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Chiral separations of amino acids by capillary electrophoresis and high-performance liquid chromatography employing chiral crown ethers

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Abstract

High-performance liquid chromatography with a chiral crown ether stationary phase and capillary electrophoresis (CE) with a chiral crown ether dissolved in the operating buffer were used for the separation of enantiomers of analogues of DOPA and tyrosine and some analogues of γ-aminobutyric acid (GABA). CE and HPLC yielded similar results for the DOPA and tyrosine analogues. However, for the analogues of GABA, three of the four compounds tested were well resolved by HPLC but only one was well resolved by CE. It was necessary to use an indirect detection scheme for the CE of GABA analogues. The influence of substituents on the different compounds on the resolution factors observed by the two methods is discussed, in addition to the advantages and disadvantages of the two methods in practical applications.

1. Introduction

High-performance liquid chromatography (HPLC) using various types of chiral stationary phases, mobile phase additives and derivatizing agents has been used extensively for the separation of amino acids [1,2]. Capillary electrophoresis (CE) has also been employed for chiral separations of amino acids, but derivatization has usually been required [3]. More recently, Kuhn and co-workers [4,5] described the use of a chiral crown ether for the chiral resolution of various amino acids, peptides and optically active amines without prior derivatization. In this work, we applied both capillary electrophoresis with a chiral crown ether in the operating buffer and HPLC using a crown ether stationary phase to the separation of two series of compounds, analogues of DOPA and tyrosine and analogues of γ-aminobutyric acid (GABA). We explored the influence of the structure of the analyte molecules in a series of analogues on the degree of separation obtained and compared the results obtained for the two series of compounds using the two different procedures.

2. Experimental

All CE experiments were carried out using a Beckman P/ACE 2100 system. The system was interfaced to an IBM-compatible personal com-

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puter and Beckman System Gold software was used for data collection and manipulation. Separations were carried out in a fused-silica capillary tube (Beckman Instruments, Neuilly, France). For the separation of DOPA and tyrosine analogues, the dimensions of the tube were 27 cm × 50 μm I.D., with the detector located 7 cm from the outlet end of the capillary. A detector slit size of 100 μm × 200 μm was used for experiments with direct detection. The detection wavelength was 280 nm. The capillary was thermostated at 20°C. Most experiments were carried out using 50 m*M* sodium phosphate buffer (pH 2.2) containing 30 m*M* 18-crown-6-tetracarboxylic acid (18-crown-6-TCA). For experiments investigating the effects of the concentration of the crown ether, the concentration of 18-crown-6-TCA was varied from 0 to 100 m*M*. Alternatively, 100 m*M* Tris buffer with the pH adjusted to 2.5 with citric acid and containing 30 m*M* 18-crown-6-TCA was used. Test compounds were dissolved in water at a concentration of ca. 0.25 mg/ml.

For chiral resolutions of the GABA analogues, which have poor UV absorbance, an electrolyte solution containing 6 m*M* benzyltrimethylammonium chloride (BTMACl) and 30 m*M* 18-crown-6-TCA and the pH adjusted to 3.7 with 1 *M* NaOH was used, with indirect detection. The detector wavelength was set at 214 nm. A slit size of 50 μm × 200 μm was used for measurements with indirect detection. A 57 cm × 75 μm I.D. capillary thermostated at 25°C was used for these experiments. Test compounds were dissolved in water at a concentration of ca. 0.5 mg/ml.

HPLC experiments were carried out using a conventional HPLC system consisting of a Waters M590 pump, a Waters WISP 710B autosampler and either a Waters M481 UV detector or a Kratos Spectroflow 773 UV detector. A Crownpak CR(+) column (15 cm × 4 mm I.D., 5-μm packing) obtained form Daicel (Tokyo, Japan) was used. The column was thermostated using either a water-bath or a methanol bath. A guard column (2.1 cm × 2.0 mm I.D.) containing glass beads (30 50 μm diameter) was used. Unless indicated otherwise, the following chromatographic conditions were used for the analogues of DOPA and tyrosine: mobile phase, 0.16 *M* $HClO_4$–10% methanol (pH 1.05); temperature, 20°C; flow-rate, 1 ml/min.; detection wavelength, 280 nm; injection volume, 5 μl. Sample solutions were prepared in the mobile phase at concentrations ranging from about 0.2 to 0.3 mg/ml. The conditions used for the GABA analogues were as follows: mobile phase, 0.11 *M* $HClO_4$ (pH 1.05); temperature, 10°C; flow-rate, 0.5 ml/min; detection wavelength, 210 nm; injection volume, 10 μl. Sample solutions were prepared in the mobile phase at a concentration of ca. 0.6 mg/ml.

Chemicals were of analytical reagent grade, unless stated otherwise. Phosphoric acid, perchloric acid, methanol, sodium hydroxide and 18-crown-6-TCA were obtained from Merck (Darmstadt, Germany). Citric acid was obtained from Prolabo (Paris, France). Benzyltrimethylammonium chloride was obtained from ICN Pharmaceuticals (Plainview, NY, USA). Tris, D,L-DOPA, D-DOPA, tyrosine, L-tyrosine, (±)-ephedrine and (±)-norephedrine were obtained form Sigma (Saint Quentin Fallavier, France). Analogues of DOPA and tyrosine (compounds **1–7**) were synthesized in-house by previously published methods [6–8]. Analogues of GABA (compounds **8–11**) were also synthesized by published procedures [9–12].

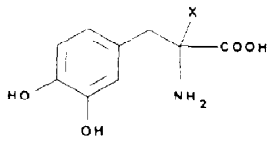
3. Results and Discussion

3.1. Results for analogues of DOPA and tyrosine

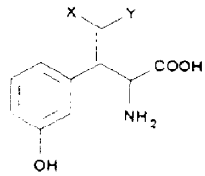
Influence of structure of analytes on chiral resolution

We investigated a series of analogues of DOPA and tyrosine with various substituents. Structures of the compounds and results of HPLC and CE experiments of this series of compounds are given in Table 1. Structures of the crown ethers used in the experiments are shown in Fig. 1. It can be seen that for compounds having three substituents on the carbon α to the amino group (**1–4**), no resolution was

Table 1
Summary of results obtained by HPLC and CE for analogues of DOPA and tyrosine



Structure A (compounds **1–4**)



Structure B (compounds **5–7**)

Compound	Structure	X	Y	HPLC results[a]			CE results[b]		
				t_{r1}	t_{r2}	R_s	t_{m1}	t_{m2}	R_s
DOPA				3.24	4.78	4.4	5.84	5.97	1.1
Tyrosine				3.95	5.77	4.4	5.86	6.00	1.0
1[c]	A	CH_2F		3.13	–	0	15.51	–	0
2[c]	A	CHF_2		2.80	–	0	26.84	–	0
3[c]	A	$CH{=}CH_2$		5.20	–	0	11.93	–	0
4[c]	A	$CH{\equiv}CH$		3.44	–	0	24.98	–	0
5	B	H	H	5.73	11.44	7.8	8.69	10.11	6.3
6	B	F	H	5.26	8.00	4.5	9.72	13.31	11.1
6[d]	B	F	H	17.25	23.38	4.3			
7[d]	B	H	F	15.97	17.39	1.1	10.57	11.09	2.0

[a] t_{r1} = Retention time of first-eluting enantiomer and t_{r2} = retention time of second-eluting enantiomer from HPLC column.
[b] t_{m1} = migration time of faster migrating enantiomer and t_{m2} = migration time of slower migrating enantiomer by CE.
[c] CE operating buffer, 100 m*M* Tris–citrate–30 m*M* 18-crown-6-TCA (pH 2.5).
[d] HPLC mobile phase, 0.06 *M* $HClO_4$ (pH 1.5); flow-rate, 0.5 ml/min; temperature, 20°C.

obtained with CE or HPLC. Even using eluents with a higher perchloric acid concentration (0.16 *M*) and no methanol and working at lower temperatures (0–10°C) did not allow the resolution of these compounds. When Behr et al. [13] measured stability constants for complexes of various amines with chiral crown ethers, including 18-crown-6-TCA, they determined that primary amines with three α-substituents formed very weak complexes as compared with analogues with only two substituents at the α-carbon. Kuhn et al. [5] compared results for ephedrine, a secondary amine that does not fit into the crown ether cavity, and norephedrine, its demethylated analogue, by CE using 18-crown-6-TCA in the buffer. They found that whereas the enantiomers of norephedrine were resolved, no resolution was observed for ephedrine. The migration time of ephedrine in the CE system was also significantly faster than those of the norephedrine enantiomers. From this, they concluded that complexation of the ammonium cation with the crown ether is necessary for chiral recognition by CE using 18-crown-6-TCA. In our laboratory, we carried out analogous experiments by HPLC using the Crownpak CR(+) column. We were also unable to resolve the enantiomers of ephedrine, whereas the enantiomers of norephedrine were resolved with longer retention times than that observed for ephedrine. Therefore, the fact that no separation was seen for the α-substituted compounds by either HPLC or CE is most likely due to very weak, if any, complexation of these compounds with the crown ethers.

The remaining compounds have a C=C double bond in the position β to the amino group. All of these compounds were resolved using both CE and HPLC, but there are significant differences in the results. Using HPLC, **5** was better resolved than DOPA and tyrosine. The resolution obtained for **6** was similar to that for DOPA and

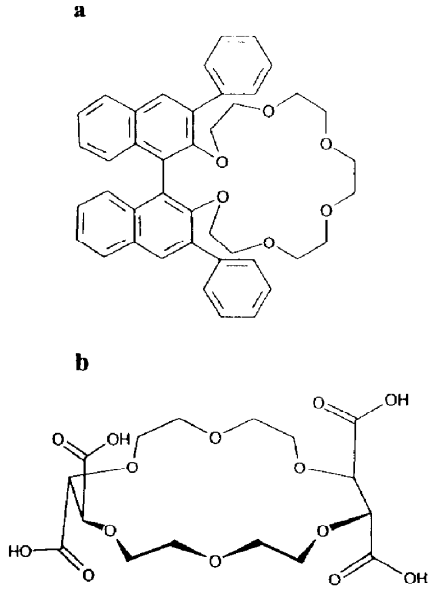


Fig. 1. (a) (*S*)-2,3:4,5-Bis(1,2-3-phenylnaphtho)-1,6,9,12,15,18-hexaoxacycloeicosa-2,4-diene crown ether incorporated into the stationary phase of the Crownpak CR(+) HPLC column; (b) (2*R*,3*R*,11*R*,12*R*)-(+)-1,4,7,10,13,16-hexaoxacyclooctadecane-2,3,11,12-tetracarboxylic acid crown ether used in the capillary electrophoresis operating buffer.

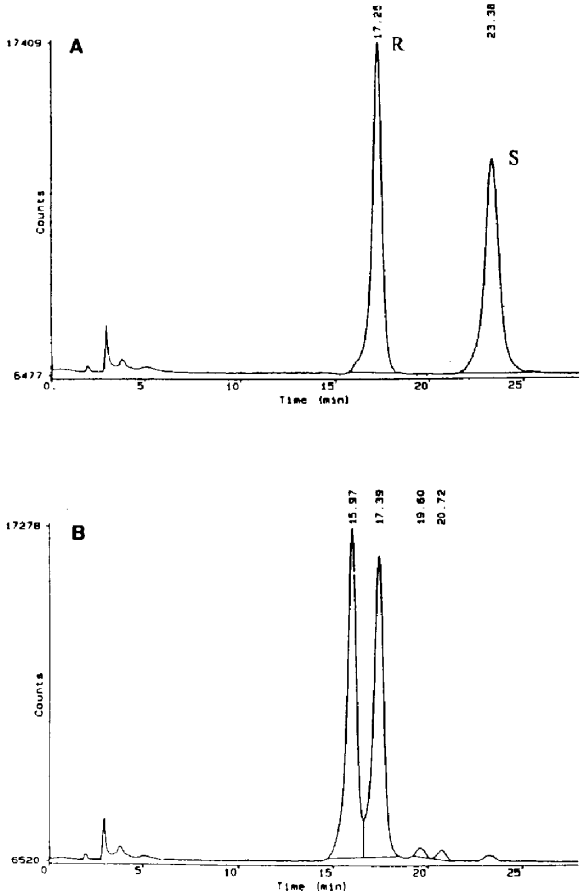


Fig. 2. Representative chromatograms obtained for analogues of tyrosine on the Crownpak CR(+) column. (A) Compound **6**, 1.05 μg injected; (B) compound **7**, 1.5 μg injected. Conditions: mobile phase, 0.06 *M* $HClO_4$ (pH 1.5); temperature, 20°C; flow-rate, 0.5 ml/min; detection wavelength, 280 nm. Peaks for **7** are not chirally identified because the individual enantiomers were not available.

tyrosine. For **7**, the resolution was much poorer than for DOPA and tyrosine. The order of elution was determined for those compounds for which at least one of the individual enantiomers was available (DOPA, tyrosine and **6**). In each instance, the *R*-enantiomer was eluted first, followed by the *S*-enantiomer. This indicates that the *S*-enantiomer forms a stronger complex with the (*S*)-(+)-crown ether. Representative chromatograms are shown in Fig. 2.

However, the CE results were clearly different. Compounds **5** and **6** were much better resolved by CE than DOPA and tyrosine, whereas only a modest increase in resolution was observed for **7**. Representative electropherograms are shown in Fig. 3. The large resolution factors observed for **5** and **6** are in contrast to earlier reports of CE using 18-crown-6-TCA as a chiral selector, where more modest resolution factors, between 0 and 3, were observed [4,5]. Large resolution factors imply that there is a large difference between the stability constants for the complexes of the two enantiomers with the crown ether. The large resolution factors observed for **5** and **6** can probably be explained by the altered geometry at the β-carbon. Apparently, this geometry leads to the stabilization of the complex of 18-crown-6-TCA with one of the two enantiomers. The proximity of the fluorine atom to the amino group in **7** most likely destabilizes this complex and therefore a more modest resolution factor is observed. The rela-

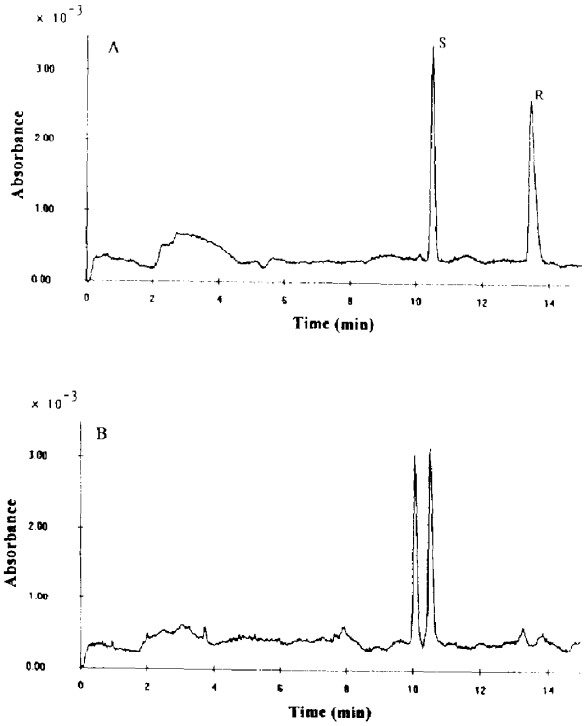


Fig. 3. Representative electropherograms obtained for analogues of tyrosine. (A) Compound **6** (12 ng injected); (B) Compound **7** (ca. 8 ng injected). Conditions: capillary, 27 cm × 50 μm I.D., fused silica; buffer, 50 m*M* sodium phosphate (pH 2.2)–30 m*M* 18-crown-6-TCA; temperature, 20°C; applied voltage, 10 kV; detection wavelength, 280 nm. Peaks for **7** are not chirally identified because the individual enantiomers were not available.

tively poor resolution observed for **7** by HPLC can probably be explained in the same way.

Again, the order of migration was determined for those compounds for which at least one single enantiomer was available. In contrast to the results observed by HPLC, in the CE system the *S*-enantiomer migrated fastest, followed by the *R*-enantiomer for the compounds tested. However, it is not possible to make generalizations with regard to migration order. At least one example exists in the literature in which the *R*-enantiomer migrates fastest in a CE system using 18-crown-6 TCA [14]. The reversal of elution order as compared with HPLC with the Crownpak CR(+) column is not surprising, since that column uses the *S*-enantiomer of the crown ether [15], whereas in the CE experiment the *R*-enantiomer of 18-crown-6 TCA is used [16].

Use of CE to study the interaction between the amino acid and crown ether

By HPLC, it is difficult to obtain information about the relative stability of complexes of analyte species with a chiral selector in the stationary phase, owing to non-stereospecific interactions of the analytes with the stationary phase. However, one can gain this sort of information by CE, in which there are few competing interactions, by varying the amount of chiral selector in the operating buffer. Penn et al. [17] have shown this to be a useful approach to gain information on the kinetics of interactions of compounds with cyclodextrins.

In this experiment, the migration times of the enantiomers of the compounds were measured and plotted as a function of the concentration of 18-crown-6-TCA in the operating buffer. Plots for DOPA and **5**–**7** are shown in Fig. 4. A calculation of the actual stability constants is not possible using these data because the contribution of electroosmotic flow, which is very low at pH 2.2, was not measured. However, it is possible to obtain information on the relative extent of complexation of the two enantiomers.

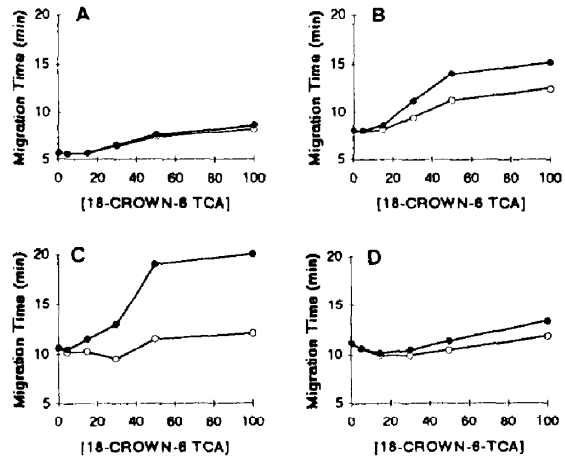


Fig. 4. Effect of concentration of 18-crown-6-TCA in operating buffer on migration times for analogues of DOPA and tyrosine. (A) DOPA; (B) **5**; (C) **6**; (D) **7**. ○ = first peak; ● = second peak.

For all compounds, the plots show that the migration times initially decrease, then increase as a function of the concentration of crown ether in the buffer, whereas the resolution between the two enantiomers always increases with the crown ether concentration. The initial decrease in migration time is probably a result of an increase in electroosmotic flow as a function of crown ether concentration. Although the electroosmotic flow was very slow and was not measured, it can have a significant effect on the migration time, particularly of slower migrating species. In a separate experiment, the migration time of pyridine, which does not interact with the crown ether, was measured as a function of crown ether in the buffer, and its migration time decreased slightly as the crown ether concentration was increased. As the crown ether is not expected to have any effect on the electrophoretic mobility of pyridine, this is a further indication that electroosmotic flow decreases with increasing crown ether concentration. In the buffer system used in these experiments, 18-crown-6-TCA complexes with sodium ion [13], reducing the amount of sodium in the buffer which is available to shield effectively silanol groups on the capillary walls. Hence the electroosmotic flow is expected to increase with increasing crown ether concentration. For compounds which form complexes with the crown ether, the migration times increase after an initial slight decrease, owing to the reduced electrophoretic mobility of the complexed amino acid compared with the free amino acid. At higher crown ether concentrations this effect dominates the effect of the crown ether on electroosmotic flow.

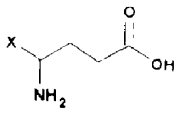
Distinct differences between the data for well resolved and modestly resolved compounds were observed. For DOPA, a compound that was separated with a modest resolution factor, the migration times of the two enantiomers increased at only slightly different rates with increasing 18-crown-6-TCA concentration. The data for **7** are similar. This indicates that both enantiomers are complexed with the crown ether and that the separation is based on a small difference in the two stability constants. However, for **5** and **6**, which showed high resolution factors under the standard conditions, the migration time of the slower migrating isomer increased much more rapidly with increasing concentration of the crown ether than that of the faster migrating isomer. This behaviour indicates that the slower migrating isomer complexes with the crown ether to a much greater extent than does the faster migrating isomer. No significant decrease in resolution with high concentrations of crown ether, as has been observed in some enantiomeric separations with cyclodextrins [18], was seen. An 18-crown-6-TCA concentration of 100 m*M* was the practical upper limit under the conditions of these experiments. At higher concentrations, the crown ether began to precipitate from the buffer solution.

3.2. Results for analogues of γ-aminobutyric acid

Chiral separations by CE with 18-crown-6-TCA and HPLC using the Crownpak CR(+) column were attempted for a series of four analogues of GABA. The structures of the compounds and the results obtained are given Table 2. The results indicated that for this family of compounds, with various substituents on the carbon attached to the amino group, HPLC is generally more useful for chiral resolution. However, some general trends were observed using both techniques. Ethynyl-GABA (**8**) was well resolved by both HPLC and CE. Vinyl-GABA (**9**) was not resolved by either technique. Methyl-GABA (**11**) was resolved by HPLC only. Although allenyl-GABA (**10**) was resolved by both techniques, the resolution obtained by CE was very poor. A representative HPLC trace for **8** is shown in Fig. 5 and a typical electropherogram is shown in Fig. 6.

It is interesting to note the effect of electrolyte pH on the peak shape obtained for these compounds by CE. When using the conditions recommended by Kuhn et al. [5] for indirect detection and chiral separation, the peak shapes observed for the analogues of GABA were very poor. The peaks were very broad with asymmetric shapes. This indicated that the peak broadening was due to concentration overloading and a

Table 2
Summary of results obtained by HPLC and CE for analogues of GABA



Compound	X	HPLC results[a]			CE results[b]		
		t_{r1}	t_{r2}	R_s	t_{m1}	t_{m2}	R_s
Ethynyl-GABA (**8**)	C≡CH	11.42	18.10	5.27	14.64	16.39	2.97
Vinyl-GABA (**9**)	CH=CH_2	14.62	–	0	14.09	–	0
Allenyl-GABA (**10**)	CH=C=CH_2	18.74	21.85	2.00	14.53	14.69	<0.5
Methyl-GABA (**11**)	CH_3	7.67	10.23	2.99	12.77	–	

[a,b] See Table 1.

poor mismatch of the conductivities of the sample and the buffer, a phenomenon that is known to cause such peak shapes [19]. The most notable difference between the GABA analogues and the α-amino acids is that the pK_a value for the acid moiety (pK_1) is about 4.1–4.2 for the GABA analogues [20], whereas it is in the range 2.1–2.3 for the α-amino acids [21]. At pH 2.5, the pH used in the work by Kuhn et al., the GABA analogues will have a higher positive charge than the α-amino acids, thus accounting for the difference in conductivity. Therefore, in order to decrease the conductivity of the GABA analogues, the pH of the buffer was increased to 3.7. This resulted in more acceptable peak shapes, as is demonstrated for ethynyl-GABA (**8**) in Fig. 6.

For those compounds for which at least one single enantiomer was available, we found that the *R*-enantiomer always eluted before the *S*-enantiomer in the HPLC system, whereas in the CE system, the *S*-enantiomer of ethynyl-GABA was observed to migrate faster than the *R*-enantiomer. No attempt was made to determine the migration order of allenyl-GABA by CE as the resolution was very poor. The elution and migration orders are the same as those observed for the analogues of DOPA and tyrosine. The effect of the substituents on resolution of the

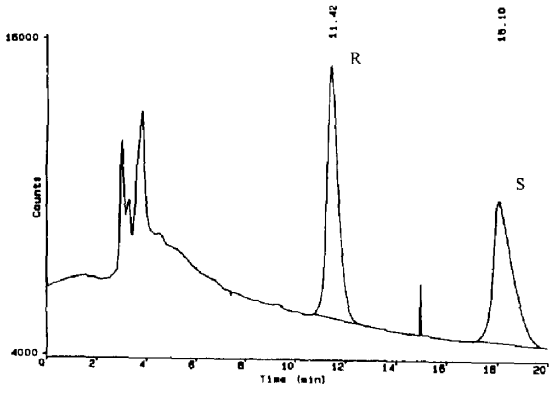


Fig. 5. Chromatogram obtained for **8** (6.5 μg injected) on the Crownpak CR(+) column. Conditions: mobile phase, 0.11 *M* $HClO_4$ (pH 1.05); temperature, 10°C; flow-rate, 0.5 ml/min; detection wavelength, 210 nm.

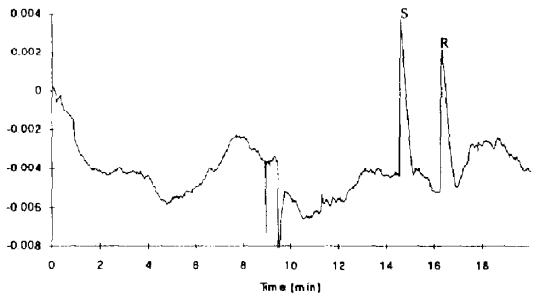


Fig. 6. Electropherogram obtained for **8** (13 ng injected). Conditions: capillary, 57 cm × 75 μm I.D., fused silica; buffer, 6 m*M* BTMACl–30 m*M* 18-crown-6-TCA (pH adjusted to 3.7 with 1 *M* NaOH); temperature, 25°C; applied voltage, 15 kV; detection wavelength, 214 nm.

different analogues is not obvious from the HPLC and CE data. Particularly puzzling is the inability to resolve vinyl-GABA by either procedure. The results were verified by analysis of the individual enantiomers. The resolution of all of these analogues of GABA, as their N-pentafluoropropyl esters, has been previously achieved by capillary GC using a Chirasil-Val stationary phase [22].

4. Conclusions

We have demonstrated the utility of crown ethers in the chiral resolution of two particular groups of amino acids in both HPLC and CE. For analogues of DOPA and tyrosine, we found that for those analogues having a substituent on the carbon α to the amino group, no separation was obtained using either method. There is evidence that this is due to lack of complexation with the crown ether, probably owing to steric hindrance. For a second set of analogues having a C=C on the carbon β to the amino group, large resolution factors were obtained in the CE experiment. The resolution factors were much larger than those observed for DOPA and tyrosine themselves and those reported in the literature for other compounds. When the amount of crown ether in the operating buffer was varied, it was discovered that for these compounds the complex of one enantiomer with the crown ether is significantly more stable than the other. Although no such pattern was observed in the HPLC experiment, these compounds were all adequately resolved by HPLC also. For analogues of GABA, we observed that three of the four compounds tested were well resolved by HPLC, whereas only one was well resolved using CE. Detection was also a significant problem in the CE analysis of GABA analogues. Because the compounds have poor UV absorbance properties, it was necessary to use an indirect detection scheme.

In the chiral resolution of amino acids with good UV absorbance properties, neither technique was found to be clearly advantageous over the other. Capillary electrophoresis is advantageous when the amount of sample available is very small, as is sometimes the case in pharmaceutical research. In routine analysis, CE may also be advantageous because it avoids the high cost of a chiral HPLC column. However, when high sensitivity is required, HPLC is advantageous owing to the larger detector cell volume. In the chiral analysis of compounds that absorb only poorly in the UV region, HPLC is clearly advantageous because of the greater sensitivity possible with this technique. Clearly, at present, HPLC and CE are complementary techniques that both provide numerous possibilities in the area of chiral separations.

Acknowledgements

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