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Original Scientific Article

Enantioseparation of Dansylated Amino Acids by Ligand-exchange Capillary Electrophoresis Using L-phenylalaninamide, L-lysine or L-threonine as Chiral Selector†

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Abstract. In recent years enantioseparation of both active pharmaceutical ingredients and bio molecules such as amino acids became more and more necessary because in most cases the two stereo forms exhibit different pharmacological effects. This article deals with the chiral separation of dansylated amino acids by ligand-exchange capillary electrophoresis using L-phenylalaninamide, L-lysine and L-threonine as chiral selectors. Experiments with different central metal ions such as Cu(II), Co(II), Cd(II), Ni(II) and Zn(II) were carried out. Optimal conditions were found out by studying the effect of the pH and the selector molarity on the chiral resolution. Best separation was obtained for the Cu(II)/L-lysine complex, showing a chiral resolution up to 17 for Dns-DL-Met. (doi: 10.5562/cca1762)

Keywords: LECE, dansylated amino acids, CZE, chiral separation, ligand-exchange, capillary electrophoresis

INTRODUCTION

The development of enantiomer separation techniques has attracted great attention since it was investigated that in some cases only one enantiomer of a racemic drug mixture shows the desired pharmacological effect (eutomer). The other form (distomer) may not show any effect *e.g.* (R)-ibuprofen,[1] unwanted side effects as it is the case with D-thyroxine, or in some cases even toxic effects *e.g.* D-DOPA. About half of the used drug substances are chiral and about 35 % out of them are administered as pure enantiomers.[2] Even if there are not that drastic effects of the distomer, it represents an unnecessary burden for the organism.

The chiral separation of underivatized and Dns-AAs is of special interest not at least since it was postulated that, in contrary to former hypothesis, D-amino acids also occur in higher animals and not only in lower species. The D-forms can have physiological effects such as D-Ser and D-Asp or they can be related to pathophysiological processes, such as Alzheimer's disease, Parkinson's disease, schizophrenia and renal disease.[3] AAs and their derivatives are used as building blocks for peptide synthesis or as drugs *e.g.* 3,4-dihydroxyphenylalanine (L-DOPA) and L-Trp. Dansylation of AAs is used on the one hand to label the N-terminal end of a peptide and on the other hand to simplify the detection of amino acids.

In recent decades multitudes of direct as well as indirect chiral separation techniques have been developed. In addition to HPLC and GC capillary electrophoresis turned out to be a powerful alternative with some unique advantages *e.g.* high peak efficiency, good compatibility with biological samples, short analysis times and the low consumption of buffer solutions. Even the flexibility in changing the method conditions played a considerable role in the rise of CE to one of the most spread separation techniques together with HPLC.

In most cases chiral separation in CE is performed by an enantioselective interaction of a so called chiral selector and the analyte[4] but there are also some groups developing new chiral derivatization procedures applied in indirect separation techniques.[5–7]

In the direct separation mode different types of chiral selectors, such as cyclodextrines, macrocyclic antibiotics, carbohydrates, chiral crown ethers, calixarenes, proteins and chiral metal complexes (Ligand-exchange) are most commonly used as chiral selectors.[8–12]

The principle of ligand-exchange is based on the formation of diastereomeric ternary mixed metal com-

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plexes between the chiral selector ligand and the analyte and was first introduced by Davankov and Roghozin in the late 1960s-early 1970s for conventional columns.[13] Zare and co-workers transformed this principle to CE using histidine or aspartame-Cu(II) complexes for the resolution of racemic mixtures of Dns-AAs in the late 1980s.[14] Schmid and Gübitz succeeded first in directly resolving underivatized AAs using L-Pro and L-Hypro as their Cu(II) complexes added to the electrolyte.[15] Meanwhile a multitude of different chiral selectors for the resolution of AAs and Dns-AAs racemates by LE were introduced by different groups, namely, Cu(II)-L-arginine,[16] Zn(II)-L-arginine,[17] Cu(II)-aspartame,[18,19] Cu(II)-L-alaninamide,[20] Cu(II)-*N,N*-didecyl-L-alanine,[21,22] Cu(II)-L-histidine,[14] Cu(II)-L-isoleucine,[23] Cu(II)-L-lysine,[24] Zn(II)-L-lysine,[25] Cu(II)-L-ornithine,[26] Cu(II)-D-penicillamine[LE-MEKC],[27] Zn(II)-L-phenylalaninamide,[28] Cu(II)-L-phenylalaninamide,[20] Cu(II)-*N*-(2-hydroxyoctyl)-L-4-hydroxyproline,[29,30] Cu(II)-L-4-hydroxyproline,[15,31–33] Cu(II)-*N*-(2-hydroxypropyl)-L-4-hydroxyproline,[29,30] Cu(II)-L-proline[15,23] and Cu(II)-L-prolinamide[20].

In this research, experiments with the three chiral selectors L-phenylalaninamide, L-lysine and L-threonine were performed under different conditions, to resolve Dns-AAs by LECE. For the optimization of the methods the effect of the pH value and the selector concentration on the resolution is shown. For the Cu(II)/L-lysine a validation referring to repeatability was carried out.

EXPERIMENTAL

Instrumentation

A fully automated ^{3D}CE system (Agilent Technologies, CA, USA) equipped with a diode array detector was used for the experiments. Measurements were performed in 50 μm ID fused silica capillaries (58.5/50 cm effective length) from Microquartz (Munich, Germany). Detection was performed via on-column measurements of the UV absorption at 208 and 254 nm. Operation temperature was set to 25 °C. Before measurement capillaries were washed with water, 0.2 mol dm^{-3} NaOH, water and electrolyte. Samples were injected hydrodynamically for 5 s at 10 mbar unless indicated otherwise.

Chemicals and Solutions

All chemicals were of analytical grade. Dns-DL-α-ABA, Dns-DL-Asp, Dns-DL-Glut, Dns-DL-Leu, Dns-DL-NLe, Dns-DL-Met, Dns-DL-Phe, Dns-DL-Thr, Dns-DL-Trp, Dns-DL-Val, Dns-DL-NVa, L-phenylalaninamide, L and D lysine HCl and L-threonine were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Ammonium acetate, sodium acetate, Cu(II) sulfate, Zn(II) sulfate, Ni(II) sulfate, Co(II) sulfate and Cd(II) chloride were obtained from VWR (Darmstadt,Germany). Water was deionized and double distilled.

The electrolyte was prepared by dissolving a desired quantity of the chiral selector and the ionic additives in double-distilled water. The pH was adjusted depending on the buffer system with a suitable reagent. The solutions were degassed for 2 minutes by ultrasonification and filtered through a 0.45 μm pore size Teflon filter (Schleicher and Schuell, Dassel, Germany) before use.

Sample solutions were prepared by dissolving the analytes (1 mg/ml) in a mixture of methanol/water (1:1).

RESULTS AND DISCUSSION

Enantioseparation in LECE is based on the formation of diastereomeric ternary mixed metal complexes between the chiral selector ligand and the analyte. Depending on the different complex stability constants of the two mixed complexes enantiomer resolution is reached. The following equilibria should be taken into account:

$$\mathrm{Cu(L\text{-}selector)_2} + \mathrm{S\text{-}analyte} \rightleftarrows \mathrm{(L\text{-}selector)Cu(S\text{-}analyte)} + \mathrm{L\text{-}selector}$$

$$\mathrm{Cu(L\text{-}selector)_2} + \mathrm{R\text{-}analyte} \rightleftarrows \mathrm{(L\text{-}selector)Cu(R\text{-}analyte)} + \mathrm{L\text{-}selector}$$

The optimum pH for the complexation differs from chiral selector and the type of analyte. The correlation between the pH and the chiral resolution for different selectors is shown later.

Use of L-phenylalaninamide as Chiral Selector

L-phenyalaninamide was already used for the resolution of Dns-AAs by Chen[34] with Cu(II) and by Qi[28] with Zn(II) as central metal ion. For the following experiments a similar background electrolyte was chosen to guarantee adequate current. Ammonium acetate was replaced by sodium acetate. A chiral selector concentration of 10 mmol dm^{-3} and a selector to ion ratio of 2:1 was used. Using Zn(II) as central ion, Dns-DL-NLeu, Dns-DL-Nval, Dns-DL-Leu and Dns-DL-α-ABA were resolved in addition to those Dns-AAs presented in the afore cited articles. With Cu(II) as metal ion no new analytes could be resolved. Table 1 shows the obtained results using Zn(II) as metal ion. In contrast to Cu(II), Zn(II) was used with negative voltage in order to detect the analytes. The change in the polarity was manifested in the enantiomer migration order (EMO). With Zn(II) the D-form migrates faster than the L-form, with Cu(II) it is the opposite.

Table 1. Additionally separated Dns-DL-AAs using Zn(II)-L-phenylalaninamide as a chiral selector

	t_1 / min	t_2 / min	α	R_s
Dns-DL-α-ABA	17.26	17.32	1.004	0.6
Dns-DL-Leu	16.17	16.29	1.007	1.3
Dns-DL-NLe	19.61	19.90	1.015	2.9
Dns-DL-NVa	17.53	17.69	1.009	1.6

Conditions: 10 mM L-phenylalaninamide, 5 mM zinc(II) sulfate, 5 mM sodium acetate, 100 mM boric acid; adjusted with 1 M tris-solution to pH 8.2; applied voltage: 23 kV to anode; Injection: 10 mbar for 6 s.

Copper and zinc are the most frequently used central ions but apart from those, other ions were investigated for their complex building ability.[35] We examined the effect of Ni(II), Cd(II) and Co(II) on the resolution of Dns-AAs. Using Ni(II) as metal ion 7 out of 11 tested analytes could be resolved as it is shown in Table 2. For Dns-DL-Met, Dns-DL-Phe and Dns-DL-Trp the resolution was higher than 1.5.

Because Ni(II) was not used before as central ion in combination with phenylalaninamide the optimum pH-value and selector concentration was to be found out. Figure 1 shows the effect of the pH on the resolution of Dns-DL-Trp. The higher the resolution the more stable is the complex at the specific pH value. The best resolution turned out to occur at a pH of 8.2 as it was used with Cu(II) and Zn(II).

The effect of the selector concentration is shown in Figure 2. The selector to metal ion ratio was set to 2:1 and the total molarity of selector plus metal ion was

Table 2. Enantioseparation of Dns-DL-AAs by Ni(II)-L-phenylalaninamide as a chiral selector

	t_1 / min	t_2 / min	α	R_s
Dns-DL-α-ABA	17.53	17.64	1.006	0.7
Dns-DL-Glut	9.51	9.58	1.008	0.8
Dns-DL-Leu	20.76	20.91	1.007	0.7
Dns-DL-NLe	18.59	18.76	1.009	1.1
Dns-DL-Met	22.90	23.85	1.041	2.4
Dns-DL-Phe	18.60	18.83	1.013	2.0
Dns-DL-Thr	19.70	-	1.000	-
Dns-DL-Trp	24.94	25.25	1.012	1.8
Dns-DL-Val	18.90	-	1.000	-
Dns-DL-NVa	18.52	18.65	1.007	0.9

Conditions: 10 mM L-phenylalaninamide, 5 mM nickel(II) sulfate, 5 mM sodium acetate, 100 mM boric acid; adjusted with 1 M tris-solution to pH 8.2; applied voltage: 23 kV to anode; Injection: 10 mbar for 6 s.

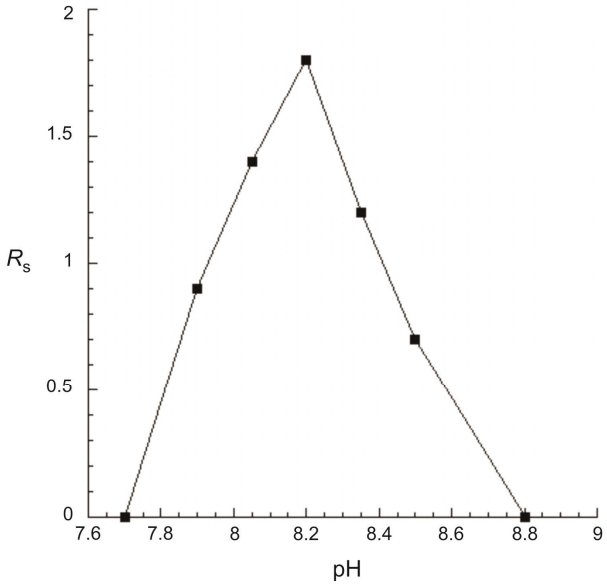

Figure 1. Effect of the pH value of the Ni(II)-L-phenylalaninamide complex on the resolution. Conditions: 10 mM L-phenylalaninamide, 5 mM nickel(II) sulfate, 5 mM sodium acetate, 100 mM boric acid; pH adjusted with 1 M tris-solution; applied voltage: 23 kV to anode; Injection: 10 mbar for 6 s.

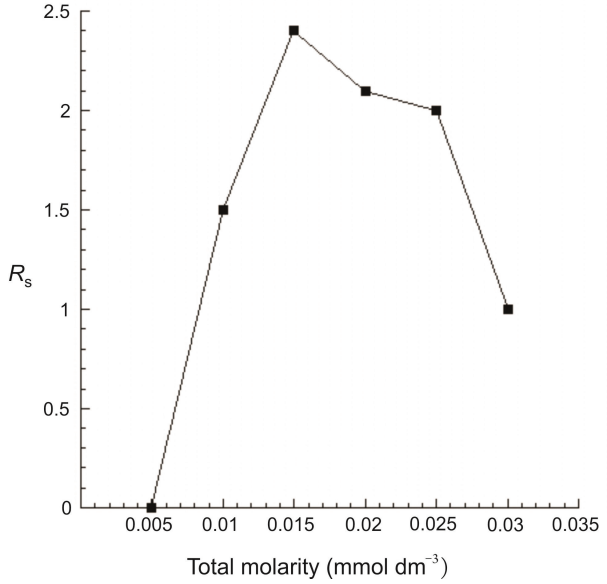

Figure 2. Effect of the total molarity of Ni(II)-L-phenylalaninamide complex. on the resolution. Conditions: L-phenylalaninamide / nickel(II) sulfate 2:1, 5 mM sodium acetate, 100 mM boric acid; adjusted with 1 M tris-solution to pH 8.2; applied voltage: 23 kV to anode; Injection: 10 mbar for 6 s.

changed. Best results were obtained at a total molarity of 15 mmol dm^{-3}, which means that 10 mmol dm^{-3} selector and 5mmol dm^{-3} Zn(II) were used. The electronic source was set to positive mode and so the EMO

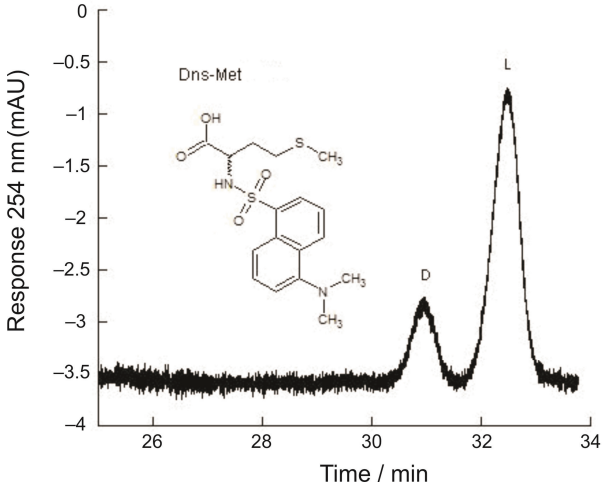

Figure 3. Determination of the EMO of Dns-DL-methionine spiked with L-enantiomer using L-phenylalaninamide as chiral selector. Conditions: 10 mM L-phenylalaninamide, 5 mM nickel(II) sulfate, 5 mM sodium acetate, 100 mM boric acid; adjusted with 1 M tris-solution to pH 8.2; applied voltage: 23 kV to anode; Injection: 10 mbar for 6 s.

was D before L. Therefore this method is applicable for purity checking of Dns-L-AAs. The determination of the EMO is shown in Figure 3 for Dns-DL-Met.

The L-phenylalaninamide-Cd(II)-system showed chiral separation for Dns-DL-NVal, Dns-DL-NLeu and Dns-DL-Asp. Obviously it was the first time resolution of a racemic mixture of Dns-AAs was obtained with Cd(II) as central metal ion. With the metal ion Co(II) no analytes were separated.

Use of L-lysine as Chiral Selector

The suitability of lysine complexes for the chiral separation of underivatized amino acids was introduced by Lu[24] for Cu(II) as central metal ion and by Qi[25] for Zn(II) complexes. To our knowledge there is no publication that deals with the enantioseparation of derivatized amino acids using this selector-ion system.

The background electrolyte consisting of 25 mmol dm^{-3} ammonium acetate was adopted from previous unpublished experiments with L-ornithin as chiral selector showing similar structure.

Copper(II) was used as central metal ion in a 1 to 2 ratio to the selector.

To optimize the conditions pH values from 5.1 to 9 were tested. Figure 4 shows the relation between the pH and the chiral resolution for the model substance Dns-DL-Trp. The use of L-Lys/Cu(II) showed a high resolution among the whole pH spectrum. Figure 4 and 5 show that even the poorest resolution for Dns-DL-Trp was higher than 10. Further the relation between the total molarity of selector plus copper(II) and the resolution was investigated as it is shown in Figure 5.

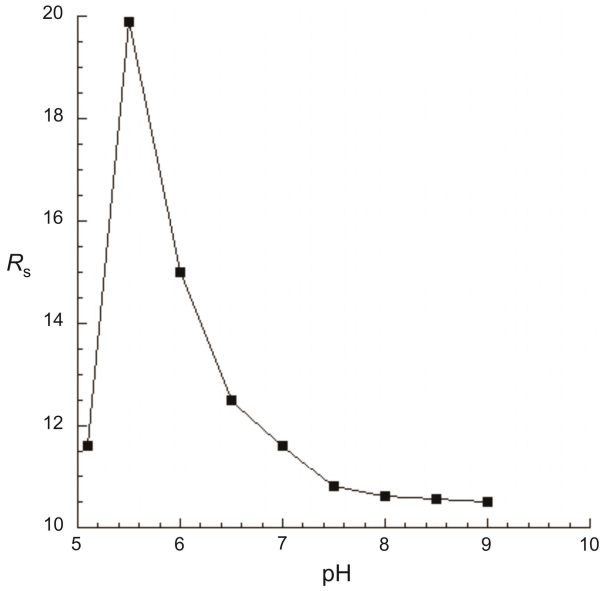

Figure 4. Correlation between the pH value and the resolution for the Cu(II)-L-lysine complex. Conditions: 10 mM L-lysine, 5 mM copper(II) sulfate, 25 mM ammonium acetate; pH adjusted with ammonia; applied voltage: 27 kV to cathode; Injection: 15 mbar for 5 s.

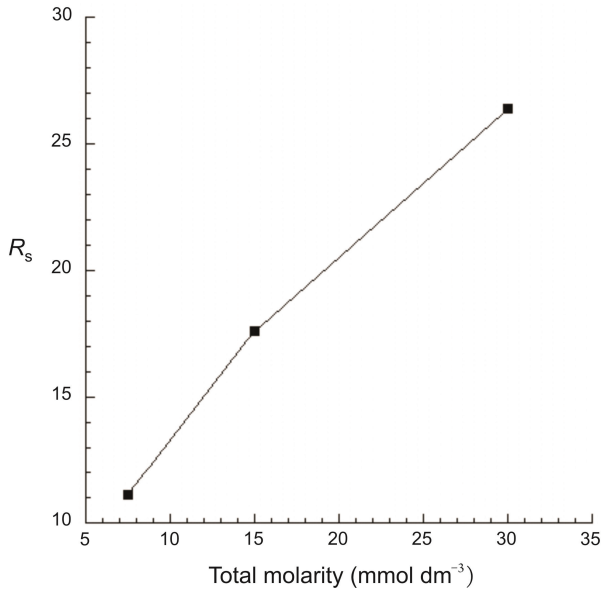

Figure 5. Correlation between the total molarity of Cu(II)-L-lysine complex and the resolution. Conditions: L-lysine/ copper(II) sulfate 2:1, 5 mM copper(II) sulfate, 25 mM ammonium acetate; pH 5.5 adjusted with ammonia; applied voltage: 27 kV to cathode; Injection: 15 mbar for 5 s.

Resolution increased with higher concentration of selector-copper(II) complex. With respect to migration time an electrolyte of 10 mmol dm^{-3} L-lysine and 5 mmol dm^{-3} Cu(II) at pH 8.0 was to screen a set of Dns-AAs in further experiments. The repeatability of the

Table 3. Repeatability data for retention time and resolution by means of Dns-DL-tryptophan

	t_1 / min	t_2 / min	R_s
Intraday $n = 5$	11.23 ± 0.58, RSD = 5.24 %	12.35 ± 0.78 RSD = 6.29 %	11.12 ± 0.91 RSD = 8.18 %
Day-to-day $n = 10$	11.43 ± 0.84, RSD = 7.35 %	12.48 ± 1.15, RSD = 9.23 %	11.42 ± 1.29, RSD = 11.26 %

Conditions: 10 mM L-lysine, 5mM copper(II) sulfate, 25 mM ammonium acetate; adjusted with ammonia to pH 5.5; applied voltage: 27 kV to cathode; Injection: 15 mbar for 5 s.

method for the analyte Dns-DL-tryptophan is shown in Table 3.

With this method 10 out of 11 tested Dns-amino acids were separated. Results are shown in Table 4. The resolution values range from 3.6 for Dns-DL-Asp and 17.0 for Dns-DL-Met. A simultaneous chiral resolution of a mixture of Dns-DL-Asp, Dns-DL-Met, Dns-DL-Asp, Dns-DL-α-ABA and Dns-DL-Leu was achieved within 30 minutes. (Figure 6)

Due to the faster migration of the L enantiomer this method is not suitable for purity studies of Dns-L-AAs because most of the time the smaller peak of the D-impurity may be overlapped by the L-peak. Simple change of the selector from L- to D-lysine inverts the EMO and purity check can be performed for L-enantiomers. (Figure 7)

Use of L-threonine as Chiral Selector

The last chiral selector tested in this study was L-Thr which was used successfully for enantioseparation of sympathomimetics and β-blockers by Hödl *et al.*[36] In this work L-Thr is shown to be applicable for the chiral resolution of Dns-AAs as well. In this case no testing of different metal ions was performed. An electrolyte consisting of 90 mmol dm^{-3} L-Thr and 45 mmol dm^{-3} Cu(II)-sulfate was used at pH 8.2. The previous results with the other two chiral selectors show that a pH of about 8.0 is suitable for the complex formation of Dns-

Table 4. Enantioseparation of Dns-AAs using Cu(II)-L-lysine as a chiral selector

	t_1 / min	t_2 / min	α	R_s
Dns-DL-α-ABA	16.29	18.55	1.289	11.2
Dns-DL-Asp	28.09	29.42	1.069	3.6
Dns-DL-Leu	23.28	27.52	1.289	15.0
Dns-DL-NLe	16.74	18.30	1.189	7.1
Dns-DL-Met	13.50	16.09	1.552	17.0
Dns-DL-Phe	14.85	17.14	1.392	10.6
Dns-DL-Thr	16.23	17.52	1.187	6.1
Dns-DL-Trp	11.09	12.22	1.453	10.8
Dns-DL-Val	33.26	35.76	1.104	5.9
Dns-DL-NVa	13.31	14.81	1.252	6.4

Conditions: 10 mM L-lysine, 5 mM copper(II) sulfate, 25 mM ammonium acetate; adjusted with ammonia to pH 8.0; applied voltage: 27 kV to cathode; Injection: 15 mbar for 5 s.

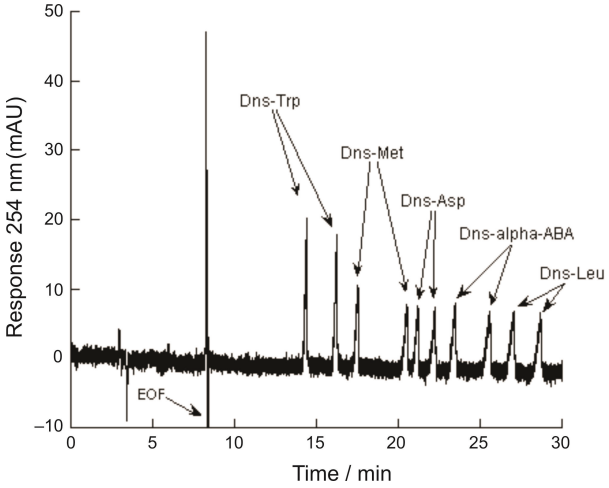

Figure 6. Simultaneous chiral separation of Dns-DL-Asp, Dns-DL-Met, Dns-DL-Asp, Dns-DL-α-ABA and Dns-DL-Leu with L-lysine as chiral selector. Conditions: 10 mM L-lysine, 5 mM copper(II) sulfate, 25 mM ammonium acetate, adjusted with ammonia to pH 5.5; applied voltage: 27 kV to cathode; Injection: 10 mbar for 5 s.

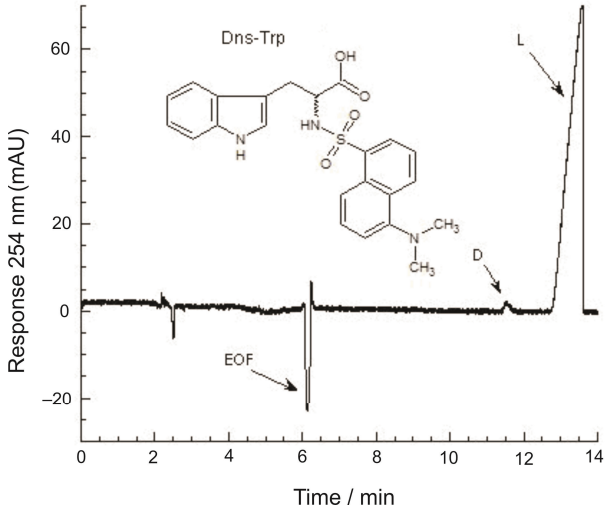

Figure 7. Purity check of Dns-L-tryptophan containing 3,5 % D-tryptophan with L-lysine as chiral selector. Conditions: 10 mM L-lysine, 5 mM copper(II) sulfate, 25 mM ammonium acetate, adjusted with ammonia to pH 5.5; applied voltage: 27 kV to cathode; Injection:10 mbar for 5 s.

Table 5. Enantioseparation of Dns-AAs using Cu(II)-L-threonine as a chiral selector

	t_1 / min	t_2 / min	α	R_s
Dns-DL-Glut	40.050	41.430	1.052	2.9
Dns-DL-Met	20.965	21.105	1.019	1.2
Dns-DL-Phe	21.390	21.684	1.039	2.2
Dns-DL-Trp	26.656	28.104	1.103	5.7

Conditions: 90 mM L-threonine, 45 mM copper(II) sulfate; adjusted with ammonia to pH 8.0; applied voltage: 10 kV to cathode; Injection: 10 mbar for 5 s.

AAs and additionally the EOF is relatively strong which results in shorter retention times. In total 4 out of 11 tested Dns-AAs were separated with this selector as it is shown in Table 5. Noticeable are the long migration times of the glutamic acid enantiomers. Because of the two carboxylic groups the molecule seems to be slightly negatively charged and tends to migrate opposite to the EMO to the anode. The EMO turned out to be L before D. The optimization of the method including pH value and selector-concentration will be subject to further investigations.

CONCLUSION

The suitability of L-phenylalaninamide, L-lysine and L-threonine as chiral selectors in LECE with different central metal ions was shown. The L-phenylalaninamide/Ni(II) complex was applicable for the enantioseparation of 8 Dns-AAs and even with Cd(II) 3 Dns-AAs were resolved. With L-Lys/Cu(II) 10 out of 11 Dns-AAs were separated with high resolution compared to the other selector/metal complexes. Additionally 5 Dns-AAs were separated simultaneously. L-Thr which up to now has been used only for the chiral separation of sympathomimetics and β-blockers showed an effect on the chiral resolution of Dns-AAs with Cu(II) as metal ion as well.

ABBREVIATIONS

AA, amino acid; LECE, ligand-exchange capillary electrophoresis; Dns-AAs, dansylated amino acids

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