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Chiral resolution of diastereomeric di- and tripeptides on a teicoplanin aglycone phase by capillary electrochromatography

The chiral separation ability of a capillary packed with teicoplanin aglycone as a chiral stationary phase was investigated. This stationary phase was used successfully for chiral resolution of both diastereomeric dipeptides and tripeptides possessing one or two chiral centers. The composition of the mobile phase was shown to be crucial for separation. The use of reversed-phase mode was clearly superior to the polar-organic mode. The nature of the organic modifier was found to have a marked influence on separation. After optimizing conditions, all diasteromeric dipeptides and tripeptides investigated were baseline-resolved, however, it was not possible to find a uniform mobile phase showing optimal results for all peptides investigated.

Keywords: Capillary electrochromatography / Chiral analysis / Dipeptides / Enantioseparation / Tripeptides
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1 Introduction

Macrocyclic antibiotics, introduced as chiral selectors by Armstrong's group [1–4, 12, 16, 22], found broad application for the chiral separation of a large variety of compounds in HPLC [1–3], CE [4–12], supercritical fluid chromatography (SFC) [13–15], and more recently in CEC [16–26]. The macrocyclic antibiotics vancomycin, ristocetin A and teicoplanin belong to the glycopeptide group of antibiotics. With the aim of investigating the role of the sugar moiety in chiral recognition, Berthod *et al.* [27] prepared a teicoplanin aglycone (TAG) phase and compared its enantioselectivity with an intact teicoplanin phase by HPLC. Interestingly, the authors observed that enantioselectivity was reduced for drugs and significantly enhanced for amino acids. In previous works, we investigated the application of a teicoplanin aglycone phase, 3.5 μm, packed into capillaries, to the chiral separation of several drug classes, amino acids [25] and glycyldipeptides [26]. The phase showed excellent resolution power, especially for amino acids and glycyldipeptides.

This paper deals with studies on the resolution of diastereomeric di- and tripeptides on a teicoplanin aglycone chiral stationary phase (CSP) by CEC. Since this chiral selector is highly UV-absorbing, addition to the background electrolyte may cause severe detection problems. Therefore, in CE analytes with high UV absorption or the partial filling method [5] can be applied with this selector. In the case of weakly UV-absorbing compounds such as amino acids, derivatives have to be prepared [28]. Since in the case of CEC the selector is immobilized and no detection interferences are therefore existing, underivatized analytes can be applied.

Oligopeptides such as di- and tripeptides are building blocks in peptide synthesis and also play a role in nature. Since it has been discovered that not only L-amino acids but also D-enantiomers are present in peptides and proteins, chiral analysis of peptides attracted increasing interest. For the chiral separation of dipeptides the use of crown ethers in HPLC [29] and CE [30, 31], cyclodextrins in CE [28, 32, 33], vancomycin and teicoplanin in CE [23], ligand-exchange chromatography [34, 35] and ligand-exchange CE [36] was described. This study focuses on the chiral separation of diastereomeric di- and tripeptides under reversed-phase conditions by CEC on a TAG phase and elucidates the role of organic modifiers on the separation.

2 Materials and methods

2.1 Chemicals and solutions

All chemicals were of analytical grade. Acetonitrile (ACN), ethanol (EtOH), methanol (MeOH), acetic acid, and triethylamine (TEA) were from E. Merck (Darmstadt, Ger-

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Abbreviations: CSP, chiral stationary phase; **TAG**, teicoplanin aglycone; **TEAA**, triethylamine acetate

* Dedicated to Prof. Dr. G. Gübitz on the occasion of his 60th birthday

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many). Water was deionized and doubly distilled. Ala-Gly, Ala-Gly-Gly, Ala-Phe, and Ala-Met were from Bachem (Bubendorf, Switzerland). Ala-Ala was purchased from Acros (Geel, Belgium). All other dipeptides were obtained from Sigma (St. Louis, MO, USA). TAG immobilized on 3.5 μm silica gel was a gift from Astec (Whippany, NJ, USA). Triethylamine acetate (TEAA) solution was prepared by adjusting appropriate pH of a 0.2% TEA solution with diluted acetic acid. 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 water or in the mobile phase.

2.2 Capillary electrochromatography

Fused-silica capillaries were from Microquartz (Munich, Germany). For CEC, a TAG-CSP packed into 100 μm ID fused-silica capillaries (36 cm/27.5 cm effective length) was used. Packing was provided by Grom (Herrenberg, Germany) using the typical procedure with frit sintering. For CEC experiments, we used a fully automated ^{3D}CE system (Agilent Technologies, CA, USA) equipped with an external CEC pressure device (12 bar) and a diode array detector. Samples were injected electrokinetically for 6 s at 12 kV unless indicated otherwise. Detection was performed *via* on-column measurement of the UV absorption at 208 nm. During the run, a pressure of 12 bar was applied at both ends of the capillary to prevent air bubble formation.

3 Results and discussion

3.1 Chiral separation of diastereomeric dipeptides

As shown in a previous study, TAG is a very suitable selector for enantioseparation of glycyl dipeptides [26]. The structure of TAG is shown in Fig. 1. Since the intact teicoplanin showed significantly lower chiral recognition ability for dipeptides, the sugar chains seemed to cause a steri-

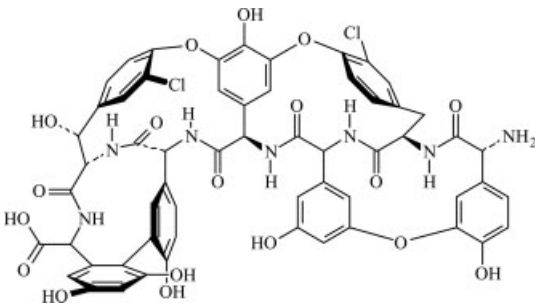

Figure 1. Chemical structures of the chiral selector TAG.

cal hindrance in this case. In the present study, both diastereomeric di- and tripeptides were investigated for their separation ability on this chiral stationary phase. The chemical structures of the compounds tested are given in Fig. 2. Generally, the peptides might dock to the basket-like peptide aglycone. Interactions between selector and analyte, hydrogen bonds, dipole stacking, ionic interactions, π-π-interactions, and steric repulsion have to be taken into account.

3.1.1 Optimization of the mobile phase

In this study, separation experiments were done with reversed-phase mode only, since it has been found that the polar-organic mode did not produce satisfactory chiral separation of glycyl dipeptides [26]. TEAA was used as a mobile-phase additive enhancing endoosmosis. Aqueous solutions of 0.2% TEAA were tested in the pH range of 3.5–7; pH 4.1 was found to be optimal with respect to retention times and resolution. This mobile phase (TEAA, pH 4.1) resulted in partial resolution of all diasteromeric dipeptides investigated. Bulky dipeptides, such as Leu-Leu or Leu-Phe were baseline-resolved but at the price of retention times up to 90 min and peak shape was unsatisfactory. The last-eluting peak in particular was very broad and thus hard to identify. Under these conditions, dipeptides consisting of short amino acids such as Ala-Ala, Ala-Ser or Ala-Val showed only two or three peaks.

Next, the influence of MeOH on retention times and resolution was studied by adding 10–20% MeOH to TEAA, pH 4.1. With increasing amounts of MeOH, retention times drastically increased, however, separation improved only in some cases. Ala-Val, for example, showed three peaks and separation of Leu-Ala produced four peaks. Addition of 30% v/v MeOH resolved Ala-Val, Leu-Ala and other dipeptides into their four possible isomers, but retention times were about 2 h and the peaks were rather broad. For example, the forth peak of Leu-Leu could hardly be detected, since this peak showed very poor efficiency. In Fig. 3, enantioseparation of Ala-Ser is shown with addition of 0, 10, 20, and 30% MeOH to TEAA, pH 4.1, as a mobile phase. In the case of Ala-Ser, addition of 10% v/v MeOH to the mobile phase showed four baseline-separated peaks within 24 min.

Previous results have shown that enantioseparation of glycyl dipeptides was influenced positively by addition of ACN to the mobile phase, producing shorter migration times and improved peak shape [26]. Addition of 10–20% v/v ACN to TEAA, pH 4.1, resulted in baseline resolution of Leu-Ala, Leu-Phe and Leu-Val. Ala-Ser, which was baseline-resolved with addition of MeOH to the

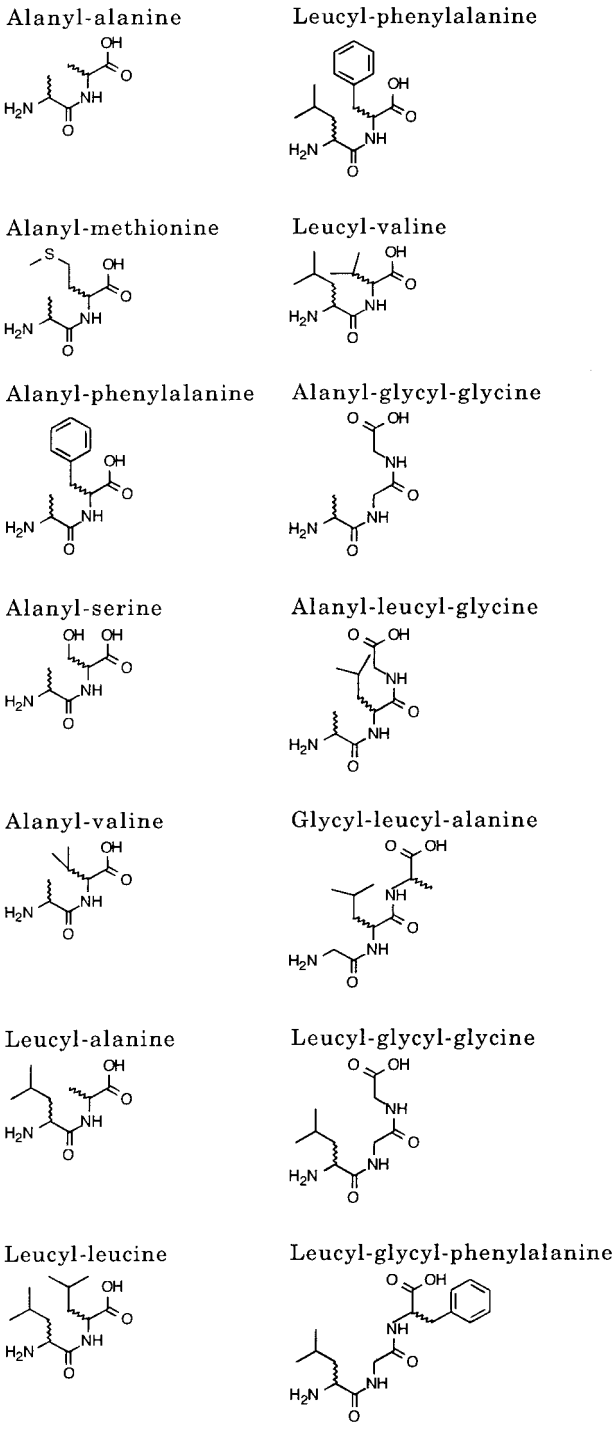

Figure 2. Chemical structures of the di- and tripeptides investigated.

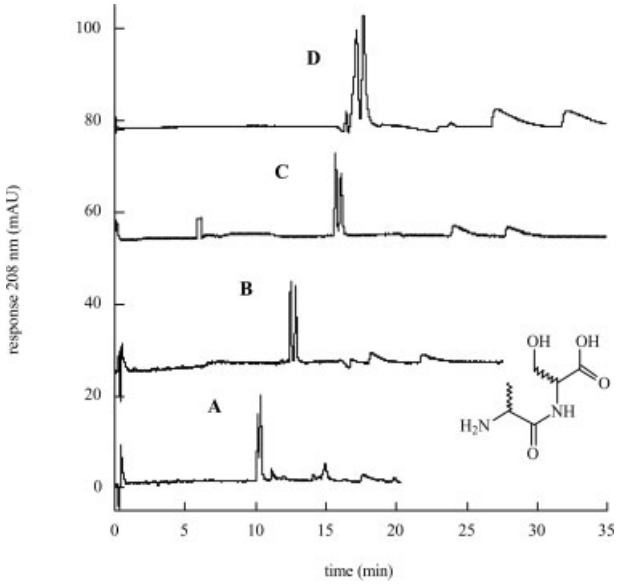

Figure 3. Chiral separation of Ala-Ser on TAG by CEC using different mobile phases, *U* = 15 kV. (A) TEAA, pH 4.1; (B) TEAA, pH 4.1/MeOH (90:10); (C) TEAA, pH 4.1/MeOH (80:20); (D) TEAA, pH 4.1/MeOH (70:30).

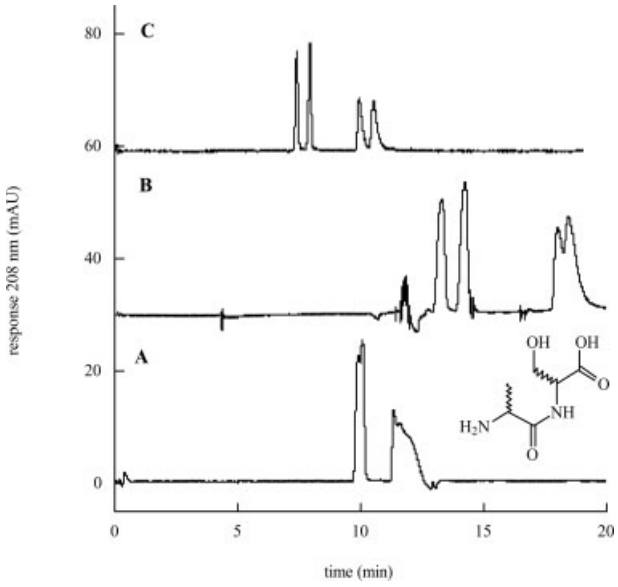

Figure 4. Chiral separation of Ala-Ser on TAG by CEC using different mobile phases, *U* = 15 kV. (A) TEAA, pH 4.1/ACN (90:10); (B) TEAA, pH 4.1/ACM/MeOH (50:30:20); (C) TEAA, pH 4.1/ACN/EtOH (50:30:20).

mobile phase, was then only partially separated, as shown in Fig. 4A. Very fast separations were obtained with 30% ACN added to the aqueous mobile phase and a voltage of 28 kV instead of 15 kV: five of nine dipeptides were baseline-resolved within 16 min (Table 1). Under these conditions theoretical plates for Ala-Phe were found to be 15 000–60 000/m. Leu-Val, Leu-Phe and Ala-Ala showed four peaks, three of them were baseline-resolved. Ala-Ser was again only partially separated. Figure 5 shows the chiral separation of Leu-Phe using

Table 1. Separation data of the diastereomeric dipeptides investigated

Dipeptide	t_1 (min)	t_2 (min)	t_3 (min)	t_4 (min)	$\alpha_{1/2}$	$\alpha_{2/3}$	$\alpha_{3/4}$
Ala-Ala	5.23	5.31	6.73	7.91	1.065	2.084	1.432
Ala-Met	6.38	6.88	11.01	11.85	1.298	2.922	1.122
Ala-Phe	7.68	8.24	12.32	14.58	1.194	2.186	1.300
Ala-Ser	5.02	5.66	–	–	1.296	–	–
Ala-Val	6.17	6.54	8.56	10.67	1.171	1.795	1.463
Leu-Ala	6.52	6.86	7.07	7.71	1.161	1.086	1.241
Leu-Leu	8.54	10.2	12.41	15.78	1.381	1.341	1.443
Leu-Phe	9.66	11.02	11.65	13.90	1.310	1058	1.315
Leu-Val	7.70	8.78	9.00	13.93	1.328	1.050	2.074

Retention times (t) and separation factors of peaks 1 and 2 ($\alpha_{1/2}$), peaks 2 and 3 ($\alpha_{2/3}$) and peaks 3 and 4 ($\alpha_{3/4}$) are given. Mobile phase: TEAA, pH 4.1/ACN (70:30), U = 28 kV

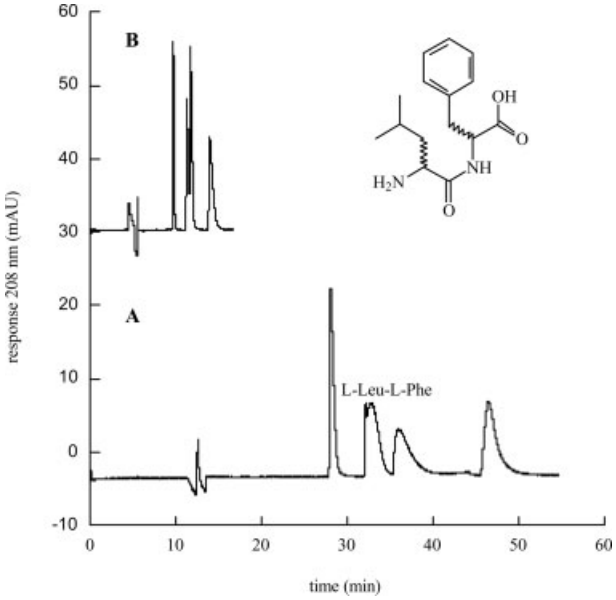

Figure 5. Chiral separation of Leu-Phe on TAG by CEC using different mobile phases, U = 15 kV; (A) TEAA, pH 4.1/ACN (80:20), U = 15 kV; (B) TEAA, pH 4.1/ACN (70:30), U = 28 kV.

20% (A) or 30% (B) ACN added to TEAA, pH 4.1 at 15 kV and 28 kV, respectively. With 30% ACN added to TEAA, pH 4.1, there was no baseline resolution of Leu-Phe. Compared to MeOH, the use of ACN as an organic modifier was found to be advantageous for bulky dipeptides with respect to retention times and peak shapes.

An alternative was the use of both ACN and MeOH or EtOH as organic modifiers added to TEAA, pH 4.1. These ternary mixtures combined higher selectivity due to MeOH or EtOH with improved peak shape due to ACN added to the mobile phase. Table 2 shows separation data of all dipeptides investigated using 50% TEAA, pH 4.1, 30% ACN and 20% MeOH. In spite of the optimal chiral resolution ability of this mobile phase for glycyl dipeptides, it did not resolve all diastereomeric dipeptides throughout. Leu-Ala, for example, was not baseline-resolved with this ternary phase (Fig. 6A), but was separated into its four enantiomers either by 30% MeOH or 10% ACN only. Ala-Ser was not baseline-resolved with TEAA, pH 4.1/ACN/MeOH (50:30:20) as shown in Fig. 4B, but with a similar mobile phase containing EtOH instead of MeOH (Fig. 4C). Leu-Ala, Leu-Phe, and Leu-Val were not baseline-resolved with ternary mixtures as mobile phases.

3.1.2 Elution order of diastereomeric dipeptides

With glycyl-dipeptides, the L-form always migrated faster than the D-enantiomer [26]. In the case of several baseline-resolved dipeptides tested, the natural LL-forms were the second-eluting isomers (see example in Fig. 5A). The complete migration order was tested with Leu-Leu, since all four enantiomers of this dipeptide are commercially available. The D-Leu-L-Leu-isomer eluted first, followed by the naturally occurring L-Leu-L-Leu, then L-Leu-D-Leu, and finally D-Leu-D-Leu (Fig. 7). The literature reports different elution orders for Leu-Leu using different selectors. With a chiral crown ether as a chiral selector added to the background electrolyte in CE, the migration order L-Leu-L-Leu, D-Leu-L-Leu, L-Leu-D-Leu and D-Leu-D-Leu was observed [30]. However, when a chiral crown ether was immobilized in a stationary-phase HPLC, elution order was D-Leu-D-Leu, D-Leu-L-Leu,

Table 2. Separation data of the diastereomeric dipeptides investigated

Dipeptide	t_1 (min)	t_2 (min)	t_3 (min)	t_4 (min)	$\alpha_{1/2}$	$\alpha_{2/3}$	$\alpha_{3/4}$
Ala-Ala	14.24	15.09	24.31	33.37	1.200	2.811	1.633
Ala-Met	17.24	19.02	42.10	49.29	1.246	3.559	1.224
Ala-Phe	19.18	21.07	41.61	47.84	1.231	3.040	1.203
Ala-Ser	13.29	14.22	17.98	18.44	1.267	1.851	1.056
Ala-Val	16.35	17.91	29.43	41.65	1.246	2.456	1.630
Leu-Ala	16.54	18.21	18.62	21.65	1.343	1.063	1.436
Leu-Leu	18.77	23.49	24.74	30.33	1.608	1.100	1.407
Leu-Phe	20.20	25.20	25.32	32.14	1.544	1.009	1.476
Leu-Val	18.21	22.03	22.10	39.05	1.530	1.006	2.527

Retention times (t) and separation factors of peaks 1 and 2 ($\alpha_{1/2}$), peaks 2 and 3 ($\alpha_{2/3}$) and peaks 3 and 4 ($\alpha_{3/4}$) are given. Mobile phase: TEAA, pH 4.1/ACN/MeOH (50:30:20), U = 15 kV

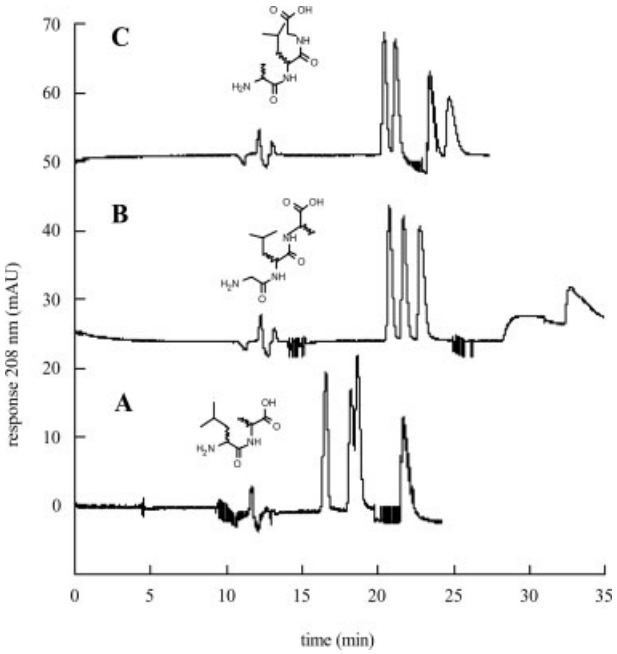

Figure 6. Chiral separation of (A) Leu-Ala, (B) Gly-Leu-Ala and (C) Ala-Leu-Gly on TAG by CEC. Mobile phase: TEAA, pH 4.1/ACN/MeOH (50:30:20), *U*=15 kV.