droisoquinoline-3-carboxylic acid (*threo*-β-MeTic) (**4b**) were prepared by literature methods [9,26] (Fig. 1). The nomenclature and abbreviations of the investigated compounds are in accordance with the IUPAC-IUB JCBN recommendations [27].

Triethylamine (TEA), glacial acetic acid (AcOH), trifluoroacetic acid (TFA), boric acid, phosphoric acid and other reagents of analytical reagent grade were from Merck (Darmstadt, Germany). α,α,α-Tris-(hydroxymethyl)methylamine (TRIS) of ultrapure grade was from Aldrich (Steinheim, Germany). Dimethylsulfoxide (DMSO) and L-amino acid oxidase type I was from Sigma (St. Louis, MO, USA). Native α-CD, sulfopropylated-α-CD sodium salt (SP2-α-CD), sulfopropylated-β-CD sodium salt (SP2-β-CD), sulfopropylated-γ-CD (SP2-γ-CD) sodium salt, all with a degree of substitution of 2 moles/mole CD, sulfopropylated-β-CD sodium salt (SP4-β-CD) with a degree of substitution of 4 moles/mole β-CD, α-CDS with a degree of substitution of 11 moles/mole α-CD and β-CDS with a degree of substitution of 12 moles/mole β-CD were from Cyclolab R&D Ltd. (Budapest, Hungary). Ultrapure water was obtained from a Millipore Milli-Q system (Milford, MA, USA) and was used for the preparation of all aqueous solutions. Milli-Q water was further purified by filtration on a 0.45-μm filter Type HV Millipore (Molsheim, France).

2.2. Solutions

Background electrolyte (BGE) solutions, 75.0 mM borate buffer (pH 9.0), 15.0 mM phosphate buffer (pH 7.2) and 15.0 mM acetate buffer (pH 4.75) were prepared with Milli-Q water by dissolving appropriate amount of boric, phosphoric and acetic acids in ca. 45 ml water in a beaker, adjusting the pH with 1.0 M NaOH and diluting with Milli-Q water to a final volume of 50 ml in a volumetric flask. The buffer solutions were purified by filtration on 0.45-μm filter Type Anotope (Millipore) and were degassed in an ultrasonic bath for 5 min.

Chiral additives were dissolved in BGE. Stock solutions of amino acids (1 mg ml^{-1}) were prepared by dissolving them in water or buffers.

2.3. Identification of enantiomers of β-methyl-amino acids

To prove the migration sequence of the amino acids, enzymatic degradation was applied to obtain enantiomerically pure or enriched isomers. For the enzymatic degradation of the β-substituted amino acids L-amino acid oxidase was used. This enzyme desaminates α-amino acids possessing the L configuration to produce α-keto acids, leaving the D forms of the α-amino acids unchanged. 0.2 mg of racemic *erythro* or *threo* compounds were dissolved in 200 μl of 0.1 M TRIS buffer (pH 7.0) in a test tube and 2 mg L-amino acid oxidase was added. The test tube was filled with oxygen, tightly capped and incubated for 1–14 days at 37 °C.

2.4. Apparatus

All experiments were performed using a HP^{3}CE full automated instrument (Agilent Technologies, Palo Alto, CA, USA) equipped with a diode array detector and Chemstation software for data handling. Fused silica capillary 64.5 cm total length, 56 cm effective length, 50 μm I.D. was purchased from Agilent Technologies. The capillary was preconditioned prior to all runs by flushing with Milli-Q water (0.5 min), 1.0 M NaOH (1.0 min), Milli-Q water (1.0 min) and BGE (10.0 min). Detection was accomplished via measurement of the UV absorption at 210 and 225 nm. The capillary was thermostated at 25 °C. Samples were injected hydrodynamically (50 mbar × 4 s) and during measurement 20 kV was applied. The electroosmotic flow (EOF) was determined by DMSO (c = 0.05% (v/v) in water).

The pH was measured with a Model 420A digital precision pH-meter (Orion, Beverly, MA, USA).

2.5. Evaluation procedure

As primary response functions we recorded the total (t_1 and t_2) and reduced migration times $t^* = t - t_{EOF}$. The selectivity (α) and resolution (R_S) were applied as the secondary response functions when evaluating the performance of the separation system. Selectivity was calculated as

$$\alpha = \frac{t_2 - t_{EOF}}{t_1 - t_{EOF}}$$

where t_2 and t_1 are the total migration times and t_{EOF} is the migration time of DMSO. Resolution was calculated as

$$R_S = \frac{2(t_2 - t_1)}{w_1 + w_2}$$

where w_2 and w_1 are the extrapolated peak widths at the baseline.

3. Results and discussion

Anionic CD derivatives have been among the most widely used types of chiral selectors for CE [28–31]. In the normal polarity mode of CE, the bulk solution moves toward the cathode in consequence of the EOF, while the anionic CD chiral selectors move toward the anode due to electrophoretic movement [29]. Neutral enantiomers (with no electrophoretic mobility themselves) display different distributions between these two countercurrent moving phases, leading to different mobilities. Synthetic amino acids, which have two ionizable groups with pK_as of around 3 and 10, will exist mainly in zwitterionic form between the two pK_as. In the appropriate pH range, therefore, enantiomeric separation can be achieved, similarly as for non-ionizable analytes. All of the analytes investigated in the present work have an aromatic ring and two stereogenic centers, and they can exist as two pairs of enantiomers (Fig. 1). However, this study started with the separation of a single pair of enantiomers (*erythro* or *threo*) on native α-CD. It was expected that the aromatic ring in these amino acids can fit into the cavity of α-CD, resulting in a separation of the enantiomers. Unfortunately, when native α-CD was applied, dissolution problems arose and reproducible results could not be obtained. To avoid fouling of the capillary, sulfopropylated CDs possessing higher water solubility were utilized as chiral selectors.

3.1. Effect of CD concentration on separation efficiency

The effect of the CD concentration on the separation has been reported to be an essential parameter in CD-modified CZE by several authors [32–34]. Accordingly, this was investigated for the enantiomers *erythro*- and *threo*-β-MeTrp over the concentration range 10–100 mM SP2-α-CD in a 75 mM borate buffer system (pH 9.0) (Table 1). With increasing CD concentration, the migration time continuously increased. However, optimum separation (highest α and R_S values) for both model substances was achieved at 50 mM SP2-α-CD. The concentration of SP2-α-CD giving maximum resolution depends on the strength of the isomer–chiral selector complex. A further increase in concentration can result in either a slow or a rapid decrease in resolution. Similar tendencies were observed for other CDs investigated. In further experiments, SP2-α-CD and SP2-β-CD were used at 50 mM, and SP4-β-CD at 25 mM. SP4-β-CD has twice the charge of SP2-β-CD. Thus, to keep the current low enough (at the same level as for SP2-β-CD), the half concentration of the other CDs was applied for SP4-β-CD.

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3.2. Effect of buffer concentration on separation efficiency

The use of buffers is essential in electrophoretic techniques. In CE, increase of the buffer concentration lowers the EOF and increases the current and the temperature, producing more Joule heat. For this reason, the maximum usable ionic strength is limited by the electrical conductivity of the buffer. For investigation of the effect of the concentration of the buffer on the separation efficiency, the concentration of borate buffer (pH 9.0) was varied in the range 25–100 mM for the separation of the *erythro*- and *threo*-β-MeTrp enantiomers. With increasing buffer concentration, the migration time changed slightly and the separation factor increased slightly. Optimum resolution was achieved at 75 mM borate buffer (Table 2). For the acetate (pH 4.75) and the phosphate buffer (pH 7.2), an optimum concentration of 15.0 mM was used.

3.3. Effect of electrolyte pH

pH effects were studied over the range 4.75–9.0 for all of the analytes. For this purpose, three buffer systems were applied, i.e. 15 mM acetate (pH 4.75), 15 mM phosphate (pH 7.2) and 75 mM borate (pH 9.0). Table 3 lists the separation data relating to the enantiomeric pairs in the presence of SP2-α-CD in the three buffer systems. The pH dependence of the migration times followed a minimum curve, i.e. the highest migration times were observed at the

Table 1
Separation data, migration times (t_1 and t_2), selectivity (α) and resolution (R_S) for β-methylTrp enantiomers by capillary zone electrophoresis using SP2-α-CD as chiral selector at different CD concentrations

CD concentration (mM)	*erythro*-β-MeTrp				*threo*-β-MeTrp			
	t_1 (min)	t_2 (min)	α	R_S	t_1 (min)	t_2 (min)	α	R_S
10	7.6	7.8	1.06	1.10	7.4	7.5	1.04	0.65
25	10.2	10.5	1.08	1.65	9.8	9.9	1.16	1.25
50	12.3	12.8	1.15	4.15	12.1	12.4	1.10	2.55
100	38.3	40.3	1.08	1.00	n.d.	n.d.	n.d.	n.d.

BGE, 75.0 mM borate buffer (pH 9.0) (see Section 2.2), n.d., not determined, broad peaks.

Table 2
Separation data, migration times (t_1 and t_2), selectivity (α) and resolution (R_S) for β-methylTrp enantiomers by capillary zone electrophoresis using SP-2-α-CD as chiral selector at different borate buffer concentrations

Buffer concentration (mM)	*erythro*-β-MeTrp				*threo*-β-MeTrp			
	t_1 (min)	t_2 (min)	α	R_S	t_1 (min)	t_2 (min)	α	R_S
25	12.1	12.3	1.07	1.63	11.1	11.3	1.08	1.96
75	12.3	12.8	1.15	4.15	12.1	12.4	1.10	2.54
100	12.2	12.7	1.14	3.71	11.7	12.0	1.11	2.50

BGE, borate buffer (pH 9.0) (see Section 2.2.).

lowest pH; increase of the pH to neutral led to shortened migration times, while further increase of the pH caused the migration times to become longer. However, the α and R_S values decreased continuously with increasing pH (the only exceptions being where resolution was obtained only in borate buffer: *threo*-β-MeTyr and *threo*-β-MeTic). These results can be explained by the pH dependence of protonation and complex formation. The p*K* of SP2-α-CD is about 2, which means that in the investigated pH range (4.75–9.0) it is negatively charged. Some of the amino acid enantiomers are positively charged at pH 4.75; they form strong complexes with the negatively charged SP2-α-CD, and these complexes "contramigrate" with EOF, resulting in high retention and good resolution. At neutral pH, the amino acids are in "zwitterionic" form, the stability of the complexes with SP2-α-CD may be lower, the "zwitterionic" amino acids migrate with EOF, and both the retention and the resolution are decreased. At high pH, both amino acid enantiomers and SP2-α-CD are negatively charged, and they "contramigrate" with EOF again, the migration time being increased; the stability and the difference in stability of the complex formed between the negatively charged amino acid enantiomers and negatively charged SP2-α-CD are small, and the resolution is decreased. (However, sulfopropylated CDs are not 100% pure; they may contain 1–10% native CD. The native CDs migrate with EOF, and therefore the observed migration is the resultant of these two effects.) Similar tendencies in migration time, α and R_S were observed for SP2-β-CD and SP4-β-CD (Tables 4 and 5).

3.4. Effect of applied voltage

The effects of applied voltage in the range 10–30 kV on the EOF, and consequently on the migration time and the separation, were studied in the 75 mM borate buffer system (pH 9.0) with Phe as model substance. Good results were obtained by setting the voltage at 20 kV. An elevated voltage setting resulted in a slight decrease in the separation factor, due to the increased Joule heating of the capillary, revealing higher diffusion velocities and peak broadening. Although use of a lower voltage results in more time for discrimination, if the migration is too slow, then the high speed of the CE separation is lost.

Table 3
Separation data, reduced migration times (t_1^* and t_2^*), selectivity (α) and resolution (R_S) for β-methyl-amino acid enantiomers by capillary zone electrophoresis using SP2-α-CD as chiral selector at different pHs

Compound	t_1^* (min)	t_2^* (min)	α	R_S	BGE**
erythro-β-MePhe	1.7	1.8	1.06	0.70	A
	0.8	0.9	1.13	0.65	B
	3.3	3.4	1.03	0.65	C
erythro-β-MeTyr	1.8	2.1	1.17	1.55	A
	0.7	0.8	1.14	1.35	B
	2.7	2.8	1.04	0.80	C
erythro-β-MeTrp	6.0	8.9	1.48	8.25	A
	1.6	2.1	1.31	6.35	B
	3.0	3.5	1.15	4.15	C
erythro-β-MeTic	9.2	9.2	1.00	0.00	A
	1.7	1.7	1.00	0.00	B
	3.4	4.0	1.00	0.00	C
threo-β-MePhe	2.0	2.4	1.20	2.20	A
	1.1	1.2	1.15	1.80	B
	3.5	3.6	1.03	0.75	C
threo-β-MeTyr	2.6	2.6	1.00	0.00	A
	1.0	1.0	1.00	0.00	B
	2.9	23.0	1.03	1.00	C
threo-β-MeTrp	7.5	9.5	1.27	6.65	A
	1.5	1.9	1.24	3.50	B
	3.2	3.5	1.10	2.55	C
threo-β-MeTic	8.9	8.9	1.00	0.00	A
	1.5	1.5	1.00	0.00	B
	3.5	3.6	1.01	0.20	C

$t_1^* = t_1 - t_{EOF}$, $t_2^* = t_2 - t_{EOF}$; BGE**: A, 15.0 mM acetate buffer (pH 4.75), B, 15.0 mM phosphate buffer (pH 7.2) and C, 75.0 mM borate buffer (pH 9.0) (see Section 2.2); SP-2-α-CD, 50 mM.

Table 4
Separation data, reduced migration times (t_1^* and t_2^*), selectivity (α) and resolution (R_S) for β-methyl-amino acid enantiomers by capillary zone electrophoresis using SP2-β-CD as chiral selector at different pHs

Compound	t_1^* (min)	t_2^* (min)	α	R_S	BGE**
erythro-β-MePhe	1.6	1.7	1.06	0.65	A
	1.0	1.0	1.00	0.00	B
	4.8	4.8	1.00	0.00	C
erythro-β-MeTyr	4.0	4.0	1.00	0.00	A
	1.2	1.2	1.00	0.00	B
	5.6	5.6	1.00	0.00	C
erythro-β-MeTrp	6.2	6.8	1.09	1.50	A
	1.4	1.5	1.07	1.10	B
	7.5	7.5	1.00	0.00	C
erythro-β-MeTic	8.0	8.0	1.00	0.00	A
	1.6	1.6	1.00	0.00	B
	5.2	5.4	1.05	1.50	C
threo-β-MePhe	2.0	2.2	1.05	0.95	A
	1.1	1.4	1.09	0.70	B
	4.2	4.2	1.00	0.00	C
threo-β-MeTyr	4.0	4.0	1.04	0.80	A
	1.4	1.5	1.03	0.90	B
	4.9	4.9	1.00	0.00	C
threo-β-MeTrp	8.8	9.7	1.10	2.10	A
	3.5	3.7	1.06	1.65	B
	3.0	3.3	1.08	1.25	C
threo-β-MeTic	6.1	7.7	1.30	2.75	A
	1.8	2.3	1.19	2.50	B
	5.6	5.9	1.05	1.70	C

$t_1^* = t_1 - t_{EOF}$, $t_2^* = t_2 - t_{EOF}$; BGE**: A, 15.0 mM acetate buffer (pH 4.75), B, 15.0 mM phosphate buffer (pH 7.2) and C, 75.0 mM borate buffer (pH 9.0) (see Section 2.2); SP-2-β-CD, 50 mM.

Table 5
Separation data, reduced migration times (t_1^* and t_2^*), selectivity (α) and resolution (R_S) for β-methyl-amino acid enantiomers by capillary zone electrophoresis using SP4-β-CD as chiral selector at different pHs

Compound	t_1^* (min)	t_2^* (min)	α	R_S	BGE**
erythro-β-MePhe	3.8	4.2	1.11	0.95	A
	1.2	1.4	1.13	0.45	B
	6.1	6.1	1.00	0.00	C
erythro-β-MeTyr	8.7	9.1	1.05	1.55	A
	1.9	1.9	1.00	0.00	B
	5.3	5.3	1.00	0.00	C
erythro-β-MeTrp	6.8	7.6	1.10	2.55	A
	2.0	2.0	1.00	0.00	B
	5.3	5.5	1.04	1.07	C
erythro-β-MeTic	7.0	7.0	1.00	0.00	A
	1.7	1.7	1.00	0.00	B
	7.4	7.7	1.05	1.90	C
threo-β-MePhe	5.1	6.5	1.07	1.15	A
	1.5	1.6	1.07	0.50	B
	5.4	5.4	1.00	0.00	C
threo-β-MeTyr	12.0	12.6	1.05	1.80	A
	2.3	2.4	1.04	0.60	B
	6.4	6.6	1.03	0.65	C
threo-β-MeTrp	6.1	7.0	1.15	2.50	A
	2.3	2.6	1.13	0.90	B
	3.3	3.6	1.09	2.15	C
threo-β-MeTic	6.4	8.2	1.30	5.55	A
	1.8	2.2	1.22	3.05	B
	6.9	7.7	1.12	4.00	C

$t_1^* = t_1 - t_{EOF}$, $t_2^* = t_2 - t_{EOF}$; BGE**: A, 15.0 mM acetate buffer (pH 4.75), B, 15.0 mM phosphate buffer (pH 7.2) and C, 75.0 mM borate buffer (pH 9.0) (see Section 2.2); SP-4-β-CD, 25 mM.

3.5. Chiral separation of erythro- and threo-β-methyl-amino acids using SP2-α-CD as selector

Separation data, reduced migration times (t_1^* and t_2^*), selectivity (α) and resolution (R_S) for β-methyl-amino acid enantiomers with SP2-α-CD as chiral selector at different pHs are presented in Table 3. Besides the BGE pH value, the separation data depend strongly on the structures of the analytes. Amino acids with two ring systems are retained more strongly than those with one aromatic ring (Table 3). In the inclusion-complexation mechanism, either the whole molecule or its hydrophobic part fits into the CD cavity, and thus the CD type plays a very important role in the separation process. The hydrophobic interaction with the cavity alone is not sufficient to allow the separation of enantiomeric compounds; the weak bonds between the substituent groups on the asymmetric center(s) of the analyte and the secondary/or primary native or substituted hydroxy groups of the CD are responsible for chiral recognition. The mechanism of this chiral separation is believed to be the formation of an inclusion complex between the hydrophobic moiety (the aromatic ring) of the analyte and the cavity of the CD. Additional interactions occur between the hydrophilic parts of the analytes and the external surfaces of the CD. The data in Table 3 reveal that the migration times for β-MeTrp and β-MeTic were higher than those for β-MePhe or β-MeTyr. Analytes with two ring systems probably fit better into the hydrophobic cavity of SP2-α-CD. However, in spite of the higher migration times, α and R_S were much lower for β-MeTic than for β-MePhe, β-MeTyr and β-MeTrp, or no separation occurred. The enantiomers of both *erythro*- and *threo*-β-MeTic are very constrained conformationally; the stabilities of complex of the two enantiomers may be very similar, resulting in no or very poor separation. The poor separation of *threo*-β-MeTyr enantiomers may also be explained in terms of structural features.

3.6. Chiral separation of erythro- and threo-β-methyl-amino acids with SP2-β-CD as selector

The native β-CD exhibited a somewhat higher cavity diameter than that of the native α-CD; 0.78 and 0.57 nm, respectively. The data in Table 4 indicate that the migration times differed only slightly on the two selectors for the β-MePhe analogs. Perceptibly higher migration times were observed on SP2-β-CD in the three buffer systems for β-MeTyr and β-MeTrp, and in the borate buffer for β-MeTic, while lower migration times were obtained for β-MeTic in the acetate buffer system. The SP2-β-CD (with the same degree of substitution as that of SP2-α-CD) exhibited a somewhat higher cavity diameter, and therefore a looser fit of the amino acids in the cavity was expected. However, in most cases the migration time did not change or increased when SP2-β-CD was applied. As concerns the separation efficiency, the resolutions were lower for the SP2-β-CD selector, especially in the case of *erythro*-β-MeTyr enantiomers. The β-MeTic enantiomers, which were not separable in the presence of SP2-α-CD selector, could be separated when SP2-β-CD was applied. In the borate buffer the *erythro*-β-MeTic enantiomers were baseline separated, and the *threo*-β-MeTic enantiomers were separated in all three buffer systems.

3.7. Chiral separation of erythro- and threo-β-methyl-amino acids with SP4-β-CD as selector

Since the SP4-β-CD with a substitution degree of four, is more negatively (twofold) charged than SP2-β-CD, the electrophoretic movement in the anodic direction increased. The complex formed with the amino acid also moves faster in the anodic direction, resulting in a wider "separation window", and higher migration times and resolutions were expected. (Naturally, to be able to detect the amino acids, they must reach the detection window, i.e. the observed electrophoretic velocity must point in the cathodic direction.) It may be seen from the data in Table 5 that the migration times in most cases increased when SP4-β-CD was applied, supporting the presumed mechanism (exceptions were *erythro*-β-MeTyr and *erythro*-β-MeTrp in the borate buffer, *erythro*-β-MeTic in the acetate buffer, and *threo*-β-MeTrp in the acetate and phosphate buffers). As compared with the data with the SP2-β-CD selector, the α and R_S values in most cases increased, and especially high resolutions were obtained for the β-MeTic analogs. However, these higher resolutions did not reach the R_S values obtained with the SP2-α-CD selector, indicating the better fit of these amino acids in the SP2-α-CD cavity (exceptions were the *threo*-β-MeTyr and *erythro*- and *threo*-β-MeTic enantiomers). The special behavior of conformationally very constrained β-MeTic enantiomers during the CE separation with the application of CDs indicates the importance of steric effects in chiral discrimination.

3.8. Optimization of separation of four enantiomers of erythro- and threo-β-methyl-amino acids

The incorporation of amino acids into peptides often causes racemization. In amino acids with two chiral centers, epimerization may occur, e.g. the (2*R*,3*R*)-*erythro* isomer could be formed from the (2*S*,3*R*)-*threo* isomer. If peptide synthesis started with racemic *erythro* or *threo* compounds as usual, all four isomers could appear in the peptide. Following separation of the peptide diastereomers, the amino acid content should be determined after a hydrolysis procedure. It is a basic task, therefore, to separate not only the enantiomers, but also the *erythro* and *threo* stereoisomers.

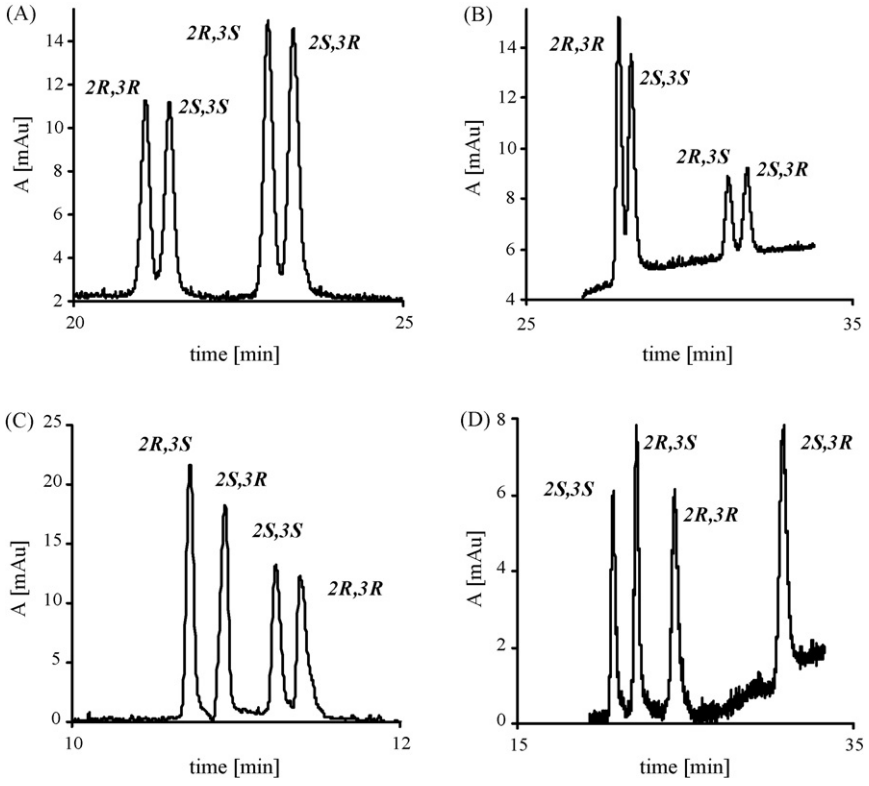

Fig. 2. Selected electropherograms of analytes **1**–**4**. (**A**) β-MePhe, (**B**) β-MeTyr, (**C**) β-MeTrp, (**D**) β-MeTic; selector: (**A**–**C**) SP4-β-CD, (**D**) β-CDS; BGE (**A**, **B** and **D**) 15 mM acetate buffer (pH 4.75), (**C**) 75 mM borate buffer (pH 9.0); applied voltage, 20 kV; detection, 210 nm.

Fig. 2 depicts the enantioseparation of all four stereoisomers of β-methyl-substituted amino acids. In the case of a mixture of *erythro*- and *threo*-β-MePhe, β-MeTyr and β-MeTrp, all four stereoisomers were baseline resolved on the application of SP4-β-CD and acetate or borate buffers, while the *erythro*- and *threo*-β-MeTic enantiomers could be separated by the application of β-CDS in the presence of acetate buffer.

3.9. Identification of enantiomers of β-methyl-amino acids

The sequence of migration of β-methyl-amino acids was checked by spiking with authentic enantiomers obtained from enzymatic digestion, and an example of the total separation of four enantiomers is indicated in Fig. 2. It is interesting to note that the sequence of elution of the *erythro* and *threo* diastereomers differs in the different background electrolytes. For most of the amino acids, change of the BGE was accompanied by a change in the elution of the diastereomers. However, such changes in most cases did not influence the sequence of elution of the enantiomers. In summary, there is no general rule for the elution sequence, which underlines the importance of the identification of individual peaks.

4. Conclusions

This study has demonstrated that the developed CE methods are quite successful for the direct enantioseparation of various β-methyl-amino acids. Of the sulfated CDs, SP2-α-CD and SP4-β-CD were most useful for the enantioseparation of β-MePhe, β-MeTyr and β-MeTrp, while for β-MeTic the β-CDS was the most applicable. The optimum BGE composition, pH and applied voltage were determined with respect to the migration, selection and resolution. The elution sequence was determined for all analytes, but no general rule could be established.

On the basis of the presented results and our earlier HPLC separation data it can be stated that the baseline resolution of the investigated compounds could be achieved in an easier way applying CE than HPLC. Depending on the compound's structure in HPLC both the column (chiral selector) and the eluent are to be changed to reach efficient separation, while the easy variation of the quality of buffer and selector in CE makes this technique a more convenient, flexible and economic method of choice in chiral analysis.

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References

[1] P. Balaram, Curr. Opin. Struct. Biol. 2 (1992) 845.
[2] R.M. Liskamp, J. Recl. Trav. Chim. Pays-Bas 113 (1994) 1.
[3] V.J. Hruby, F. Al-Obeidi, W. Kazmierski, Biochem. J. 268 (1990) 249.
[4] V.J. Hruby, Acc. Chem. Res. 34 (2001) 389.
[5] D. Tourwé, E. Mannekens, T.N.T. Diem, P. Verheyden, H. Jaspers, G. Tóth, A. Péter, I. Kertész, G. Török, N.N. Chung, P.W. Schiller, J. Med. Chem. 11 (1998) 5167.
[6] A. Péter, G. Tóth, E. Cserpán, D. Tourwé, J. Chromatogr. A 660 (1994) 283.
[7] A. Péter, G. Tóth, D. Tourwé, J. Chromatogr. A 668 (1994) 331.
[8] A. Péter, G. Tóth, G. Török, D. Tourwé, J. Chromatogr. A 728 (1996) 455.
[9] A. Péter, G. Tóth, Anal. Chim. Acta 352 (1997) 335.
[10] A. Péter, G. Török, G. Tóth, W. Van Den Nest, G. Laus, D. Tourwé, J. Chromatogr. A 797 (1998) 165.
[11] A. Péter, G. Török, G. Tóth, W. Van Den Nest, G. Laus, D. Tourwé, D.W. Armstrong, Chromatographia 48 (1998) 53.
[12] G. Török, A. Péter, E. Vékes, J. Sápi, M. Laronze, J.-Y. Laronze, Chromatographia 51 (2000) 165.
[13] A. Péter, M. Péter, F. Fülöp, G. Török, G. Tóth, D. Tourwé, J. Sápi, Chromatographia 51 (2000) 148.
[14] A. Péter, G. Török, J. Chromatogr. A 793 (1998) 283.

[15] A. Péter, G. Török, D.W. Armstrong, G. Tóth, D. Tourwé, J. Chromatogr. A 828 (1998) 177.
[16] A. Péter, G. Török, W.D. Armstrong, G. Tóth, D. Tourwé, J. Chromatogr. A 904 (2000) 1.
[17] N. Grobuschek, M.G. Schmid, C. Tusher, M. Ivanova, G. Gübitz, J. Pharm. Biomed. Anal. 27 (2002) 599.
[18] L.M. Nogle, C.W. Mann, W.L. Watts Jr., Y. Zhang, J. Pharm. Biomed. Anal. 40 (2006) 901.
[19] M. Alias, M.P. López, C. Cativiela, Tetrahedron 60 (2004) 885.
[20] A. Péter, G. Török, G. Tóth, W. Lindner, J. High Resolut. Chromatogr. 23 (2000) 628.
[21] Zs. Darula, G. Török, G. Tóth, E. Mannekens, K. Iterbeke, D. Tourwé, A. Péter, J. Planar. Chromatogr. 11 (1998) 346.
[22] A. Rizzi, Electrophoresis 22 (2001) 3079.
[23] B. Chankvetadze, J. Chromatogr. A 1168 (2007) 45.
[24] J. Érchegyi, B. Penke, L. Simon, S. Michaelson, S. Wenger, B. Waser, R. Cescato, J.-C. Schaer, J.C. Reubi, J. Rivier, J. Med. Chem. 46 (2003) 5587.
[25] C. Jiang, D.W. Armstrong, A.W. Lantz, A. Péter, G. Tóth, J. Liq. Chromatogr. Relat. Technol. 30 (2007) 1421.
[26] L. Jeannin, T. Nagy, L. Vassileva, J. Sápi, J.-Y. Laronze, Tetrahedron Lett. 36 (1995) 2057.
[27] IUPAC-IUB JCBN recommendations, J. Biol. Chem. 260 (1985) 14.
[28] S. Gratz, A.M. Stalcup, Anal. Chem. 70 (1998) 5166.
[29] S. Fanali, J. Chromatogr. A 875 (2000) 89.
[30] M.D. Egger, Y. Liu, J. Sevčik, E. Tesařova, R. Rozhkov, R.C. Larock, D.W. Armstrong, Electrophoresis 25 (2003) 2650.
[31] A. Lantz, R.V. Rozhkov, R.C. Larock, D.W. Armstrong, Electrophoresis 25 (2004) 2727.
[32] S. Birnbaum, S. Nilsson, Anal. Chem. 64 (1992) 2872.
[33] S. Terabe, Y. Miyashita, Y. Ishihama, O. Shibata, J. Chromatogr. 636 (1993) 47.
[34] S. Fanali, J. Chromatogr. 545 (1991) 437.