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Enantioseparation of α-amino acids and dipeptides by ligand-exchange capillary electrophoresis of various L-4-hydroxyproline derivatives

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Abstract

The principle of ligand exchange has been applied to the enantioseparation of underivatized aromatic and aliphatic amino acids as well as dipeptides. Two non commercially available *N*-alkyl-L-4-hydroxyproline derivatives were compared to underivatized L-4-hydroxyproline for their ability to resolve α-amino acids and dipeptides. *N*-(2-hydroxyoctyl)-L-4-hydroxyproline and *N*-(2-hydroxypropyl)-L-4-hydroxyproline were used as their copper(II) complexes as chiral selectors. With these selectors, several aliphatic amino acids and dipeptides, in addition to aromatic amino acids, were resolved. The pH optimum was found to be 4.3 for amino acids and 6.0 for dipeptides.

Keywords: Enantiomer separation; Ligand-exchange capillary electrophoresis; Chiral selectors; Amino acids; Peptides; Hydroxyproline

1. Introduction

This paper represents a continuation of our studies on chiral ligand-exchange capillary electrophoresis (LECE). The principle of LECE was first applied by Gozel and Zare et al. [1,2] to the resolution of dansyl amino acids. In many cases it is necessary to have a method for the direct enantioseparation of underivatized amino acids. Based on our experience from HPLC [3,4], we previously used L-proline and L-4-hydroxyproline Cu(II) complexes for the chiral resolution of amino acids by CE. However, using this method, only amino acids containing aromatic moieties were resolved [5]. To increase the interactions between the chiral selector and the analyte, we synthesized *N*-(2-hydroxyoctyl)-L-4-hydroxyproline. This selector showed improved selectivity [6].

The present paper deals with comparative studies on the chiral resolution of amino acids using L-4-hydroxyproline, *N*-(2-hydroxypropyl)-L-4-hydroxyproline and *N*-(2-hydroxyoctyl)-L-4-hydroxyproline as chiral selectors. Contrary to L-4-hydroxyproline, *N*-(2-hydroxypropyl)-L-4-hydroxyproline and *N*-(2-hydroxyoctyl)-L-4-hydroxyproline also showed enantioselectivity for aliphatic amino acids and dipeptides.

2. Experimental

2.1. Instrumentation

A PrinCE capillary electrophoresis system (PrinCE Technologies, Emmen, The Netherlands) equipped with a Lambda 1000 UV–Vis detector (Bischoff Analysentechnik, Leonberg, Germany)

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was used. Sample detection was performed at 208 nm. An Axxiom Chromatography 737 system, v3.91 (Moorpark, CA, USA) was used to collect data. Fused silica capillary tube (70 cm×50 μm I.D.) was purchased from Composite Metal Services (Hallow, UK). The detection window was at 59.5 cm.

2.2. Chemicals and solutions

All chemicals were of analytical grade. 1,2-Epoxypropane and dimethyl sulfoxide (DMSO) were purchased from Aldrich and L-4-hydroxyproline (L-Hypro), copper sulfate, 1,2-epoxyoctane and methanol (twice distilled) were from Fluka (Buchs, Switzerland). Dipeptides and amino acids were from Sigma (St. Louis, MO, USA). *Ortho*-phosphoric acid and sodium hydroxide were obtained from E. Merck (Darmstadt, Germany). *N*-(2-Hydroxyoctyl)-L-4-hydroxyproline (HO-L-Hypro) was synthesized as described previously [6]. *N*-(2-Hydroxypropyl)-L-4-hydroxyproline (HP-L-Hypro) was prepared the same way using 1,2-epoxypropane instead of 1,2-epoxyoctane.

Sample solutions were prepared by dissolving the analytes (1 mg) in double distilled, deionized water or methanol (1 ml). Samples were injected hydrodynamically (from 10 to 25 mbar) for 6 s, for an injection volume between 5 nl and 10 nl. Applied voltage was 10–30 kV. Phosphate solution (5 m*M*) was prepared by diluting *ortho*-phosphoric acid and adjusting pH by addition of 2 *M* sodium hydroxide solution. Mobility of the electroosmotic flow was determined by injecting DMSO under the same conditions.

2.3. Separation conditions

Amino acids (Table 1) were resolved using following conditions (a): 10 m*M* selector (L-Hypro, HP-L-Hypro and HO-L-Hypro), 5 m*M* copper sulfate and 5 m*M* phosphate solution pH 4.3, U=10 kV, analyte: 10 mbar, 6 s. Condition (b): 20 m*M* selector (L-Hypro, HP-L-Hypro and HO-L-Hypro), 10 m*M* copper sulfate and 5 m*M* phosphate solution pH 4.3, U=10 kV, analyte: 25 mbar, 6 s. Using HO-L-Hypro, 35% methanol were added and voltage was 20 kV.

Dipeptides (Table 2) were resolved using following conditions: sample injection 15 mbar, 6 s, electrolyte: 20 m*M* HO-L-Hypro, 10 m*M* Cu(II), 5 m*M* phosphate solution, pH 6.0, 35% CH_3OH, U= 30 kV.

2.4. Calculation of separation data

Effective mobility, selectivity factor α and resolution R_s was calculated by the following equations:

$$\mu_{\text{eff.}} = \mu_{\text{app.}} - \mu_{\text{EOF}} \quad \alpha_{\text{eff}} = \mu_{\text{eff.D}}/\mu_{\text{eff.L}}$$

$$R_s = \frac{2(t_2 - t_1)}{w_1 + w_2}$$

where w is the width of the peak.

3. Results and discussion

Separation in LECE is based on the formation of diastereomeric ternary mixed metal complexes possessing different stability constants. The equilibria taking place in the solution are given in the following equations:

$$\text{Cu (L–Ax)}_2 + \text{L–Ay} \leftrightarrow \text{Cu (L–Ax)(L–Ay)}$$

$$\text{Cu (L–Ax)}_2 + \text{D–Ay} \leftrightarrow \text{Cu (L–Ax)(D–Ay)}$$

To study the influence of derivatizing L-Hypro on enantioselectivity, two alkyl derivatives of L-Hypro were prepared, HP-L-Hypro and HO-L-Hypro, Fig. 1.

These derivatives showed significant advantages over L-Hypro regarding chiral recognition. Hydrophobic interactions of the alkyl chain of the selector with the alkyl- or aryl groups of the analytes might be one contribution to enhanced selectivity. The major role among the stereoselectivity supporting interactions is probably played by the hydroxy group in the side chain of the selector, which is formed by opening of the epoxide ring, and is assumed to participate in complex formation. This assumption is supported by the fact that only small differences in selectivity were observed between the HO-L-Hypro and HP-L-Hypro, indicating that the chain length is not a crucial point. A possible structure for the mixed selector–analyte complex is shown in Fig. 2. A similar complex was proposed for HPLC sepa-

Table 1
Separation data of the amino acids investigated. Migration times of the eluted enantiomers (t_1 and t_2), effective separation factor α_{eff} and resolution (R_s) are given

Amino acid	L-4-Hydroxyproline				*N*-(2-Hydroxypropyl)-L-4-hydroxyproline				*N*-(2-Hydroxyoctyl)-L-4-hydroxyproline			
	t_1 (min)	t_2 (min)	α	R_s	t_1 (min)	t_2 (min)	α	R_s	t_1	t_2 (min)	α	R_s
Arginine[a]	15.22	-	1.00	0.00	14.54	-	1.00	0.00	14.99	-	1.00	0.00
Asparagine[a]	21.82	-	1.00	0.00	20.16	20.56	1.28	1.10	22.80	23.33	1.29	1.43
DOPA[a]	16.20	16.60	1.35	0.89	14.88	15.65	2.62	2.03	15.69	16.42	2.34	2.70
Threo-Dihydroxyphenylserine[a]	15.91	16.10	1.19	0.86	14.79	15.36	1.50	1.29	15.55	16.09	1.47	1.83
Histidine[a]	12.49	13.54	1.34	1.84	11.35	12.06	1.24	2.12	12.29	12.90	2.23	2.49
5-Hydroxytryptophan[a]	16.91	17.34	1.40	0.92	15.70	16.63	3.14	2.16	15.46	16.28	2.85	2.60
Leucine[a]	23.06	-	1.00	0.00	21.39	-	1.00	0.00	23.96	-	1.00	0.00
Lysine[a]	21.70	-	1.00	0.00	18.88	-	1.00	0.00	15.10	-	1.00	0.00
Methionine[a]	22.92	-	1.00	0.00	21.61	21.73	1.08	0.49	23.98	24.13	1.09	0.63
α-Methyl-DOPA[a]	16.98	17.59	1.90	1.46	15.66	16.56	3.33	2.40	15.45	16.25	2.80	2.85
α-Methylphenylalanine[a]	16.87	16.87	1.00	0.00	15.51	16.31	2.85	2.08	18.14	19.21	2.62	2.00
α-Methyltyrosine[a]	18.01	18.37	1.35	1.02	15.10	15.88	2.85	2.40	15.57	16.27	1.84	3.09
o-Methyltyrosine[a]	16.69	16.92	1.15	0.79	14.85	15.70	2.67	1.95	15.57	16.27	2.17	2.22
Norleucine[a]	25.12	-	1.00	0.00	21.43	-	1.00	0.00	21.80	-	1.00	0.00
Norvaline[a]	23.42	-	1.00	0.00	20.01	-	1.00	0.00	24.58	-	1.00	0.00
Phenylalanine[a]	16.06	16.16	1.07	0.34	14.57	15.33	2.27	2.05	16.17	16.74	1.73	1.34
Phenylserine[a]	15.99	16.09	1.05	0.55	14.81	15.27	1.36	1.80	15.51	16.00	1.33	1.96
Proline[a]	20.02	-	1.00	0.00	21.44	21.88	1.55	1.22	22.21	22.61	1.43	1.31
Serine[a]	22.18	-	1.00	0.00	20.72	21.12	1.32	1.54	22.81	23.26	1.30	1.31
Threonine[a]	22.40	-	1.00	0.00	21.25	21.65	1.29	1.18	24.51	25.01	1.25	1.39
Tryptophan[a]	17.69	18.08	1.29	0.82	15.07	15.97	3.28	2.65	15.78	16.68	2.78	3.27
m-Tyrosine[a]	20.27	20.64	1.19	0.82	15.22	16.04	2.40	2.19	15.97	16.72	2.02	2.94
o-Tyrosine[a]	17.74	17.97	1.15	0.72	15.13	16.00	2.50	2.76	15.88	16.72	2.26	3.50
p-Tyrosine[a]	16.92	17.13	1.13	0.59	15.45	16.27	2.55	3.22	15.42	16.13	2.15	3.02
Valine[a]	24.48	-	1.00	0.00	20.56	20.69	1.11	0.58	25.48	25.78	1.15	0.82

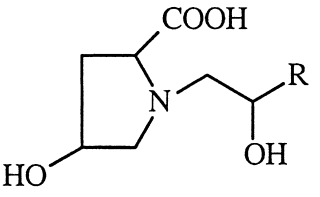
[a] For conditions see Experimental part.

ration making use of a stationary LE phase prepared using 3-glycidoxypropyltrimethoxysilane [7].

In Table 1, a comparison of separation data obtained with L-Hypro, HP-L-Hypro and HO-L-Hypro under the same conditions is given. The effective selectivity factor (α_{eff}) based on the quot-

Table 2
Separation data of glycyldipeptides by CE. Migration times of the eluted enantiomers (t_1 and t_2), effective separation factor α_{eff} and resolution (R_s) are given. For conditions, see Experimental part

Analyte	t_1	t_2	α_{eff}	R_s
Glycyl-DL-alanine	16.90	–	1.00	0.00
Glycyl-DL-asparagic acid	16.83	–	1.00	0.00
Glycyl-DL-asparagin	16.73	16.82	1.09	0.62
Glycyl-DL-leucine	15.31	–	1.00	0.00
Glycyl-DL-methionine	15.42	15.58	1.18	0.84
Glycyl-DL-norleucine	15.38	–	1.00	0.00
Glycyl-DL-phenylalanine	15.80	16.19	1.98	1.24
Glycyl-DL-serine	16.50	16.57	1.06	0.54
Glycyl-DL-threonine	16.57	16.71	1.17	0.81
Glycyl-DL-valine	16.72	–	1.00	0.00



R= CH_3 HP-L-Hypro

R=C_6H_{13} HO-L-Hypro

Fig. 1. Chemical structures of the chiral selectors investigated.

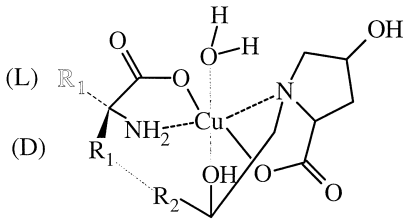


Fig. 2. Possible structure of the copper complex.

ient of effective mobilities (μ_{eff}) was used for calculation instead of α (t_2/t_1), since these data are independent on the electroosmotic flow. The difference in resolution power of the different selectors is demonstrated by means of the separation of DL-α-methylphenylalanine (Fig. 3).

As can be seen from Table 1, 10 m*M* selector concentration was sufficient in the case of HO-L-Hypro and HP-L-Hypro to obtain excellent separation for aromatic amino acids. With L-Hypro at this selector concentration only partial resolution was observed. For obtaining baseline resolution up to 80 m*M* L-Hypro was found to be necessary [5]. Fig. 4 shows the resolution of a mixture of two aromatic amino acids using HO-L-Hypro as chiral selector. The elution order was determined by spiking the racemates with the L-enantiomers.

With HP-L-Hypro and HO-L-Hypro, aliphatic amino acids were also resolved. In this case the optimal selector concentration was 20 m*M* (Fig. 5). In order to reach this concentration, the addition of 35% (v/v) methanol was necessary in the case of HO-L-Hypro. Higher concentrations, however, were not possible for solubility reasons. Generally, the addition of organic modifiers did not significantly affect resolution. At this selector concentration, it was still possible to detect aliphatic amino acids, although it was necessary to inject higher amounts.

Fig. 6 shows the resolution of DL-serine.

A 5 m*M* phosphate solution pH 4.3 was found to be the optimal electrolyte composition for the separation of amino acids. The molar ratio of selector to copper(II) was always 2:1. Despite of the fact that phosphate has no buffer capacity in this pH range, phosphate was found to be superior over other buffers regarding resolution and detection. Although histidine showed sufficient resolution at pH 4.3, the optimal pH for this basic amino acid was found to be 6.

In addition to amino acids, some dipeptides were also resolved. Baseline separation, however, was only obtained with glycyl-DL-phenylalanine. Obviously, the bulky substituent enhances chiral discrimi-

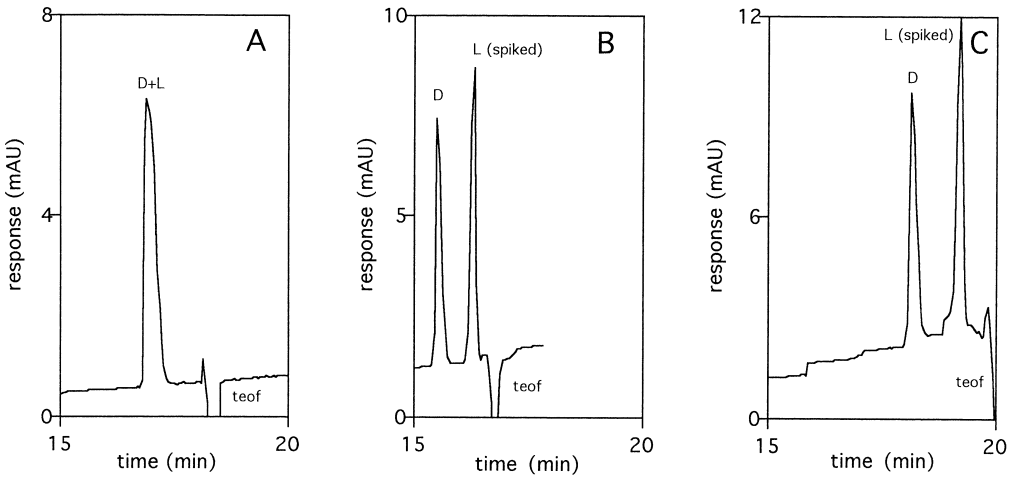


Fig. 3. Comparison of different selectors for the separation of α-methylphenylalanine. Racemate was spiked with L-enantiomer. Conditions: U=10 kV, analyte: 10 mbar, 6 s. (A) 10 m*M* L-Hypro, 5 m*M* Cu(II) in 5 m*M* phosphate solution pH 4.3. (B) 10 m*M* HP-L-Hypro, 5 m*M* Cu(II) in 5 m*M* phosphate solution pH 4.3. (C) 10 m*M* HO-L-Hypro, 5 m*M* Cu(II) in 5 m*M* phosphate solution pH 4.3.

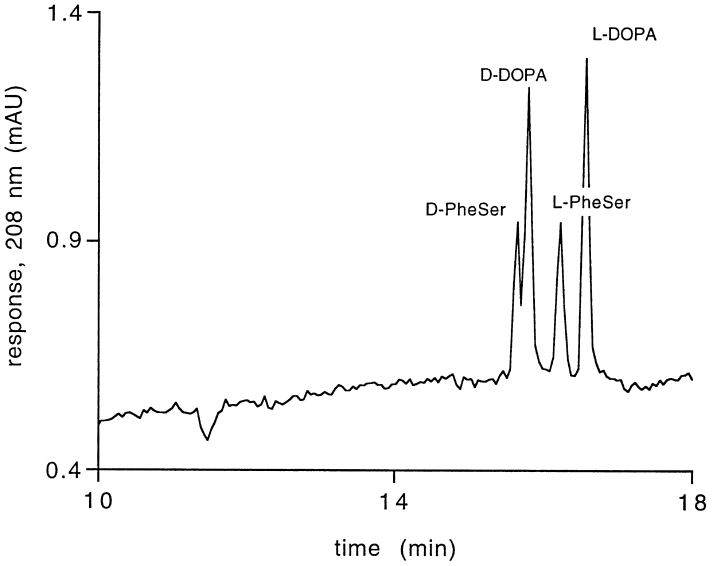


Fig. 4. Resolution of DL-DOPA and DL-phenylserine. Conditions: 10 m*M* HO-L-Hypro, 5 m*M* Cu(II) in 5 m*M* phosphate solution pH 4.3, U = 10 kV, analyte: 10 mbar, 6 s.

nation. Stronger hydrophobic interaction of the aromatic residue with the side chain of the selector might also contribute to an improved resolution. Contrary to that found for amino acids, the optimal pH for the resolution of dipeptides was found to be 6 (Fig. 7). Similar observations regarding pH were made by HPLC using chiral stationary LE phases [7,8]. In Table 2, the separation data for glycyldipep-

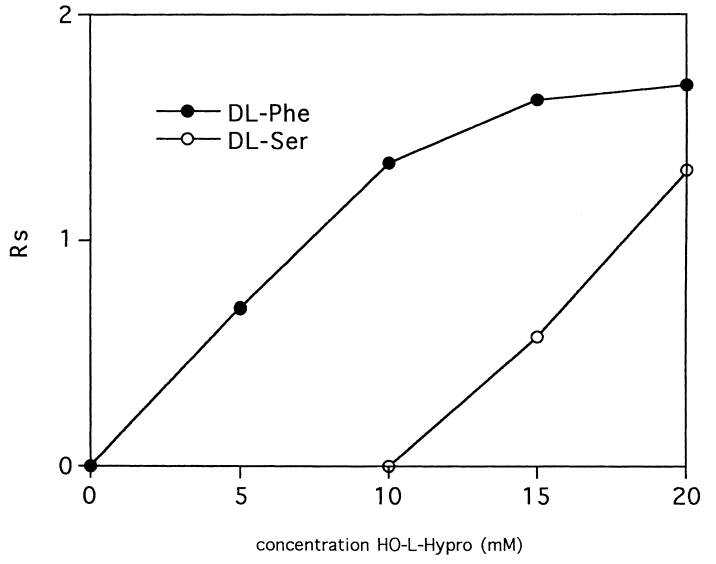


Fig. 5. Dependence of the resolution of aromatic and aliphatic amino acids on concentration of HO-L-Hypro (electrolyte: phosphate solution, pH 4.3).

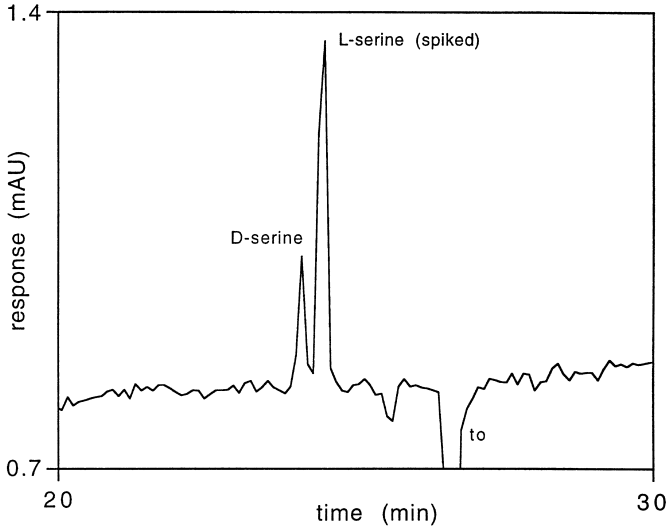


Fig. 6. Electropherogram of the chiral resolution of DL-serine. Conditions: 20 m*M* HO-L-Hypro, 10 m*M* Cu(II) in 5 m*M* phosphate solution pH 4.3, 35% methanol. U=20 kV, analyte: 25 mbar, 6 s.

tides are listed. With some diastereomeric dipeptides partial resolution was obtained. DL-Leucyl-DL-phenylalanine, for example, was split into three peaks.

4. Conclusion

N-Alkyl derivatives of L-Hypro have been shown

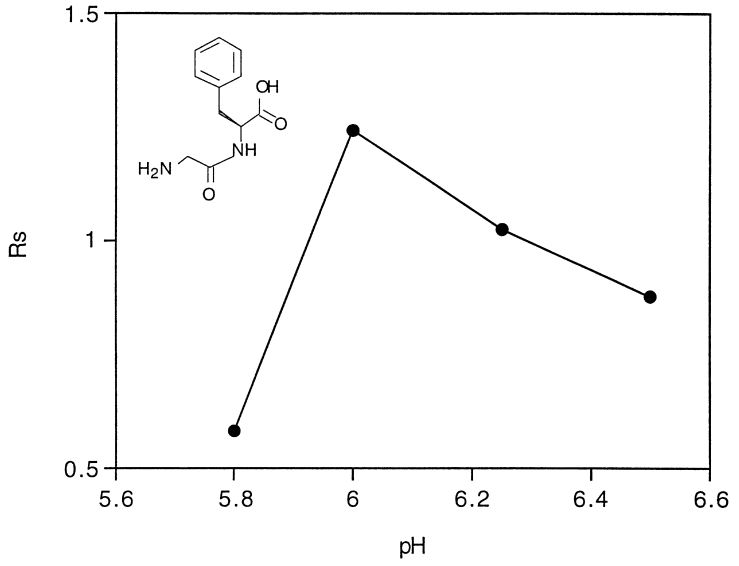


Fig. 7. Influence of the pH value on the enantiomer separation of glycyl-DL-phenylalanine. Conditions: 20 m*M* HO-L-Hypro, 10 m*M* Cu(II) in 5 m*M* phosphate solution pH 5.8–6.5, 35% methanol. U=30 kV.

to display improved enantioselectivity for aromatic and aliphatic amino acids compared to L-Hypro. 13 underivatized aromatic and 6 aliphatic amino acids were successfully separated into their enantiomers. This principle was found to be applicable at a different pH for the optical resolution of dipeptides.

The influence of sodium dodecylsulfate on resolution and elution order using the principle of electrokinetic chromatography will be the subject of further investigations.

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