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Enantioseparation of dipeptides by capillary electrochromatography on a teicoplanin aglycone chiral stationary phase

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Abstract

This work deals with investigations on the enantioseparation of glycyl-dipeptides by capillary electrochromatography (CEC) on a capillary packed with teicoplanin aglycone immobilized on 3.5 μm silica gel. The results were compared to those obtained with micro-HPLC using the same chiral stationary phase. Polar organic and reversed-phase mode were checked, whereby the latter showed better results. Out of 12 glycyldipetides investigated, all compounds showed baseline separation with R_s values up to 20. Plate numbers were in the range of 10 000–300 000/m. The choice of organic modifier was found to be crucial. While methanol increased retention time, acetonitrile reduced it. A ternary mixture of ethanol–acetonitrile–aqueous triethylamine acetate solution pH 4.1 was found to be a useful compromise, providing excellent resolution with retention times less than 25 min. Efficiency and resolution were generally found to be higher in CEC than with micro-HPLC.
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Keywords: Enantiomer separation; Chiral stationary phases, electrochromatography; Peptides

1. Introduction

Among a big variety of chiral selectors, macrocyclic antibiotics of the glycopeptide type have been shown to be powerful selectors in HPLC [1,2], CE [3,4] supercritical fluid chromatography [5–7] and more recently in capillary electrochromatography (CEC) [8–17]. Macrocyclic antibiotics such as vancomycin, ristocetin A and teicoplanin are glycopeptides, whereby both the peptide aglycone part and the sugar moities are believed to contribute to chiral recognition. Recently, Berthod et al. [18] compared a teicoplanin- and a teicoplanin aglycone HPLC phase for their chiral recognition ability. Teicoplanin aglycone lacks the D-glucosamine and the D-mannose moiety (Fig. 1). The authors observed that for the chiral recognition of a series of drugs the carbohydrate moieties are of importance while for amino acids the aglycone showed superior enantioselectivity. In a previous paper we described the application of a silica based teicoplanin aglycone phase, 3.5 μm, to the CEC separation of several drug classes and amino acids, whereby excellent selectivity was observed especially for the latter class of compounds [17].

This paper deals with the chiral separation of

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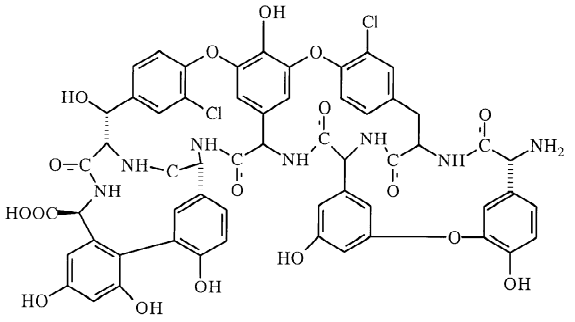


Fig. 1. Chemical structure of teicoplanin aglycone.

glycyl dipeptides by CEC using this teicoplanin aglycone chiral stationary phase (CSP).

Dipeptides represent basic and essential compounds in biological systems and are used as building blocks in peptide synthesis. The chiral separation of dipeptides is of interest when checking for racemization in peptide synthesis and investigating peptide hydrolysates. Although L-amino acids are predominant in nature, there are several examples of the existence of D-amino acids in proteins [19,20].

Only few methods have been described to date for the chiral separation of dipeptides including, for example, the use of chiral crown ethers in HPLC [21] and CE [22,23], chiral ligand-exchange chromatography [24], ligand-exchange CE [25] and cyclodextrins by CE [26–28].

We investigated a teicoplanin aglycone CSP for its ability to resolvinge dipeptides by CEC, checking different mobile phases under aqueous and non-aqueous conditions. Furthermore, results were compared to separation experiments on micro-HPLC columns containing the same CSP.

2. Experimental

2.1. Chemicals and solutions

All chemicals were of analytical grade. Methanol, ethanol, acetonitrile (ACN), acetic acid, ammonium acetate and triethylamine (TEA) were purchased from E. Merck (Darmstadt, Germany). Water was deionized and doubly distilled. All dipeptides were from Sigma (St. Louis, MO, USA). Teicoplanin aglycone (TAG) immobilized on 3.5 μm silica gel was a gift from Astec (Whippany, NJ, USA). All mobile phases were degassed for 10 min by helium 5.0, aqueous solutions were filtered through a 0.2-μm filter. Samples were prepared in a concentration of 1 mg/ml and dissolved in the mobile phase.

2.2. Materials and methods

The material for CEC was packed into fused-silica capillaries [36 cm (effective length 27.5 cm) ×100 μm I.D.] by Grom (Herrenberg, Germany). Fused silica capillaries were from Microquartz (Munich, Germany) and microbore stainless steel columns (10 or 30 cm length×1 mm I.D.) were purchased from Vici (Schenkon, Switzerland) and packed with a slurry of TAG in methanol at 390 bar.

2.3. Instrumentation

For CEC experiments, a fully automated ^{3D}CE system (Agilent Technologies, CA, USA) equipped with an external CEC pressure device (12 bar) and a diode array detector were used. Detection was performed via on-column measurement of the UV absorption at 208 nm and samples were injected electrokinetically for 6 s at 12 kV unless indicated otherwise. During the run, 12 bar were applied on both ends of the capillary to prevent air bubble formation.

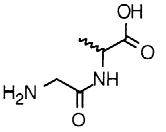
For μ-HPLC we used an L-7150 A pump (Merck–Hitachi, Darmstadt, Germany) with a passive split, an injection valve with a sample loop of 200 nl (Upchurch Scientific, Oak Harbour, WA, USA) and an L-7400 UV-detector (Merck–Hitachi) with a micro-flow cell (140 nl, 150 μm I.D. of the connecting capillary). Detection was at 208 nm.

3. Results and discussion

3.1. Enantioseparation of glycyl-dipeptides by CEC

Dipeptides might dock to the basket-like peptide aglycone whereby hydrophobic interactions, hydrogen bonds, dipole stacking, ionic and $\pi-\pi$ interactions as well as steric repulsion are assumed to be interactions responsible for chiral recognition. The sugar chains seem to be a hindrance in the case of

glycyl-alanine



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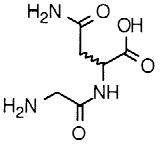

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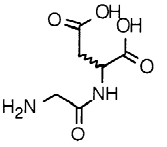

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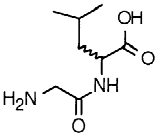

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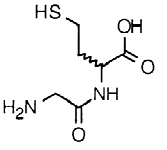
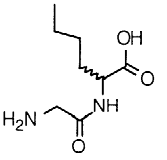


glycyl-norleucine



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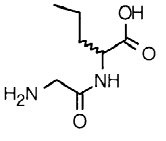

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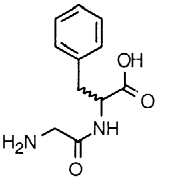

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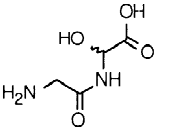

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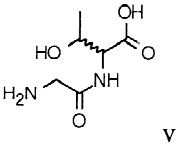
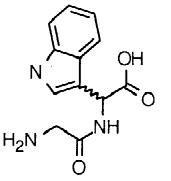


glycyl-tryptophan



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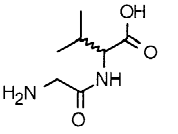
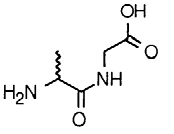


alanyl-glycine



leucyl-glycine

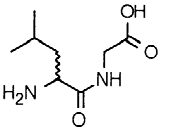


Fig. 2. Chemical structure of compounds investigated.

Table 1
Separation data of dipeptides investigated by CEC

	0.2% TEAA in water, pH 4.1						0.2% TEAA in water, pH 4.1–methanol (80:20)					
	t_L	t_D	k'_L	k'_D	α	R_s	t_L	t_D	k'_L	k'_D	α	R_s
Glycyl-alanine	9.97	21.24	0.262	1.688	6.44	8.74	15.93	50.08	0.098	2.453	24.88	16.22
Glycyl-asparagine	11.10	13.65	0.405	0.727	1.79	2.04	17.04	24.06	0.078	0.522	6.66	5.40
Glycyl-aspartate	13.23	19.12	0.674	1.420	2.10	3.36	21.06	34.58	0.324	1.174	3.62	7.72
Glycyl-leucine	15.31	34.73	0.937	3.396	3.62	3.46	23.50	72.46	0.473	3.542	7.48	12.08
Glycyl-methionine	15.80	50.34	1.00	5.372	5.37	7.94	23.44	77.08	0.474	3.848	8.12	12.98
Glycyl-norleucine	16.70	47.30	1.113	4.987	4.47	6.12	24.01	89.77	0.510	4.646	9.11	13.04
Glycyl-norvaline	13.90	43.20	0.759	4.468	5.88	9.01	20.74	82.08	0.346	4.329	12.48	17.98
Glycyl-phenylalanine	21.67	51.56	1.743	5.526	3.17	5.97	33.32	102.14	1.076	5.363	4.98	10.27
Glycyl-serine	11.19	13.59	0.401	0.705	1.76	2.35	15.12	23.53	0.115	0.736	6.35	5.60
Glycyl-threonine	10.02	11.75	0.268	0.487	1.81	2.47	15.38	18.97	0.135	0.400	2.96	4.81
Glycyl-tryptophan	46.45	101.48	4.879	11.846	2.42	3.33	58.77	154.24	2.957	9.386	3.17	9.54
Glycyl-valine	12.59	19.77	0.593	1.502	2.53	3.77	19.26	38.69	0.242	1.496	6.16	13.87

t = retention time (min), k' = retention factor, α = separation factor, R_s = resolution.
Conditions: Injection: 15 kV, 12 s, applied voltage: 15 kV.

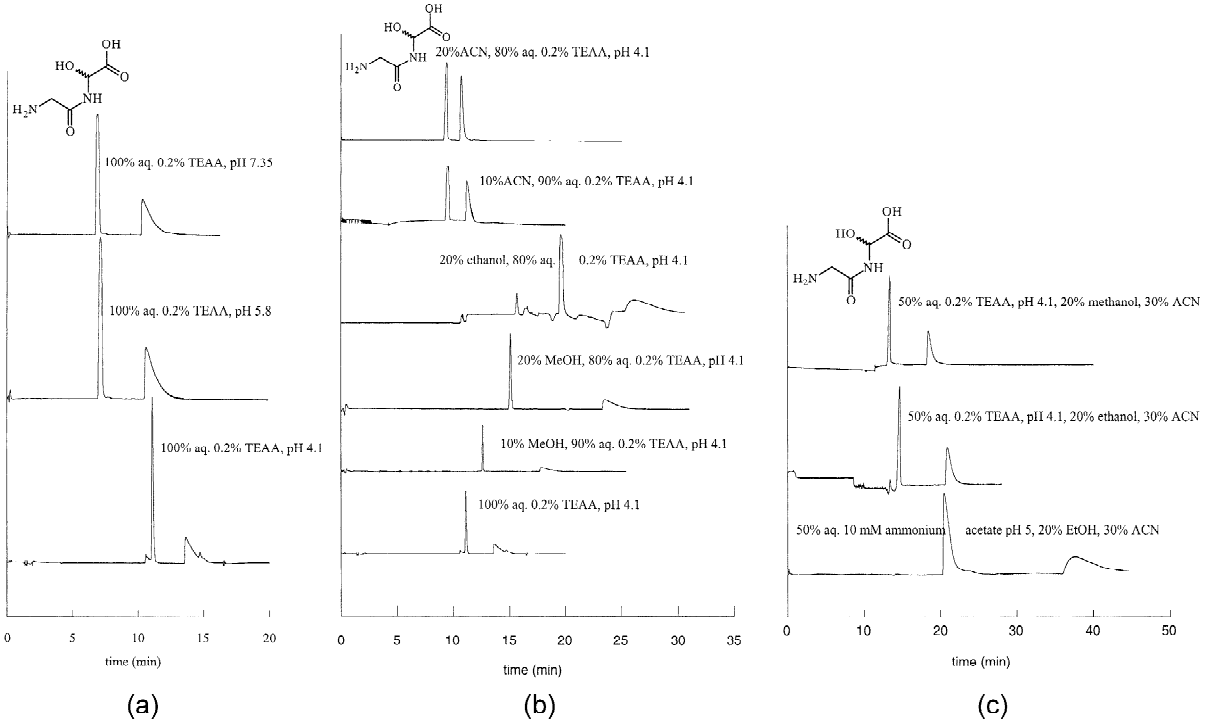


Fig. 3. (A) Chiral separation of Gly–Ser by CEC using aqueous 0.2% TEAA buffer as a mobile phase with different pH. Injection: 15 kV, 15 s (analyte dissolved in mobile phase, 1 mg/ml) Run: 15 kV. (B) Chiral separation of Gly–Ser by CEC using different mobile phases containing increasing amounts of methanol, ethanol or ACN. Injection: 15 kV, 15 s (analyte dissolved in mobile phase, 1 mg/ml). Run: 15 kV. (C) Chiral separation of Gly–Ser by CEC using different ternary mobile phases. Injection: 15 kV, 15 s (analyte dissolved in mobile phase, 1 mg/ml). Run: 15 kV.

amino acids and dipeptides, since the intact teicoplanin phase showed less chiral recognition ability for those compounds.

Encouraged by the successful resolution of different chiral drugs and amino acids [17], we tested the teicoplanin aglycone CSP in polar organic mode and under reversed-phase conditions for its ability to resolve glycyl-dipeptides (Fig. 2).

3.1.1. Use of polar-organic mode

First, enantioseparation was tried using the polar-organic mode. Mobile phases containing methanol–ACN without water showed very broad peaks and only partial or no resolution. Obviously, for successful enantioseparation of this class of compounds, the mobile phase has to contain water.

3.1.2. Use of reversed-phase mode

An aqueous phase containing 0.2% triethylamine acetate (TEAA) pH 4.1 without any organic modifier was used. Under these conditions all analytes were baseline resolved, but retention times were rather high. Elution order of enantiomers was checked by applying the pure enantiomers and was found to be L before D in all cases. Table 1 shows separation data for several samples.

3.1.2.1. Optimization of pH

The next step studied the effect of pH on retention times and separation. Increasing pH in the mobile phase slightly reduced retention times, but resolution was poorer. Fig. 3A shows the influence of pH on the separation of Gly–Ser.

3.1.2.2. Influence of organic modifiers in the mobile phase

Addition of an organic modifier, such as methanol, ethanol or acetonitrile often results in a change of selectivity or efficiency. To check the influence of organic modifiers, we added different amounts of methanol or ethanol to the mobile phase. Increasing concentrations of methanol resulted in much higher resolution, but retention times also increased (Fig. 3B). Tables 1 and 2 shows the corresponding separation data of 20% methanol or 20% ethanol added to the aqueous mobile phase, respectively.

Interestingly, addition of acetonitrile produced contrary results. With 10 or 20% acetonitrile and the same voltage, separations became faster and efficiency improved. Although separation itself became slightly poorer, each compound was still baseline resolved. Separation data using 20% ACN are shown in Table 2. A further interesting step forward was to apply ternary mixtures with the goal of combining good selectivity with high efficiency. Table 3 illustrates the effect of a ternary mixture containing only 50% (0.2%) TEAA buffer pH 4.1, 20% methanol, 30% ACN as a mobile phase. In this case, retention times were significantly reduced while excellent chiral resolution was maintained.

Table 2
Separation data of dipeptides investigated by CEC

	0.2% TEAA in water, pH 4.1–ethanol (80:20)						0.2% TEAA in water, pH 4.1–ACN (80:20)					
	t_L	t_D	k'_L	k'_D	α	R_s	t_L	t_D	k'_L	k'_D	α	R_s
Glycyl-alanine	19.26	50.60	0.115	1.930	16.69	15.26	10.65	15.43	0.121	0.624	5.15	4.78
Glycyl-asparagine	19.32	26.46	0.098	0.505	5.11	4.25	9.86	10.94	0.186	0.316	1.69	2.88
Glycyl-aspartate	24.17	36.58	0.241	0.879	3.63	10.23	11.17	13.53	0.199	0.453	2.26	4.72
Glycyl-leucine	25.33	75.07	0.293	2.834	9.65	9.690	14.37	24.39	0.393	1.365	3.46	6.07
Glycyl-methionine	24.92	83.12	0.273	3.249	11.86	11.33	14.12	24.79	0.369	1.404	3.80	7.90
Glycyl-norleucine	27.64	92.41	0.410	3.715	9.05	11.56	15.86	31.46	0.538	2.051	3.81	8.91
Glycyl-norvaline	22.12	79.57	0.143	3.114	21.65	20.52	12.98	24.49	0.258	1.375	5.31	12.11
Glycyl-phenylalanine	33.58	103.92	0.706	4.281	6.05	9.42	16.22	27.18	0.573	1.636	2.85	6.35
Glycyl-serine	19.91	25.14	0.158	0.462	2.91	2.15	9.41	10.71	0.107	0.260	2.42	2.60
Glycyl-threonine	20.60	24.43	0.126	0.335	2.66	3.04	9.74	10.45	0.145	0.229	1.57	1.29
Glycyl-tryptophan	53.42	129.90	1.792	5.789	3.23	8.19	24.63	44.87	1.059	2.751	2.59	7.36
Glycyl-valine	20.77	49.85	0.129	3.090	2.39	10.51	12.51	15.93	0.213	0.545	2.55	4.27

t = retention time (min), k' = retention factor, α = separation factor, R_s = resolution.
Conditions: Injection: 15 kV, 12 s, applied voltage: 15 kV.

Table 3
Separation data of dipeptides investigated by CEC

	0.2% TEAA in water, pH 4.1/methanol/ACN (50/20/30)						0.2% TEAA in water, pH 4.1/ethanol/ACN (50/20/30)					
	t_L	t_D	k'_L	k'_D	α	R_s	t_L	t_D	k'_L	k'_D	α	R_s
Glycyl-alanine	7.27	14.76	0.346	1.733	5.00	8.32	8.41	19.02	0.249	1.826	7.31	9.64
Glycyl-asparagine	7.19	9.05	0.398	0.760	1.90	5.16	7.44	10.44	0.155	0.621	4.00	5.45
Glycyl-aspartate	7.44	9.83	0.444	0.908	2.04	5.03	8.90	11.83	0.260	0.675	2.59	5.32
Glycyl-leucine	8.85	16.51	0.688	2.150	3.12	10.5	9.01	17.49	0.421	1.758	4.17	9.16
Glycyl-methionine	8.69	18.30	0.652	2.479	3.80	8.73	9.62	22.63	0.460	2.434	5.29	11.07
Glycyl-norleucine	9.18	19.09	0.613	2.355	3.83	11.3	10.11	24.17	0.764	3.218	4.21	11.24
Glycyl-norvaline	8.39	18.69	0.553	2.461	4.44	8.95	9.52	23.71	0.446	2.603	5.82	9.46
Glycyl-phenylalanine	9.38	17.09	0.705	2.107	2.98	6.70	10.67	20.92	0.583	2.103	3.60	6.83
Glycyl-serine	6.74	8.96	0.283	0.706	2.48	5.92	7.52	10.63	0.136	0.605	4.45	6.22
Glycyl-threonine	6.89	8.04	0.343	0.567	1.65	4.60	7.75	9.270	0.178	0.408	2.29	4.67
Glycyl-tryptophan	11.42	17.32	1.154	2.267	1.96	4.53	12.66	20.36	0.906	2.066	2.27	4.40
Glycyl-valine	8.19	12.51	0.575	1.405	2.44	8.64	9.41	15.79	0.410	1.367	3.32	7.50

t = retention time (min), k' = retention factor, α = separation factor, R_s = resolution.
Conditions: Injection: 15 kV, 12 s, applied voltage: 28 kV.

Optimal results were obtained using a similar ternary mixture containing 50% (0.2%) TEAA buffer pH 4.1, 20% ethanol and 30% ACN (Table 3). Plate numbers were determined for Gly–L-Ser and Gly–D-Ser and were found to be 40 000 and 21 000/m, respectively.

Since current was relatively low with a buffer content of 50% in the ternary mixtures, we were able to speed up separations by increasing the voltage to 28 kV. In this case, all 12 compounds were baseline resolved within 25 min. Furthermore, other mobile phases, containing 10 m*M* ammonium acetate instead of TEAA were tested, but neither efficiency nor selectivity were comparable to the results obtained with TEAA (Fig. 3C).

3.1.2.3. Influence of the structure of the glycyl-dipeptides on resolution

As can be seen from Tables 1–3, bulky and more hydrophobic dipeptides such as Gly–Nva, Gly–Nle, Gly–Phe and Gly–Trp are better resolved than short and hydrophilic compounds. Fig. 4 shows a mixture of three baseline separated glycyl-dipeptides in one run. Since resolution is remarkabley high, this approach is well suited to impurity studies. Fig. 5 shows 0.07% Gly–L-Phe in a commercially available sample of Gly–D-Phe.

Moreover, chiral separation of Gly–Leu and Gly–Ala were compared to that of Leu–Gly and Ala–Gly, respectively. Under all separation conditions tested,

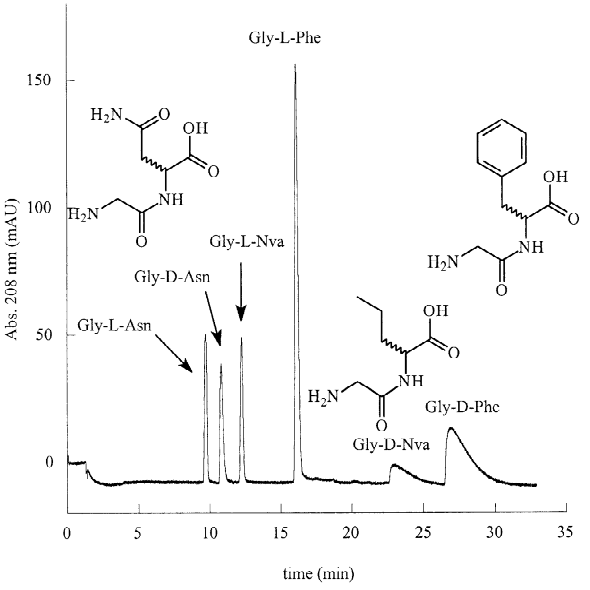


Fig. 4. Chiral separation of Gly–Asn, Gly–Nva and Gly–Phe by CEC. Conditions: Mobile phase: ACN–aq. 0.2% TEAA pH 4.1 (20:80). Injection: 15 kV, 15 s (analytes dissolved in mobile phase, 1 mg/ml). Run: 15 kV (7 μA).

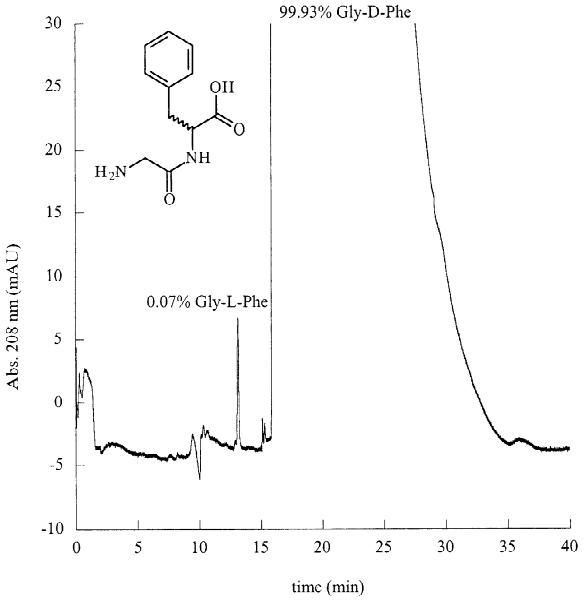


Fig. 5. Impurity study of a commercially available sample of Gly–D-Phe by CEC. Conditions: Mobile phase: aq. 0.2% TEAA pH 4.1–ethanol–ACN (50:20:30), U = 22 kV (6 μA) Injection: 18 kV, 18 s (analyte dissolved in mobile phase, 8 mg/ml).

resolution of Gly–Leu and Gly–Ala were resolved much better. The linkage of the amino acid glycine is obviously crucial: If Gly is on the amino terminal end, there is better chiral recognition by teicoplanin aglycone than vice versa.

3.2. Enantioseparation of glycyl-dipeptides by micro-HPLC

The same set of glycyl-dipeptides was tested under conditions similar to those in CEC using micro-HPLC columns with 10-fold inner diameter compared to capillaries for CEC. Using water–ethanol–ACN (50:20:30) as a mobile phase and a 10-cm column, only some of the compounds were resolved. Obviously, the column was not long enough to show equal resolution. When a 30 cm column was used, most of the dipeptides were resolved with the same mobile phase, but however, with relatively long retention times. Elution order of enantiomers was found to be same than with CEC. Table 4 shows separation data of glycyl-dipeptides, Fig. 6 shows chiral separation of Gly–Phe under these conditions. These separations were hardly optimized and could certainly could be improved with an appropriate instrument.

4. Conclusion

Enantioseparation of glycyl-dipeptides was demonstrated using a teicoplanin aglycone CSP. The mobile phase was optimized with respect to pH, organic solvent and electric field. Addition of methanol increases retention time, whereas increasing amounts of ACN speed up separation. Ternary

Table 4
Separation data of dipeptides resolved by micro-HPLC

	t_L	t_D	k'_L	k'_D	α	R_s
Glycyl-alanine	23.58	61.30	2.03	6.89	3.39	3.02
Glycyl-asparagine	20.60	25.87	1.65	2.33	1.41	0.72
Glycyl-aspartate	15.35	–	0.98	–	1.00	0.00
Glycyl-leucine	23.58	42.33	2.03	4.45	2.19	1.42
Glycyl-methionine	22.74	51.82	1.93	5.67	2.94	2.15
Glycyl-norleucine	26.34	70.03	2.39	8.01	3.35	2.80
Glycyl-norvaline	24.02	66.08	2.09	7.50	3.59	3.03
Glycyl-phenylalanine	24.72	45.07	2.18	4.80	2.20	1.51
Glycyl-serine	20.44	28.74	1.63	2.70	1.66	0.85
Glycyl-threonine	20.98	23.47	1.70	2.02	1.19	0.36
Glycyl-tryptophan	26.47	46.84	2.41	5.03	2.09	1.73
Glycyl-valine	23.73	43.90	2.05	4.65	2.27	1.71

t = retention time (min), k' = retention factor, α = separation factor, R_s = resolution.
Conditions: water–ethanol–ACN (50:20:30).

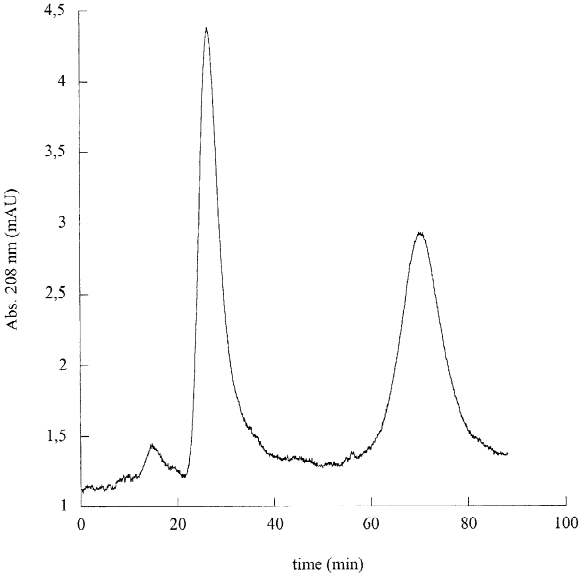


Fig. 6. Enantioseparation of Gly–DL-Nle by micro-HPLC. Conditions: Mobile phase: water–ethanol–ACN (50:20:30), flow: 15 μl/min, Injection: 200 nL (analyte dissolved in mobile phase, 1 mg/ml).

mixtures were found to be optimal as mobile phases. All glycyl dipeptides tested were baseline resolved.

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