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Enantioseparation of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate-derivatized-amino acids by capillary zone electrophoresis using native and substituted β-cyclodextrins as chiral additives I. Discussion of optimum separation conditions

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Abstract

Enantioseparation of all 19 natural occurring chiral protein amino acids was investigated after derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) reagent by using capillary zone electrophoresis (CZE). Native and substituted β-cyclodextrins were used as chiral selectors added to the background electrolyte: native β-cyclodextrin, (2-hydroxy)propyl-β-cyclodextrin, heptakis(2,6-di-*O*-methyl)-β-cyclodextrin, heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin, β-cyclodextrin polymer and carboxymethyl-β-cyclodextrin polymer. These selectors were compared with respect to enantioselectivity and analysis time, and the best suited selector specified for each amino acid. Optimum selector concentrations were experimentally determined for six of the amino acids.

Keywords: Buffer composition; Enantiomer separation; Enantioselectivity; Amino acids; Cyclodextrins

1. Introduction

Amino acid analysis has attracted broad interest from the very beginning of enantioseparation. With a few exceptions such as in bacterial cell walls [1,2], L-amino acids are the natural occurring forms in proteins and quantitation of D- and L-amino acids is therefore of certain importance e.g., in biochemistry, biotechnology and food chemistry [3]. Furthermore, it provides a method for dating [4,5] proteinaceous materials. For all these purposes sensitive and enantioselective methods are required. Derivatization of amino acids with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) reagent (cf. Fig. 1) [6] turned out to be a potent method for sensitive detection as the formation of the AQC derivatives proceeds very rapidly yielding stable fluorescent products with only weakly fluorescent side products.

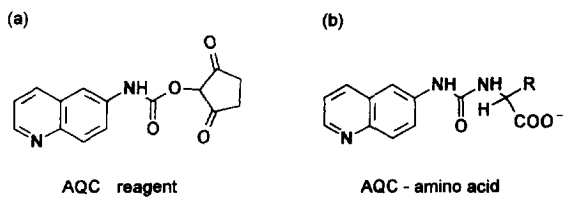

Fig. 1. Chemical structures of (a) 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) reagent and (b) AQC-derivatized amino acids.

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High-performance liquid chromatography (HPLC) separations of AQC-derivatized amino acids have been reported for amino acid analysis in protein hydrolysates [7] and enantioselective separations by means of β-cyclodextrin (β-CD) bonded stationary phases [8,9]. However, the enantiomeric resolution of the AQC-amino acids achieved was not totally satisfactory when using native β-cyclodextrin stationary phases. As capillary electrophoresis (CE) has become a more commonly used analytical technique for separation of enantiomers, resolutions of several AQC-amino acids have been reported, either by employing the chiral surfactant *N*-dodecoxycarbonylvaline [10] or using β-cyclodextrin as chiral buffer additive [11,12].

In this paper CE with native and five modified β-cyclodextrins as buffer additives is used for enantioseparation the 19 natural chiral amino acids after derivatization with AQC reagent. The various modified β-CD molecules differ in shape and hydrophobicity allowing different host–guest interactions. Selector additives best suited for each AQC-amino acid are selected on the base of the achieved enantioselectivities and analysis times. From the measured decrease in mobilities with increasing selector concentrations the optimum concentrations were determined for six selected AQC-amino acids and all non-charged selectors and its variation with EOF is discussed.

2. Experimental

2.1. Chemicals

Amino acid standards and the buffer 1,3-bis-[tris(hydroxymethyl)methylamino]propane (BTP) were obtained from Sigma (Deisenhofen, Germany); the AQC derivatization reagent (trade name AccQ.Tag.Fluor Reagent) from Waters (Bedford, MA, USA). Native β-CD was a gift from the Department of Chemistry of the Polish Academy of Sciences (Warsaw, Poland). Heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (DM-β-CD) containing about 14 methoxy-groups per β-CD molecule), heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin (TM-β-CD) containing 21 methoxy groups per β-CD molecule, (2-hydroxy)propyl-β-cyclodextrin (HP-β-CD) containing about 6.3 hydroxypropyl groups per β-CD ring, β-CD polymer crosslinked with 1-chloro-2,3-epoxypropane and carboxymethyl-β-cyclodextrin polymer (CM-β-CD) with a total carboxy content of 4.1% and crosslinked with 1-chloro-2,3-epoxypropane, were purchased from Cyclolab R and D Laboratory (Budapest, Hungary). Sodium hydroxide and hydrochloric acid were purchased from E. Merck (Darmstadt, Germany).

2.2. Apparatus and electrophoretic conditions

All experiments were carried out using an HP-3D capillary electrophoretic instrument (Hewlett-Packard, Waldbronn, Germany), equipped with a diode array detector monitoring a wavelength of 245 nm. A non-coated fused-silica capillary (Hewlett-Packard) was used with 48.5 cm total length (40 cm effective length) and internal diameter of 50 μm which was kept at a constant temperature of 20±0.1°C and to which a voltage of 30 kV was applied.

The background electrolyte (BGE) consisted of an aqueous solution of 10 m*M* BTP at pH 7.0 (adjusted with hydrochloric acid) and 5 m*M* selector (β-CD or derivatives).

2.3. Derivatization procedure

Up to about 3 mg amino acid standard were resolved in 70 μl AccQ.Fluor Borate Buffer. 20 μl of the reconstituted AccQ.Fluor Reagent (approximately 10 m*M* AccQ.Fluor in acetonitrile) was added and immediately vortex mixed for several seconds. After 1 min at room temperature and 10 min at 55°C the samples were ready to inject.

3. Results and discussion

With the chosen pH of 7.0, most of the amino acid derivatives are negatively charged: the carboxyl groups of the amino acids are fully dissociated whereas the nitrogen in the quinolyl group of the derivatization label is non-charged according to a pK_a of approximately 4.8. At this pH and using non-coated capillaries, the amino acid derivatives are dragged towards the cathode by the electroosmotic flow (EOF) generated. Thus, detection is carried out

at the cathodic side of the capillary, if not mentioned otherwise. The mono-derivatized basic amino acids Arg and Lys are zwitterionic at the given pH, whereas His carries a very low net charge. These derivatives are thus detected together with or close to the EOF signal, respectively. Migration of the zwitterionic analytes is non-selective unless a charged selector is employed. Lys, however, can be enantioseparated as doubly labelled derivative. For the AQC derivatives of Arg no other signals were found beyond that coinciding with the signal of the EOF marker; obviously only a zwitterionic derivative is obtained which cannot be enantioseparated with non-charged selectors. Arg is thus discussed in the upcoming tables only as far as charged selectors are addressed.

The two acidic amino acids Glu and Asp which carry two negative charges exhibit effective electrophoretic mobilities higher than and opposite to the mobility caused by the EOF. They are analyzed with the detector at the anodic side using a voltage of −30 kV with β-CD concentrations ranging from 0.5 to 5 m*M*. Best enantioselectivity for Glu was obtained using 2.5 m*M* β-CD achieving nearly baseline separation within 10 min, whereas Asp did not reach the detector within 30 min under the chosen EOF conditions. These two acidic amino acids are not included in the upcoming tables and discussions.

3.1. Enantioresolution by different selectors

Enantioseparation of the AQC derivatized amino acids by use of the various selectors is characterized in Table 1 by the apparent enantioselectivity coefficients, α^{app}, which are the ratios of the apparent mobilities, μ^{app}, of the faster migrating enantiomer to those of the slower migrating ones which are given in Table 2. Apparent selectivity coefficients

Table 1
Apparent enantioselectivity coefficients, α^{app}, of AQC-amino acids measured with native and differently substituted β-CDs

Amino acid	β-CD	β-CD polymer	HP-β-CD	DM-β-CD	TM-β-CD
Ala	1.021	1.048	1.041	1.040	1.069
Val	1.020	1.048	1.039	1.066	1.094
Leu	1.034	1.064	1.066	1.076	1.053
Ile[a]	1.018	1.044	1.051	1.063	1.095
		1.050		1.039	1.070
Met	1.022	1.053	1.041	1.052	1.043
Pro	1.008	1.064	1.050	1.00	1.00
Cys[b]	1.014	1.019	1.014	1.023	1.027
	1.00	1.007	1.00	1.020	1.027
Lys[c]	1.010	1.013	1.018	1.00	1.00
Ser	1.009	1.028	1.023	1.053	1.039
Thr	1.025	1.061	1.047	1.055	1.103
Asn	1.014	1.057	1.035	1.025	1.031
Gln	1.007	1.040	1.032	1.036	1.041
Phe	1.015	1.011	1.031	1.008	1.010
Trp	1.011	1.00	1.021	1.016	1.007
Tyr	1.013	1.018	1.029	1.015	1.00
His	1.00	1.00	1.00	1.00	1.00
	CM-β-CD polymer[d]				
Arg	1.040				
Lys[c]	1.053				
His	1.046				

Electrophoretic conditions: capillary: 40 cm effective length, 48.5 cm total length; BGE: aqueous solution of 10 m*M* BTP, pH 7.0, selector concentration of 5 m*M*, applied voltage: 30 kV.

[a] Two diastereomeric pairs of enantiomers.

[b] Two differently labelled pairs of enantiomers.

[c] Doubly AQC-labelled derivative.

[d] 0.5% (w/w) CM-β-CD polymer; other conditions as described above.

Table 2
Apparent mobilities of the first detected enantiomers, μ_1^{app} ($\cdot 10^{-5}$ cm^2 V^{-1}s^{-1}), of AQC-amino acids measured with native and differently substituted β-CDs

Amino acid	β-CD	β-CD polymer	HP-β-CD	DM-β-CD	TM-β-CD
Ala	7.9	9.7	8.7	7.5	3.9
Val	9.3	10.8	11.6	8.5	4.1
Leu	10.6	11.2	9.7	9.2	5.5
Ile[a]	9.4	11.3	8.8	10.1	4.5
		11.6		9.6	4.6
Met	9.9	9.4	11.6	8.4	4.9
Pro	7.6	6.5	8.3	5.9	2.1
Cys[b]	11.9	13.0	12.1	10.2	4.2
	11.7	12.5	11.6	9.9	4.3
Lys[c]	11.1	17	10.2	11.3	8.6
Ser	7.2	8.0	8.4	5.6	3.6
Thr	9.4	9.7	10.9	9.1	2.8
Asn	9.0	8.8	9.3	6.9	2.4
Gln	8.7	8.9	9.2	6.7	2.8
Phe	10.2	13.3	11.9	9.6	7.3
Trp	11.1	14.8	10.8	9.2	6.1
Tyr	10.6	13.2	11.7	7.7	4.8
His	16.2	15.2	17.4	14.6	13.9
	CM-β-CD polymer[d]				
Arg	9.5				
Lys[c]	5.4				
His	8.8				

Electrophoretic conditions as in Table 1.
[a]Two diastereomeric pairs of enantiomers.
[b]Two differently labelled pairs of enantiomers.
[c]Doubly AQC-labelled derivative.
[d]0.5% (w/w) CM-β-CD polymer.

are decisive for the enantiomeric resolution under given EOF conditions. For choosing suitable selectors by means of the data given in Table 1, as an approximate rule, baseline separation is achieved with α^{app} values exceeding 1.020–1.035, depending on the migration time, as efficiencies are reduced for analytes migrating against the EOF. All eighteen chiral amino acids investigated in this mode are enantioseparated under the given conditions as AQC derivatives by at least one of the β-CD selectors. The data given in Table 1 were measured at a constant selector concentration of 5 m*M*. The dependence of α^{app} upon selector concentration, [*S*], was experimentally determined for six amino acids, i.e., Ala, Leu, Ser, Thr, Phe and Trp, employing all the noncharged selectors. The selector concentrations corresponding to the maximum in the α^{app} vs. [*S*] curve, i.e., $[S]^{opt,app}$, were found between 2 and 5 m*M* for most analytes and selectors as given in Table 3, the lower values for the aromatic amino acids. Typical examples for α^{app} vs. [*S*] curves are shown in Fig. 2. As the optimum selector concentration is dependent on the strength of complexation [13–17], a certain variation in these values is obtained. The concentration of 5 m*M* chosen for the data in Table 1 is a reasonable compromise, although certain improvements in resolution are obtained upon reducing the selector concentration in certain cases. Clearly, the $[S]^{opt,app}$ values are strongly dependent on the strength (and direction) of the EOF and thus on even small changes in pH, in ionic strength and in capillary surface conditions. When correcting the apparent mobilities for the contributions caused by the EOF, the effective selectivity coefficients, α^{eff}, are independent of the EOF and the same holds for the dependence of α^{eff} on the selector concentration and the value of $[S]^{opt,eff}$. Values of α^{eff} are calculated here as the ratios of the corresponding effective

Table 3
Optimum apparent selector concentrations, $[S]^{opt,app}$ (m*M*), of six AQC-amino acids measured with native and differently substituted β-CDs

Amino acid	β-CD	β-CD polymer	HP-β-CD	DM-β-CD	TM-β-CD
Ala	5	–	1.3	1.3	20
Leu	2.5	2.5	1.3	2.5	30
Ser	5	–	2.5	10	30
Thr	5	2.5	5	5	30
Phe	1	–	1.3	1.3	10
Trp	1	2.5	1.3	1.3	10

Electrophoretic conditions: capillary: 40 cm effective length, 48.5 cm total length; BGE: aqueous solution of 10 m*M* BTP, pH 7.0, selector concentration between 1.25 and 40 m*M*, applied voltage: 30 kV.

mobilities μ_2^{eff}/μ_1^{eff}, subscripts 1 and 2 indicating the first and second detected enantiomer, respectively. EOF corrected data are shown in Fig. 3a–e. On the bases of these curves estimation of optimum selector concentrations can be determined for any conditions knowing strength and direction of the EOF. Clearly, for analytes migrating against the EOF, the $[S]^{opt,app}$ values are lower than the corresponding $[S]^{opt,eff}$ values, the α^{app} values at the maximum are higher and the maxima are sharper, as demonstrated by comparison of the data in Fig. 2 and Table 3 with those in Fig. 3.

A guideline for choosing the best suited selector for each amino acid is given in the following discussion and is summarized in Table 4.

3.1.1. Native β-CD

Native β-CD allows one to resolve all AQC-amino acids under the given conditions at least partially within 15 min. Exceptions are His and Arg, the two amino acids mentioned above. However, baseline separation is achieved in a few cases only (i.e., Ala, Leu, Ile, Met and Thr) as peak broadening is considerable. This group of analytes is only partially identical with that separated by means of a chiral stationary phase with immobilized "native" β-CD and HPLC [9]. For the resolution of aromatic amino acids a lower selector concentration of approximately 1.5 m*M* is recommended, when working with an EOF of about $20 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1}.

3.1.2. β-CD polymer and HP-β-CD

The β-CD polymer is the selector which provides highest selectivities in most instances. Both pairs of enantiomers corresponding to the two diastereomers of isoleucine are separated, as shown in Fig. 4.

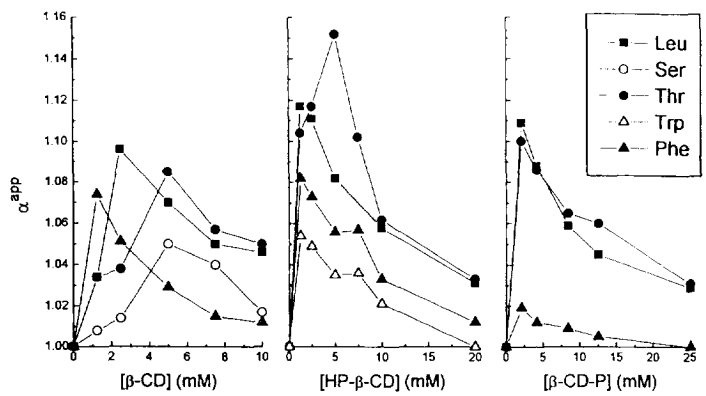

Fig. 2. Dependence of the apparent selectivity coefficients on selector concentrations using (a) native β-CD, (b) HP-β-CD and (c) β-CD polymer (β-CD-P). Experimental conditions: non-coated fused-silica capillary with internal diameter of 50 μm, total length of 48.5 cm and effective length of 40 cm. Detection wavelength of 245 nm. BGE: aqueous solution of 10 m*M* BTP at pH 7.0, temperature of 20°C, applied voltage of 30 kV as given in Section 2.2.

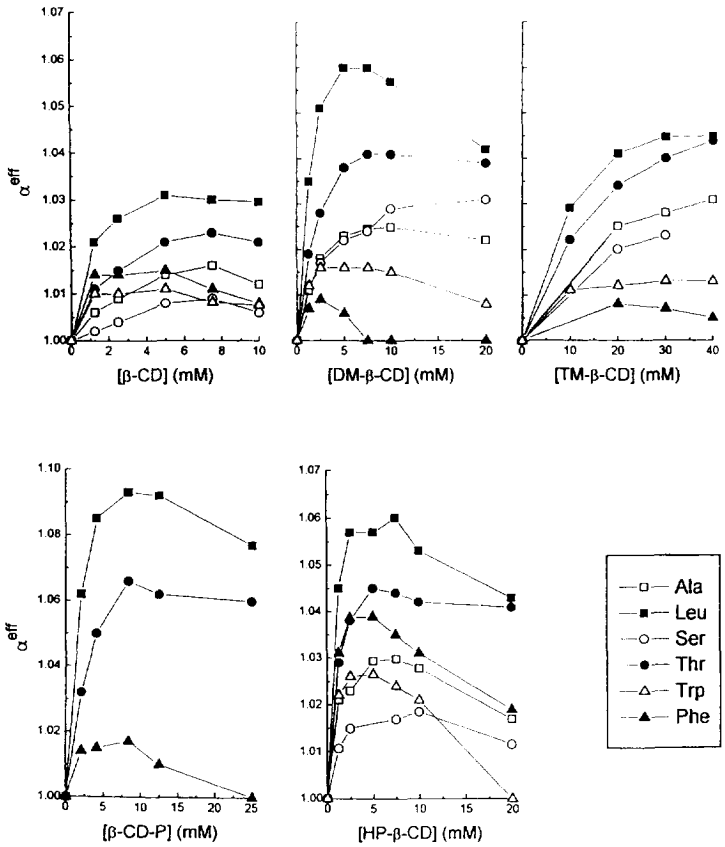

Fig. 3. Dependence of the effective selectivity coefficients on selector concentrations using (a) native β-CD, (b) DM-β-CD, (c) TM-β-CD, (d) β-CD-P and (e) HP-β-CD. Experimental conditions see Fig. 2.

Compared with the native β-CD monomer, significantly higher selectivities are achieved particularly for polar (i.e., hydroxy- and amido-) amino acids as well as non-polar aliphatic ones (Fig. 5a). However, aromatic amino acids are insufficiently resolved, e.g., for Trp no separation is obtained.

HP-β-CD is an effective selector in all cases, too, with the usual exceptions of Arg and His. This selector effects particularly good resolution of the aromatic amino acids Phe, Trp and Tyr. Analysis times of about 15 min under the given conditions and $[S]^{opt,app}$ values of about 2 to 5 m*M* are comparable with those obtained with all other substituted selectors, except TM-β-CD.

3.1.3. DM-β-CD and TM-β-CD

These two methylated CD selectors are very similar with respect to enantioselectivity. However, the migration times observed with TM-β-CD are about 2.5 times longer than those obtained with DM-β-CD, when working at selector concentrations of 5 m*M*. It is obvious that the complexing constants with TM-β-CD are much lower than with the other selectors mentioned. Employing higher concentrations of TM-β-CD, analysis time can be reduced significantly (cf. Fig. 6). The $[S]^{opt,app}$ values of TM-β-CD range from 10 to 30 m*M*, again the lower values for the aromatic amino acids. $[S]^{opt,app}$ is strongly dependent on even small variations in the EOF. However, as TM-β-CD is a very potent selector in most instances, sufficient resolution is achieved even with selector concentrations far from optimum.

Particularly, the methylated CDs are excellent selectors for the aliphatic non-polar amino acids (Figs. 5b and 6). Both pairs of enantiomers corre-

Table 4
Enantiomeric resolution of AQC-amino acids with differently substituted β-CDs

Amino acid	β-CD	β-CD polymer	HP-β-CD	DM-β-CD	TM-β-CD
Ala	+	+	+	++	++
Leu	+	++	++	++	++
Ile[a]	+	+	++	++	++
		+			
Val	(+)	++	+	++	++
Met	+	++	++	++	+
Pro		++	++		
Cys		(+)			
Lys[b]			(+)		
Ser		+	(+)	++	+
Thr	+	++	+	++	++
Asn	(+)	++	+	+	+
Gln		++	+	+	+
Phe			+		
Trp			(+)	(+)	
Tyr			+		
	CM-β-CD polymer[c]				
Arg	+				
Lys[b]	+				
His	+				

Electrophoretic conditions as in Table 1; (+) nearly baseline separation, + baseline separation, ++ separation with very high resolution.
[a]Two diastereomeric pairs of enantiomers.
[b]Doubly AQC-labelled derivative.
[c]0.5% (w/w) CM-β-CD polymer; other conditions as described above.

sponding to the two diastereomeric forms of Ile are separated. Polar amino acids like Ser, Thr and the two amides Asn and Gln are excellently resolved, too. No separation is obtained for Pro and Lys, and

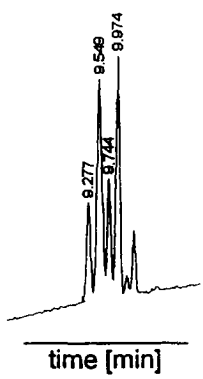

Fig. 4. Electropherograms of a mixture of AQC-Ile and AQC-*allo*-Ile. Peaks not identified by migration times are impurities. Selector concentration: 0.5% (w/v) of β-CD polymer; all other experimental conditions as in Fig. 2. Ordinate: absorbance at 245 nm.

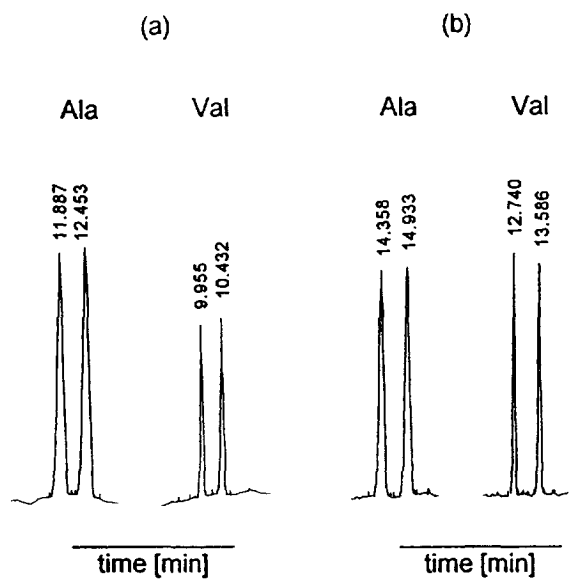

Fig. 5. Electropherograms of AQC-Ala and -Val using (a) 0.5% (w/v) of β-CD polymer and (b) 5 m*M* DM-β-CD, all other conditions as in Fig. 2. Ordinate: absorbance at 245 nm.

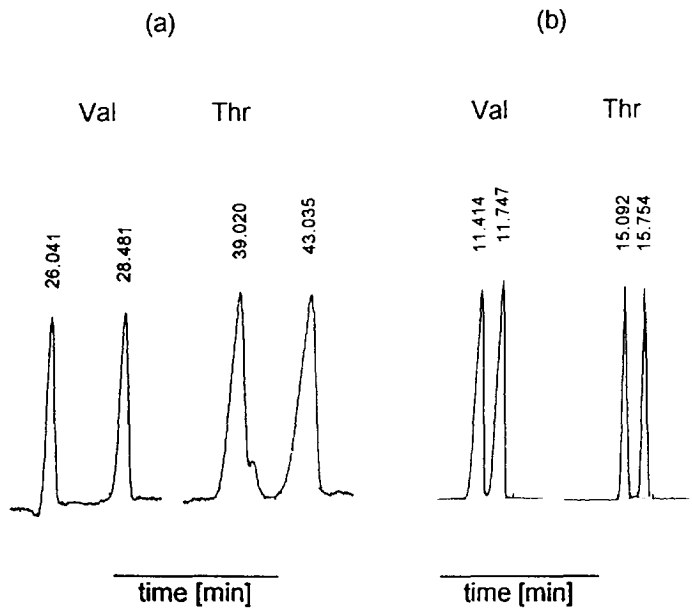

Fig. 6. Electropherograms of AQC-Val and -Thr with (a) 5 m*M* TM-β-CD and (b) 30 m*M* TM-β-CD, all other conditions as in Fig. 2. Ordinate: absorbance at 245 nm.

selectivities achieved for the aromatic amino acids are very low, especially with TM-β-CD.

3.1.4. CM-β-CD polymer

This selector is negatively charged at the given pH. This results in long analysis times and therefore rather broad peaks in most instances. This selector is of special interest only for those zwitterionic amino acids for which no baseline resolution is obtained by all other selectors discussed, i.e., Arg and His and mono-derivatized Lys. With 0.5% (w/v) of the carboxymethylated β-CD polymer, Arg, Lys and His are resolved with enantioselectivity coefficients of 1.040, 1.053 and 1.046, respectively, which means separation to baseline in all three cases. The electropherograms of Arg and His are shown in Fig. 7. The carboxymethylated β-CD polymer is assumed to be well suited for these basic amino acids primarily because of its function as a charged carrier for electrophoretically immobile zwitterions. In addition, ion-pairing interactions involved are supposed to contribute to good selectivity. For all other amino acids the CM-β-CD polymer is unsuited not only because of the very long analysis times but also due to low enantioselectivity coefficients obtained. The negative charges in both, analyte and selector, presumably prohibit selective complexation.

Enantioresolution of the AQC-amino acids has been discussed in this context on the base of apparent selectivities which determine the electrophoretic resolution in the presence of an EOF. The

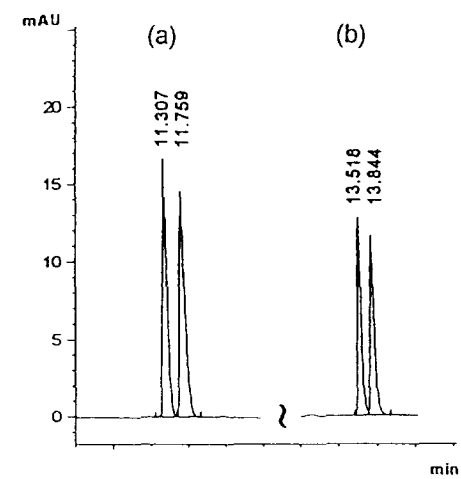

Fig. 7. Electropherograms of (a) AQC-Arg and (b) AQC-His with 0.5% (w/v) CM-β-CD polymer, all other conditions as in Fig. 2. Ordinate: absorbance at 245 nm.

EOF in all measurements reported ranged between 15 and $20 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1}. Structure–enantioselectivity relationships, which might be of interest for this set of analytes, cannot be discussed in terms of the apparent selectivity but require effective selectivity coefficients. Complexation constants between analyte and selector can be determined from effective mobilities measured at various selector concentrations by means of a curve fitting procedure. A discussion of these complexation constants is postponed to a following paper which particularly focuses on aspects of structure vs. enantioselectivity relationships, addressing structural elements in the analyte as well as in the selectors [18].

The aim of the present paper, however, is the presentation of optimized conditions for the electrophoretic enantioresolution of AQC-amino acids performed under conditions which do not exclude EOF. As it has already been stated in the introduction, these separations are of considerable interest in amino acid analysis, as AQC derivatization is very fast and simple without need for further sample clean up, finally yielding stable and highly UV absorbing and fluorescent derivatives.

Acknowledgments

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