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Enantiomeric separations in capillary zone electrophoresis using a chiral crown ether

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ABSTRACT

Chiral separations of optically active amines using 18-crown-6 tetracarboxylic acid ($18C6H_4$) as pseudo-stationary phase in capillary zone electrophoresis were studied. The buffer composition and pH strongly affected the performance of the separation. Although good separations were achieved using tertiary (triethylamine) or quaternary amines (tetramethylammonium) as cationic buffer constituents, with respect to speed of analysis, separation factor and efficiency, the best results were obtained with Tris. pH was shown to be an important factor for method optimization. Although inclusion complexation of primary amines is enhanced with increasing pH, the difference in the Gibbs free energy of the complexation of D,L-tryptophan was highest at pH 2.5. A synergistic effect on the resolution of chiral amines was observed when $18C6H_4$ and cyclodextrin were dissolved in the same buffer. With this system the enantiomers of noradrenaline could be baseline separated even though neither of the two chiral selectors by itself was able to resolve the enantiomers. The separation factor depends strongly on the concentrations of the two selectors. It was demonstrated that simply complexation of the cyclodextrin rather than chiral recognition was the basis for the improved separation.

INTRODUCTION

Stereoisomers are molecules that are identical in atomic constitution and bonding, but differ in the three-dimensional arrangement of the atoms [1]. Biological systems readily distinguish between stereoisomers so that different pharmacokinetic (absorption, distribution, etc.) and pharmacological profiles occur. Thus, the enantiomeric composition of a chiral drug is an important purity criterion in pharmaceutical analysis. For this purpose, chromatography has been established as a routine technique since chiral stationary phases became commercially available [2,3]. While HPLC sometimes shows poor efficiency and consequently low sensitivity, GC is limited to volatile compounds.

After Gassmann *et al.* [4] demonstrated the suitability of capillary zone electrophoresis (CZE) for chiral separation, this method has been developed into a powerful tool for this purpose [5,6]. Usually chiral separation is accomplished by dissolving an optically active selector in the running buffer. Enantiomers form diastereomeric complexes with the chiral selector and are separated on the basis of their different association constants. According to the kind of chiral selector, four different separation principles are used. The first is ligand-exchange electrophoresis, which is based on multi-component chelate complexes with a central ion (*e.g.*, Cu^{2+}) and two chiral bifunctional ligands [7]. Second, enantioseparation is achieved by solubilization by optically active micelles such as mixed mi-

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celles of sodium dodecyl sulphate and dodecylvaline [8] or bile salts [9]. Recently, the use of proteins such as bovine serum albumin or ovomucoid as buffer additive has been described [10]. Although these proteins should have a broad application range, their use as buffer additive seems to be limited with respect to their high UV absorbance. The fourth separation principle is based on inclusion complexes (host–guest complexes) between a chiral ligand (host molecule) and an analyte (guest molecule). In CZE two different types of host compounds are used: (i) cyclodextrins and their derivatives [11,12] and (ii) 18-crown-6 tetracarboxylic acid ($18C6H_4$).

In previous papers [13,14], the potential of $18C6H_4$ for enantioseparation and the recognition mechanisms were investigated. The aim of this work was to study the influence of experimental conditions such as buffer composition and pH. In addition, a synergistic effect of cyclodextrin and crown ether in the same buffer system was systematically investigated.

EXPERIMENTAL

Instrumentation

All separations were carried out using a P/ACE 2000 capillary electrophoresis instrument (Beckman, Palo Alto, CA, USA). Electrophoresis was performed in an untreated fused-silica capillary tube [50 cm (to detector) × 75 μm I.D.], applying a potential of 15 kV. The sample was injected with high pressure for 1 s and detected by measuring the UV absorbance at 254 nm. If not mentioned otherwise, the capillary temperature was maintained constant at 25°C.

Chemicals

All chemicals were of analytical-reagent grade if not stated otherwise and were used as obtained. Citric acid, 18-crown-6 tetracarboxylic acid ($18C6H_4$) (purum grade) and tris(hydroxymethyl)aminomethane (Tris) were obtained from Merck (Darmstadt, Germany). All D,L-amino acids, (±)-noradrenaline, (±)-norephedrine and (±)-phenylglycinol were purchased from Sigma (St. Louis, MO, USA). Hydroxypropyl-β-cyclodextrin was obtained from Janssen Chimica (Beerse, Belgium).

RESULTS AND DISCUSSION

Chiral 18-crown-6 tetracarboxylic acid was first synthesized by Lehn and co-workers [15,16]. The polyether ring system builds a cavity which is able to form stable inclusion complexes with alkali and alkaline earth metal cations, and particularly with potassium, ammonium and protonated alkylamines. In a complex the six oxygens of the ring system are oriented to the centre of the cavity and roughly define a plane. Ammonium or primary alkylamines held inside the cavity are bound by three $^{+}NH\cdots O$ hydrogen bonds in a tripod arrangement (Fig. 1). Although complexation of the primary amine in the cavity is essential for chiral separation, it is insufficient for chiral recognition. Additional interactions between the substituents of the crown ether ring and the ligand are necessary for chiral discrimination. In an earlier investigation [14] we proposed two mechanisms for chiral recognition. First, the carboxylic acid pairs on both sides of the ring plain act like chiral barriers dividing the space available for the substituents of the chiral carbon atom into two cavities. According to the size and the spatial arrangement of these substituents, diastereomeric complexes with different formation constants are formed. A second mechanism is given by the carboxylic acids which may show electrostatic interactions (hydrogen bonds or repulsion forces) with polar substituents of the ligand.

In CZE, the chiral crown ether is simply added to the buffer system where it behaves like a homogeneous distributed pseudo-phase for the analytes. Although complexation of the solutes

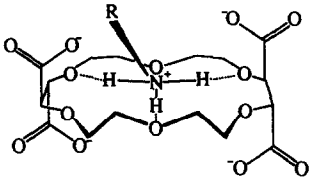

Fig. 1. Structure of a complex of $18C6H_4$ with a primary amine.

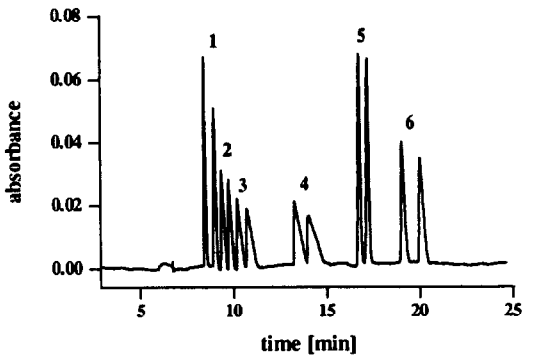

Fig. 2. Chiral separation of six primary amines by CZE with $18C6H_4$ as chiral selector. Peaks: 1 = azatryptophan; 2 = norephedrin; 3 = phenylglycinol; 4 = 3-amino-3-phenylpropionic acid; 5 = tryptophan; 6 = homophenylalanine. Experimental conditions: 10 m*M* 18C6H4–10 m*M* Tris, adjusted to pH 2.5 with citric acid.

with buffer constituents always decreases the efficiency of a separation, high theoretical plate numbers are usually found in CZE. Fig. 2 shows the separation of six primary amines. Most peaks show symmetric peak shapes, but peak pair 4 has a striking tailing which probably originates from a strong interaction of both enantiomers with the crown ether ring. A high Gibbs free energy, ΔG, of the complexation of an enantiomer with the crown ether causes not only long migration times but also broad peaks. Chiral selectivity, however, is determined by the difference in the Gibbs free energies, $\Delta(\Delta G)$, of two enantiomers. Thus, best resolution is obtained if $\Delta(\Delta G)$ is maximized while ΔG is minimized [17]. For peak pair 4, a relatively high ΔG of the complex formation with the crown ether is assumed to be responsible for the peak shape. In addition to the complex formation between host and guest, a number of experimental conditions such as buffer composition and pH exert an influence on the separation.

Influence of buffer composition

Although chiral separation is in principle possible in a buffer solution consisting solely of $18C6H_4$ [13] or in citric acid buffer, only poor results were obtained with respect to peak shape and efficiency because of electrophoretic dispersion of the cationic analytes. As explained by the theory, electrophoretic dispersion is minimized if the mobility of the corresponding ionic species of the buffer matches the mobility of the analyte [18] as closely as possible. A buffer composed of $18C6H_4$ contains only protons, which have a much higher mobility that the cationic analytes. Consequently, all sample components elute with strong tailing. $18C6H_4$ is well known to form stable complexes with sodium, potassium and ammonium ions. All experiments using background electrolytes with these cations failed because they form complexes with the crown ether which are approximately ten times more stable than those with primary amines [16].

On the other hand, tertiary or quaternary amines such as triethylamine or tetramethylammonium should be well suited as cationic buffer constituents as they do not bind to the crown ether ring and have mobilities similar to those of the analytes. As expected, the peak symmetry was much improved using these cations but the efficiency still remained poor. Moreover, the concentration of triethylamine showed a dramatic influence on the migration time which cannot be explained only by the impact of the higher ionic strength in the buffer (Table I). It is very likely that the complex formation constants between host and guests are also influenced by the buffer concentration. As shown in Table I, no more peaks could be recorded within a reasonable time for triethylamine concentrations higher than 50 m*M*. With the increasing migration time the separation factor and resolution also increased. Similar results were obtained using tetramethylammonium as cationic buffer component.

Although Tris is a primary amine which competes with the analytes for the crown ether sites, excellent separations were obtained in a Tris–citric acid buffer. The complex stability of $18C6H_4$ with Tris is probably lower than those with the analytes that replace Tris from the complexes. In this system only a moderate increase in the migration time occurred while the separation factor and the resolution remained approximately constant (Table I). Usually theoretical plate numbers of more than 50 000 were found.

TABLE I

INFLUENCE OF THE BUFFER CONCENTRATION OF TRIETHYLAMINE (TA) AND TRIS ON MIGRATION TIMES OF THE FIRST-ELUTED ENANTIOMER (t_R), SEPARATION FACTORS (α'), RESOLUTION (R_s) AND THEORETICAL PLATE NUMBERS (N) FOR THE CHIRAL SEPARATION OF D,L-TRYPTOPHAN

Experimental conditions: 10 mM 18C6H$_4$ and the given concentration of TA or Tris were titrated to pH 2.5 with citric acid.

Buffer	Concentration (mM)	t_R (min)	α'	R_s	N
TA	10	27.6	1.072	2.42	19 200
	30	59.1	1.176	6.95	31 500
	50	117.8	1.406	8.17	11 300
	80	>180	–	–	–
Tris	10	22.9	1.070	3.38	58 400
	30	36.7	1.084	3.88	37 100
	50	43.7	1.091	4.72	61 000
	80	46.3	1.080	4.09	50 600

Influence of pH

As 18C6H$_4$ is a weak acid, the complex formation with a ligand is strongly dependent on the buffer pH. With increasing pH the negative charge of the crown ether will increase by successive dissociation of the four carboxylic acid groups depending on their individual pK values (2.13, 2.84, 4.29, 4.88 [19]). This is shown in Fig. 3. The apparent mobility, μ_{app}, of a protonated amine complexed by the crown ether depends on the mobility of the free amine, μ_{amine}, the degree of dissociation, α, of the complex and the mobility of the complex, $\mu_{complex}$, according to the equation

$$\mu_{app} = \alpha\mu_{amine} + (1 - \alpha)\mu_{complex} \quad (1)$$

All three factors on the right-hand side of the equation depend strongly on the pH of the buffer system. The mobility of the free amine is influenced by the net charge, which varies with the pH as shown in Fig. 3 for tryptophan. The degree of dissociation of the crown either complex with an amine is known to depend on the degree of dissociation of the crown ether (Fig. 3) because carboxylate groups additionally stabilize the complex by cooperative crown ether and carboxylate binding interactions [19]. Thus complex formation is enhanced with increase in pH. In addition, the overall charge of the complex will become more and more negative with increasing pH values because the net charge of tryptophan decreases from +1 to zero whereas the net charge of the crown ether increases from zero to −4. Whereas μ_{Trp} is the same for both enantiomers, α and the mobility of the complex will differ for D- and L-Trp. Fig. 4 shows the separation of D,L-Trp at different pH values of the buffer. Baseline separations were obtained at all pH values. The best resolution could be calculated at pH 3.5 although efficiency was the lowest (Table II). In terms of migration time, efficiency and resolution, pH values of 2.5 and 4.6 gave equivalent results even though the enantiomers migrate as cations in the former instance and as anions in the latter.

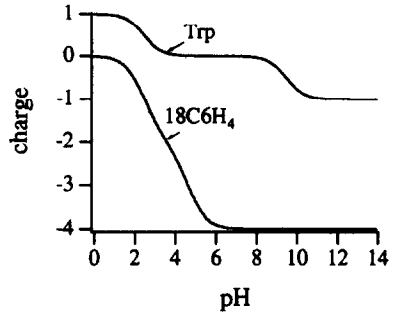

Fig. 3. Plot of the net charge of tryptophan and 18C6H$_4$ *versus* the pH calculated from the pK values.

The influence of pH on the difference in the Gibbs free energy of complexation was also studied. As one can conclude from the relationship between $\Delta(\Delta G)$ and the separation factor

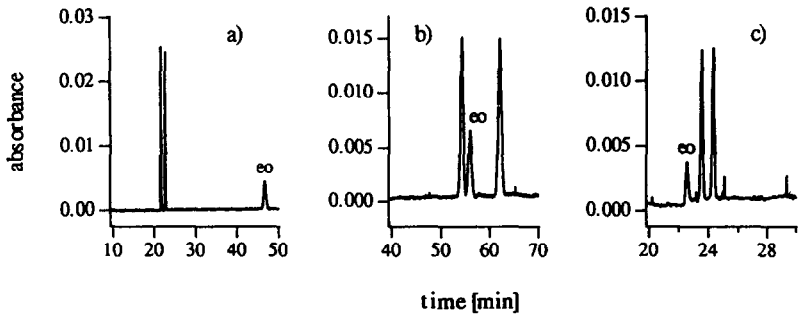

Fig. 4. Separation of D,L-tryptophan at pH (a) 2.5, (b) 3.5 and (c) 4.6. Buffer system: (a) 10 m*M* Tris–10 m*M* $18C6H_4$, adjusted to pH 2.5 with citric acid; (b) 30 m*M* phosphoric acid–10 m*M* $18C6H_4$, adjusted to pH 3.5 with Tris; (c) 10 m*M* phosphoric acid–10 m*M* $18C6H_4$, adjusted to pH 4.6 with Tris.

α', which is defined arbitrarily by the term t_2/t_1, where t_2 represents the migration time of the second-eluted enantiomer and t_1 that of the antipode, the more negative $\Delta(\Delta G)$ is the higher is the separation factor:

$$-\Delta(\Delta G) = RT \ln \alpha' \quad (2)$$

where T is the absolute temperature and R is the gas constant. According to

$$\Delta(\Delta G) = \Delta(\Delta H) - T\,\Delta(\Delta S) \quad (3)$$

the difference in the Gibbs free energy is the sum of the enthalpy difference, $\Delta(\Delta H)$, and the negative entropic contribution, $-T\,\Delta(\Delta S)$. In order to calculate the thermodynamic data for the separation. Van't Hoff plots at different buffer pHs were constructed according to the procedure described in ref. 14. The results are shown in Fig. 5. The chiral separation of D,L-Trp is controlled by the highly negative values of the enthalpy difference, whereas the entropic contribution, $T\,\Delta(\Delta S)$, has an unfavourable influence on $\Delta(\Delta G)$, and, hence, on the separation. The biggest difference in the complex formation was found at pH 2.5 where $\Delta(\Delta H)$ takes the most negative value. With increasing pH $\Delta(\Delta H)$ also increases, but, as the entropy difference decreases at the same rate, $\Delta(\Delta G)$ is only slightly affected.

TABLE II

RETENTION TIMES OF THE FIRST ELUTED ENANTIOMER (t_R), SEPARATION FACTORS (α'), RESOLUTION (R_s) AND THEORETICAL PLATE NUMBERS (N) FROM THE SEPARATION OF D,L-TRYPTOPHAN WITH $18C6H_4$ AT DIFFERENT pH VALUES

Experimental conditions as described in Fig. 4. Buffer for pH 1.0, 10 m*M* Tris–10 m*M* $18C6H_4$, adjusted to pH 1 with 1 *M* HCl; buffer for pH 6.5, 10 m*M* phosphoric acid–10 m*M* $18C6H_4$, adjusted to pH 6.5 with Tris.

Parameter	pH				
	1.0	2.5	3.5	4.6	6.5
t_R (min)	89.0	21.6	54.3	23.6	13.7
α'	1.011	1.050	1.143	1.033	1.011
R_s	0.94	3.24	6.18	2.64	0.77
N	119 500	77 700	35 100	113 200	75 300

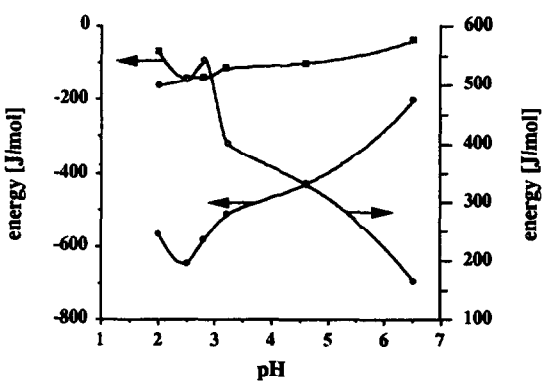

Fig. 5. Influence of the pH on the difference in (■) Gibbs free energy, $\Delta(\Delta G)$, (●) enthalpy difference, $\Delta(\Delta H)$, and (○) the entropy term $-T\,\Delta(\Delta S)$, all at 298 K, for the complex formation of D,L-Trp. Buffer system: 10 m*M* Tris–10 m*M* $18C6H_4$, adjusted to the given pH value with citric acid. Thermodynamic data were calculated from Van't Hoff plots at 20, 25, 30, 35 and 40°C.

Synergistic effects of 18C6H$_4$ with cyclodextrins

A synergistic effect on the separation of enantiomers was found if 18CH$_4$ and cyclodextrin were dissolved in the same buffer. This is shown in Fig. 6. Whereas none of both chiral selectors by itself was able to resolve the enantiomers of noradrenaline, a mixture of 18C6H$_4$ and hydroxypropyl-β-cyclodextrin (HP-β-CD) allowed baseline separation. Because HP-β-CD did not show any chiral recognition, mere complexation of the aromatic functionality of noradrenaline by the cyclodextrin cavity enhanced the chiral recognition of the crown ether. A ternary complex of the form [HP-β-CD · noradrenaline · 18C6H$_4$] is assumed to be responsible for these results. According to the law of mass action, the separation factor is strongly affected by both the crown ether and the cyclodextrin concentration, as shown in Fig. 7a. Without any cyclodextrin in the buffer the influence of the crown ether concentration on the separation factor was small. Interestingly, for all crown ether concentrations investigated the separation factor of noradrenaline first decreased to 1 on adding 5 m*M* HP-β-CD. However, with higher cyclodextrin concentrations the resolution improved markedly. Finally, baseline separation was obtained at 20 m*M* cyclodextrin. Although two complexing agents were present in the buffer with each contributing to peak broadening, the efficiency did not decrease significantly.

In contrast to noradrenaline, increasing concentrations of HP-β-CD did not change the migration time of D,L-Trp. Hence it can be concluded that Trp does not form measurable inclusion complexes with HP-β-CD. Fig. 7b shows plots of separation factor *versus* cyclodextrin concentration. As can be seen, the cyclodextrin concentration has almost no effect on the separation factor. From the results discussed above, complexation of the enantiomers with the cyclodextrin rather than chiral recognition is essential to obtain a synergistic effect with 18C6H$_4$.

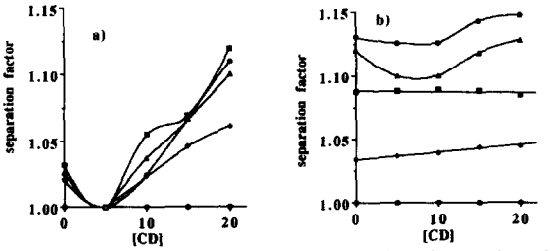

Fig. 7. Plots of the separation factor (a) (±)-noradrenaline and (b) D,L-Trp *versus* the concentration of HP-β-CD in the buffer system: 30 m*M* Tris with the appropriate concentrations of 18C6H$_4$ and HP-β-CD, adjusted to pH 2.5 with citric acid. 18C6H$_4$ concentration: ● = 0; ◆ = 5; ■ = 10; ▲ = 15; ○ = 20 m*M*.

CONCLUSIONS

This paper has demonstrated the suitability of 18C6H$_4$ for chiral separations of primary amines

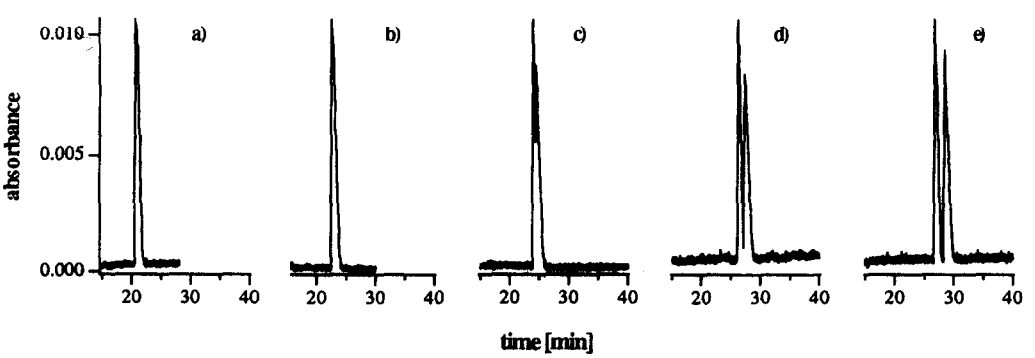

Fig. 6. Chiral separation of noradrenaline at different concentrations of HP-β-CD in the buffer system: (a) 0, (b) 5, (c) 10, (d) 15 and (e) 20 m*M*. Buffer system: 5 m*M* 18C6H$_4$–30 m*M* Tris with the appropriate concentration of HP-β-CD, adjusted to pH 2.5 with citric acid.

in CZE. The performance of the separation is strongly influenced by the buffer composition. Because of their strong complexation with $18C6H_4$, alkali and alkaline earth metal cations have to be avoided as buffer constituents. Quaternary and tertiary ammonium cations are well suited for many separations but have a great impact on the migration times of the enantiomers, which increase dramatically at higher concentrations. With respect to speed of analysis, selectivity and efficiency, Tris provides the best results as a cationic buffer constituent.

In addition to the buffer composition, pH is the most effective parameter for method optimization. Although one could expect better separations at higher pH values, where the crown ether is more negatively charged and has a stronger effect on the migration of the analytes, this assumption could not be confirmed by our results. More separation problems in our laboratory could be solved at a buffer pH of 2.5. Thermodynamic data for the complex formation confirmed these results. The difference in the complex formation of D,L-Trp with $18C6H_4$ was most pronounced at pH 2.5.

$18C6H_4$ and cyclodextrins dissolved in the same buffer showed a synergistic effect on the chiral separation of aromatic amines. Chiral recognition of noradrenaline was weak when using $18C6H_4$ as the only chiral selector but could be improved significantly by adding cyclodextrin to the buffer. Mere complexation of the analytes by the cyclodextrin rather than chiral recognition was necessary to improve the resolution. This principle is a simple approach to optimizing a separation if one chiral selector gives insufficient results. From these results it can be assumed that together with a chiral selector, also non-chiral complexing agents could improve the resolution.

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