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Capillary zone electrophoretic separation of the enantiomers of dipeptides based on host–guest complexation with a chiral crown ether

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Abstract

The enantiomeric separation of racemic glycyldipeptides and diastereomeric dipeptides by using capillary zone electrophoresis and (+)-18-crown-6-tetracarboxylic acid ($18C6H_4$) as a chiral selector added to the electrolyte is described. The separation of dipeptides with two stereogenic centres into four peaks by using capillary zone electrophoresis is reported for the first time. Chiral discrimination is attributed to the formation of a diastereomeric host–guest complex which leads to different interactions for each enantiomer. Owing to the differences in stability of the complexes, the four optical isomers elute at different migration times, allowing the chiral separation. The influence of buffer composition, crown ether concentration and the addition of organic modifiers was studied. All glycyldipeptides were resolved and for most of the diastereomeric dipeptides four baseline-separated peaks were observed.

1. Introduction

Capillary electrophoresis has been found to be a very useful tool for the separation of enantiomers [1–3]. The principle of host–guest complexation using chiral crown ethers, successfully applied in the HPLC separation of enantiomers [4–10], has recently been adapted also to capillary zone electrophoresis for the separation of enantiomers of amino acids [11–13] and amino alcohols [13,14].

This paper describes the optical resolution of dipeptides by (+)-18-crown-6-tetracarboxylic acid ($18C6H_4$) added as a chiral selector to the electrolyte. Hilton and Armstrong [7] resolved some dipeptides by HPLC using a crown ether column at different temperatures. Obviously, dipeptides are basic and essential components in biological systems either as individual compounds or as constituents of proteins [15,16]. The development of methods for the direct resolution of compounds having more than one chiral centre is therefore of great interest for enantiomeric purity control. Further, peptide syntheses often require the presence of only one enantiomer.

In previous papers [11–14], the potential of $18C6H_4$ for enantioseparation and the recognition mechanisms were described. The aim of this study was to investigate the influence of the buffer composition, the crown ether concentra-

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tion and organic modifiers such as methanol on the chiral separation of dipeptides.

2. Experimental

2.1. Methods

Capillary electrophoresis was performed using a Prince capillary electrophoresis instrument (Lauerlabs, Netherlands) equipped with an on-column UV detector (Lambda 1000; Bischoff Analysentechnik, Leonberg, Germany). Separations were carried out at ambient temperature in a fused-silica capillary tube (80 cm × 75 μm I.D., effective length 71 cm). Samples were injected hydrodynamically (20 mbar) for 6 s, which led to an injection volume of about 12 nl. The potential during analysis was 20 kV unless stated otherwise. Dipeptides were detected by UV absorption at 206 nm. An Axxiom 737 system (Moorpark, CA, USA) was used for data acquisition.

2.2. Materials

All reagents were of analytical-reagent grade unless indicated otherwise. (+)-18-Crown-6-tetracarboxylic acid, citric acid, tartaric acid, sodium dihydrogenphosphate and methanol (additionally doubly distilled) were purchased from Merck (Darmstadt, Germany). All dipeptides, N-tris(hydroxymethyl)methylglycine (tricine), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) and tris(hydroxymethyl)aminomethane (Tris) were obtained from Sigma (Deisenhofen, Germany).

Sample solutions were prepared by dissolving the dipeptides (1 mg) in doubly distilled, deionized water (1 ml); samples and buffer solutions were filtered through a 0.45-μm pore-size filter (Schleicher & Schüll, Dassel, Germany) and degassed with helium 5.0.

3. Results and discussion

18-Crown-6 is a macrocyclic polyether ring [17,18]. Six oxygen atoms link six ethylene groups to build a cavity suitable to potassium ions (K^+), ammonium ions (NH_4^+) or protonated primary alkyl amines (RNH_3^+). The mechanism of chiral recognition has already been widely discussed [4–6,8,10–14,19]. Briefly, hydrogen bonds forming a tripod arrangement and an optical barrier caused by the asymmetric substituents are responsible for the chiral discrimination. Lehn and co-workers [20,21] synthesized a useful derivative, (+)-18-crown-6-tetracarboxylic acid (Fig. 1), which seems to be a very suitable chiral additive. In this case, in addition to the chiral barrier formed by the carboxylic acid pairs, electrostatic interactions might support the chiral separation [13].

3.1. Optimization of the buffer system

Generally, separations were carried out either with the crown ether only or with an additional electrolyte. Different buffer systems were investigated to optimize the separation conditions.

Using a background electrolyte, 10 m*M* $18C6H_4$ with 10 m*M* Tris–citrate, gave the fastest and best results concerning the peak sharpness. According to Kuhn et al. [12], the mobility of this buffer system fits the chiral selector because of the minimization of electrorphoretic dispersion. Tris was considered to have similar migration properties to amino acids without forming a complex with the crown ether. CAPS adjusted to pH 2.0 with tartaric acid gave peak tailing and a poor peak shape. A solution of 1 m*M* sodium dihydrogenphosphate (higher phosphate levels resulted in too high current values) failed, possibly because of interaction of the Na^+ cation with $18C6H_4$. A test experiment with DL-leucyl-DL-leucine using 10 m*M* Tris–citrate without the chiral selector resulted in only

Fig. 1. Structure of (+)-18-crown-6-tetracarboxylic acid.

two peaks: the first peak consisted of L-leucyl-L-leucine (L-Leu-L-Leu) and D-leucyl-D-leucine (D-Leu-D-Leu), the second peak of L-leucyl-D-leucine (L-Leu-D-Leu) and D-leucyl-L-leucine (D-Leu-L-Leu). The migration order was verified by sampling the four pure enantiomers.

3.2. *Resolution of glycyldipeptides*

The elution sequence for glycyl-DL-valine was investigated by sampling the enantiomers. Gly-L-Val migrated faster, indicating that $18C6H_4$ forms stronger complexes with glycyl-D-enantiomers than with Gly-L-antipodes. Kuhn et al. [11] found amino acids to show same behaviour.

Separation data are given in Table 1 and an example is shown in Fig. 2. Generally, each glycyldipeptide tested was separated although the chiral centre is in the δ-position to the primary amine. Using HPLC, dipeptides with a C-terminal glycine, where the chiral C atom is located in the α-position to the primary amine, are found to be resolved much better [7]. This type of dipeptide was not included in our investigations. Fig. 3 demonstrates the relationship between the molecular mass (M_r) of each glycyldipeptide and its separation factor $\alpha(t_2/t_1)$. The experiment was carried out using 25 m*M* $18C6H_4$ without any additional background electrolyte. The higher the M_r, the more efficient was the resolution of the enantiomers. Moreover, the comparison of Gly-DL-Leu with Gly-DL-Nle and of Gly-DL-Val with Gly-DL-Nva [observed when using 10 m*M* $18C6H_4$ and 10 m*M* tris–citrate (pH 2.0)] showed another effect: straight-chain alkyl groups were resolved worse than their branched isomers.

3.3. *Separation of diastereomeric dipeptides*

We examined the chiral resolution of a series of different dipeptides having two optically active centres. Analyses were performed using either 10 m*M* $18C6H_4$ and 10 m*M* Tris–citrate (pH 2.0) as a buffer system or 25 m*M* $18C6H_4$ without any additional background electrolyte.

Most of the diastereomeric dipeptides were separated into their four enantiomers. Separation data are given in Table 2. As the pure enantiomers for the other dipeptides were not available, the elution order was only investigated for Leu-Leu. In contrast to the glycyldipeptides, the molecular masses do not seem to be correlated with separation factors. Dipeptides consisting of amino acids with bulky substituents were better resolved than those with small substituents, e.g. DL-leucyl-DL-leucine or DL-leucyl-DL-phenylalanine compared with DL-alanyl-DL-valine. In some cases the use of only 10 m*M* $18C6H_4$ with additional background electrolyte resulted in baseline resolution, connected with a decreased migration time and sharper peaks. For example, Fig. 4 shows the separation of DL-leucyl-DL-phenylalanine into its four enantiomers using 10 m*M* $18C6H_4$ and 10 m*M* Tris–citrate (pH 2.0) as a buffer system. In this case, the number of theoretical plates was about 30 000.

We optimized the chiral resolution of a DL-leucyl-DL-leucine racemate, applying different amounts of $18C6H_4$ in water without adding any additional electrolyte. At concentrations below 5 m*M* $18C6H_4$, only three completely resolved peaks were obtained: L-Leu-L-Leu migrating fastest, followed by a mixture of D-Leu-L-Leu and L-Leu-D-Leu. The third peak was D-Leu-D-Leu. At a concentration of about 5 m*M* $18C6H_4$, a partial separation of L-Leu-D-Leu and D-Leu-L-Leu was observed. Optimum resolution was obtained at 25 m*M* $18C6H_4$, whereby D-Leu-L-Leu migrated faster than L-Leu-D-Leu. A further increase in the chiral additive resulted in a decrease in the resolution factor. Using an $18C6H_4$ concentration higher than 55 m*M*, the three-peak constellation was reobtained. This is generally confirmed by a theoretical model, indicating that the degree of separation depends on the concentration of the chiral selector and that there is an optimum concentration [22]. Fig. 5 shows the change in R_s (L-Leu-D-Leu/D-Leu-L-Leu) versus the concentration of the crown ether.

Besides increasing the concentration of the chiral selector, the addition of methanol was found to improve the resolution in some cases (Table 3). Without adding methanol, a 25 m*M* $18C6H_4$ solution was necessary for the resolution

Table 1
Separation data for optically active glycyldipeptides using chiral 18C6H$_4$ (25 mM): resolution (R_s), separation factor ($\alpha = t_2/t_1$) and migration time of the first-eluted enantiomer (t_1)

Compound	Formula	t_1 (min)	α (t_2/t_1)	R_s
Glycyl-DL-aspartate		28.11	1.040	1.57
Glycyl-DL-apraragine		26.53	1.028	0.96
Glycyl-DL-leucine		28.03	1.041	1.57
Glycyl-DL-methionine		28.97	1.048	1.79
Glycyl-DL-norleucine		28.63	1.032	1.47
Glycyl-DL-norvaline		27.91	1.026	1.15
Glycyl-DL-serine		27.72	1.019	0.90
Glycyl-DL-threonine		28.14	1.029	1.21

Table 1 (continued)

Compound	Formula	t_1 (min)	α (t_2/t_1)	R_s
Glycyl-DL-tryptophan		33.07	1.064	1.94
Glycyl-DL-valine		32.56	1.026	0.98
Glycyl-DL-alanine		28.20	1.006	0.30
Glycyl-DL-phenylalanine		30.99	1.056	1.75

of DL-leucyl-DL-leucine to obtain four baseline-separated peaks, whereas the addition of 20% (v/v) methanol to a 15 mM solution of $18C6H_4$ gave the same result (Fig. 6).

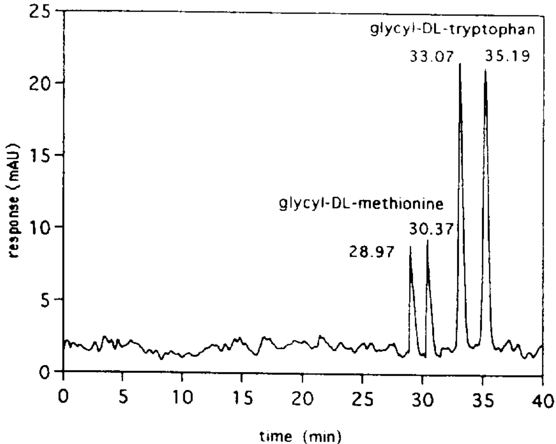

Fig. 2. Separation of the enantiomers of glycyl-DL-methionine and glycyl-DL-tryptophan using 25 mM $18C6H_4$ (pH 2), 20 kV.

Using HPLC and a Crownpak CR(+) column, Hilton and Armstrong [7] obtained at ambient temperature only three peaks for the separation of Leu-Leu, whereas at low temperature the dipeptide was completely resolved. Interestingly,

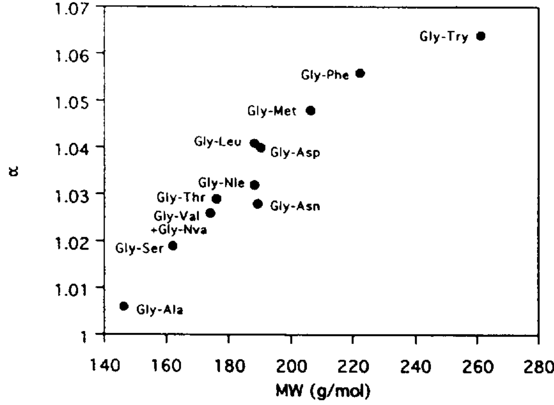

Fig. 3. Relationship between the molecular mass of a glycyldipeptide and its separation factor $\alpha(t_2/t_1)$. Buffer system: 25 mM $18C6H_4$ without any background electrolyte.

Table 2
Separation data for optically active dipeptides (buffer: 25 m*M* 18C6H$_4$): migration time for the first-eluted enantiomer (t_1) and separation factors of the 1st and 2nd, the 2nd and 3rd and 4th peaks, $\alpha_{1/2}$, $\alpha_{2/3}$ and $\alpha_{3/4}$ respectively

Compound	Formula	Peaks	t_1 (min)	$\alpha_{1/2}$	$\alpha_{2/3}$	$\alpha_{3/4}$
DL-Alanyl-DL-serine		4	22.81	1.031	1.017	1.028
DL-Alanyl-DL-valine		2	19.27	1.104	–	–
DL-Leucyl-DL-alanine		4	21.41	1.026	1.043	1.122
DL-Leucyl-DL-leucine		4	27.38	1.030	1.032	1.195
DL-Leucyl-DL-phenylalanine		4	21.16	1.123	1.078	1.116
DL-Leucyl-DL-tyrosine		2	26.26	1.166	–	–
DL-Leucyl-DL-valine		3	23.44	1.035	1.073	1.015

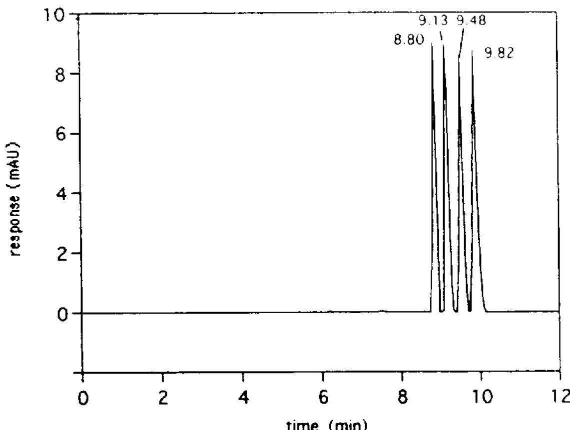

Fig. 4. Electropherogram of the chiral resolution of DL-leucyl-DL-phenylalanine [buffer: 10 mM 18C6H$_4$–10 mM Tris–citrate (pH 2.0)], U = 30 kV.

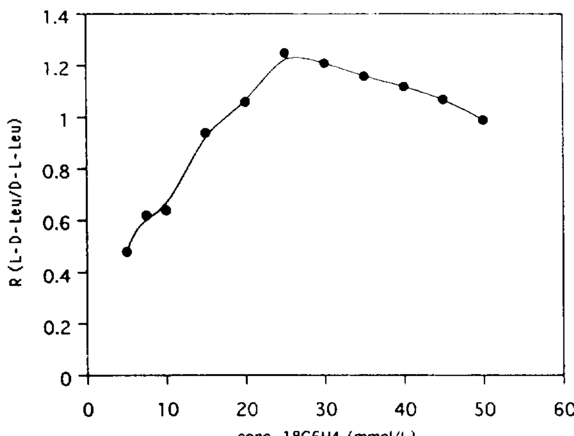

Fig. 5. Resolution of the leucylleucine enantiomer L-leucyl-D-leucine and D-leucyl-L-leucine as a function of 18C6H$_4$ concentration.

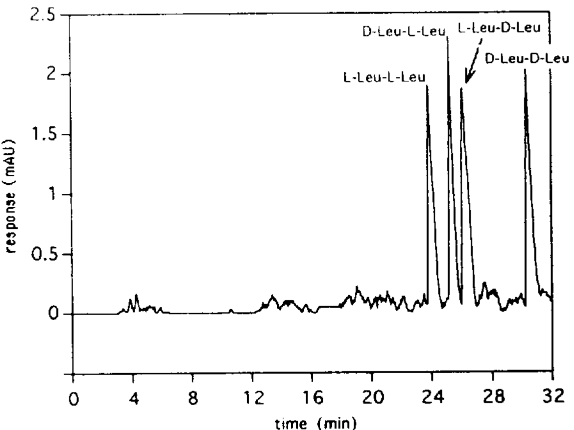

Fig. 6. Electropherogram of the chiral resolution of DL-leucyl-DL-leucine [buffer: 15 mM 18C6H$_4$, –10 mM Tris–citrate, 20% (v/v) methanol], U = 30 kV.

compared with our results, the elution order was reversed.

4. Conclusion

The potential of 18C6H$_4$ as a selector for chiral separation of dipeptides into their enantiomers using capillary zone electrophoresis has been demonstrated. The formation of diastereomeric inclusion complexes determines different migration times for each enantiomer of a racemic compound.

Twelve glycyldipeptides and seven dipeptides with two chiral centres were resolved by a simple separation system. At ambient temperature each glycyldipeptide was at least partially resolved and more than half of the diastereomeric dipeptides were separated into four peaks. Our

Table 3
Separation data for resolving the peaks of D-leucyl-L-leucine and L-leucyl-D-leucine by increasing the proportion of methanol in 15 mM 18C6H$_4$ (pH 2)

Methanol added to 15 mM 18C6H4 (%)	Resolution factor, R_s(L-D-Leu/D-L-Leu)	Separation factor, α(L-D-Leu/D-L-Leu)
0	0.921	1.020
5	0.978	1.027
20	1.14	1.035

investigations have shown that in addition to the concentration of 18C6H$_4$, the addition of organic modifiers and added electrolytes has a marked effect on the resolution.

We also investigated the influence of the structure of glycyldipeptides on the resolution. The selectivity is usually optimum when the amino functionality is adjacent to the stereogenic centre. However, such dipeptides have their chiral centre in the δ-position to the amino group. As the NH_3^+ residue fitting into the cavity of the crown ether is located at the glycyl part, no steric hindrance can be expected from branched alkyl groups. A relationship between the molecular mass of glycyldipeptides and their separation factor α was demonstrated.

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