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Enantioseparation of selected *N-tert.*-butyloxycarbonyl amino acids in high-performance liquid chromatography and capillary electrophoresis with a teicoplanin chiral selector

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Abstract

Enantioseparation of *N-tert.*-butyloxycarbonyl amino acids (*N-t*-Boc-Aas) with teicoplanin chiral selector was performed in two different separation systems: A teicoplanin-based chiral stationary phase (CSP-TE) was used in reversed-phase HPLC, and the same chiral selector (CS) was added into a background electrolyte (BGE) in HPCE. The enantioselective interaction with the same CSP/CS can be influenced by several factors, such as mobile phase/background electrolyte composition: the buffer concentration, pH, the CS concentration, the presence of organic modifiers. In addition, the charge of the chiral selector related to the charge of the analyte and to EOF are important variables in CE. The effect of these parameters on enantioselectivity and enantioseparation of selected *N-t*-Boc-Aas was studied. The presence of a sufficient concentration (1% solution) of a triethylamine acetate buffer in the mobile phase was shown to be essential for enantioseparation of these blocked amino acids in HPLC. A certain concentration of teicoplanin aggregates (along with teicoplanin molecules) in the BGE is required to obtain enantioseparation of *N-t*-Boc-Aas in HPCE.

Keywords: Chiral selectors; Enantiomer separation; Teicoplanin; Amino acids, butyloxycarbonyl derivatives

1. Introduction

Macrocyclic antibiotics (MAs) have become widely known and used as chiral selectors (CS) since 1994 [1]. Glycopeptides (teicoplanin, vancomycin, ristocetin A and avoparcin) exhibit excellent separation performance in both liquid chromatography and capillary electrophoresis [2–8]. The glycopeptides combine the advantageous properties of protein-based CSs with the properties of other CS types (e.g., the formation of π-donor π-acceptor interactions or inclusion complexes).

The basic structure of teicoplanin (Fig. 1) consists of a substituted peptide backbone forming fused macrocyclic rings, to which sugar moieties and a hydrophobic chain are attached. Closely spaced stereogenic centers enable simultaneous interactions that are required for chiral recognition. A teicoplanin-based chiral stationary phase (CSP-TE) has been shown to be suitable for enantioseparation of

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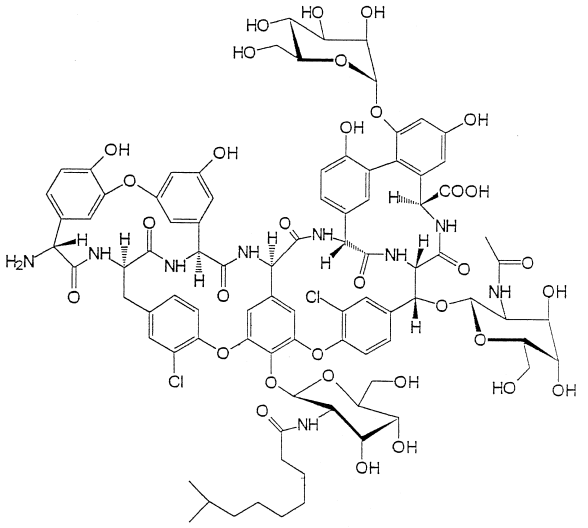

Teicoplanin

Fig. 1. The structure of teicoplanin, the main component (A_2-2) of a mixture of five forms of teicoplanin with different fatty acid chains.

various groups of compounds [9] including native amino acids (Aas) [10] and their derivatives [9,11–13].

Teicoplanin has also been found to be a powerful CS in HPCE [5,14,15]. When using it in capillary electrophoresis, its certain properties must be considered: its UV absorption, solubility, a limited stability in solution, formation of aggregates, a limited thermal stability, and partial interaction with the capillary wall. Nevertheless, due to the many interaction sites in the molecule, low concentrations of teicoplanin are sufficient for enantioseparation of a variety of compounds including variously derivatized amino acids [9,16,17].

Blocked amino acids are important precursors in syntheses of peptides or peptide-based drugs [18]. The amino (or carboxyl) groups are protected during the synthesis and then the blocking group is released. *N-tert*.-butyloxycarbonyl amino acids (*N-t*-Boc-Aas) are most often employed for this purpose. The enantiomeric purity of the precursors is essential for good quality of the products.

Amino acids are often derivatized prior to analysis to improve both the enantioseparation and the detection limits. Substituents with an aromatic ring (dansyl-, benzoyl-, dinitrophenyl-, and others) mostly improve enantioseparation while non-aromatic blocking groups usually yield poorer results. Therefore, it is difficult to find a suitable separation system for *N-tert*.-butyloxycarbonyl amino acids. Enantioseparation of a few *N-t*-Boc-Aas has been performed in various HPLC and HPCE separation systems [9,19–31]. Silica gel-bonded amide and urea derivatives [20] or an anion-binding CS based on metalloporphyrin [21] have been used to separate some *N-t*-Boc-Aas in HPLC. Some derivatives have been enantioseparated on teicoplanin-based CSP [22]. We have also shown that the CSP-TE is suitable for enantioseparation of several *N-t*-Boc-Aas [23]. A few enantiomers of *N-t*-Boc-Aas have been separated using polysaccharide-derived CSPs (mainly with Chiralpak AD) [24]. Chiral stationary phases derived from (*S*)-A-(3,5-dinitrobenzoyl) leucine have been demonstrated to be able to separate various *N*-derivatized amino acids [25]. Some *N-t*-Boc-Aas have also served as model compounds to investigate the effect of the amide connecting tether type in this study. A methacrylic acid—trimethylolpropane trimethacrylate copolymer imprinted with *N-t*-Boc-L-phenylalanine has been evaluated as a stationary phase for the chromatographic separation of *N-t*-Boc-D/L-Phe [26]. Quinine and quinidine and their carbamoyl-derivatives have been systematically studied as novel enantioselective anion exchangers for the use in HPLC [27,28], capillary electrophoresis [29] and capillary electrochromatography [30,31]. In order to evaluate the enantioselectivity and retention characteristics of these CSs, a large set of *N*-derivatized amino acids has been studied, including few *N-t*-Boc-Aas. However, hydroxypropyl-β-cyclodextrin seems to be the only suitable chiral selector that is capable of separating most of these blocked amino acids. Hydroxypropyl-β-cyclodextrin-bonded chiral stationary phase has been successfully employed in HPLC [32], but the same chiral selector has yielded poorer results in HPCE [33].

Enantioseparation in HPLC and HPCE results from differences in stereoselective interaction of individual enantiomers with a chiral selector. The enantioselective interaction with the same CSP/CS can be influenced by several factors, namely, the mobile phase or BGE composition, pH, organic

additives, and the CS concentration [2,6–8,10,14,5,16]. The charge on the CS related to the solute charge and to EOF plays a significant role in CE [34]. The temperature is an important variable in an enantioselective HPLC, as compared to normal HPLC separations [7]. Unfavorable effects of Joule heating can cause problems in electrically driven systems [35,36].

In the present work we demonstrate the possibility of using a teicoplanin-bonded CSP in HPLC and teicoplanin as a chiral additive to the background electrolyte and HPCE. To obtain deeper knowledge on the interaction and enantiorecognition mechanism, we have studied the influence of the mobile phase and/or BGE composition on the enantioseparation of *N-t*-Boc-Aas. The studied derivatives were selected in accordance with the requirement of their good detectability in both the separation systems. The results obtained with the same chiral selector by using the two separation methods have been compared.

2. Experimental

2.1. Chemicals

The test enantiomers of *N-tert.*-butyloxycarbonyl amino acids were obtained from Sigma (St. Louis, MO, USA).

The mobile phases and background electrolytes were prepared from the following compounds and solvents: triethylamine, purity >99% (Sigma), and glacial acetic acid, sodium hydrogenphosphate, phosphoric acid, all analytical-grade purity (Lachema, Brno, Czech Republic). Teicoplanin was obtained from ASTEC (Whippany, NY, USA). Methanol, purity for chromatography, and acetonitrile, gradient-grade purity, were purchased from Merck (Darmstadt, Germany). Distilled and deionized water was used in the experiments.

2.2. HPLC

The HPLC equipment (Pye Unicam, Cambridge, UK) consisted of an LC-XPD pump, a Model 7125 Rheodyne injection valve (Cotati, CA, USA) with a 10-μl sample loop, and a LC-UV detector. The detection wavelength was 254 nm. The signal acquisition and data handling were performed with a PC 4880 software.

A commercially available steel column 250×4.6 mm I.D. was used, packed with the teicoplanin-bonded chiral stationary phase, particle size 5 μm, Chirobiotic T (ASTEC).

The mobile phases contained 0.1 or 1.0% triethylamine acetate buffer (TEAA), pH 4.1, and various amounts of methanol or acetonitrile. The buffer pH was adjusted with acetic acid and measured before addition of organic modifiers. The buffers were filtered through a 0.45-μm filter, and the mobile phases were degassed before use. The flow-rate was 0.7 ml/min.

The void volume of the column was determined with an aqueous solution of KI.

The measurements were carried out at a temperature of 25°C.

The samples were dissolved in methanol at a concentration of 0.5–1.0 mg/ml and 2.0–5.0 μl of these solutions were injected onto the chromatographic column.

2.3. HPCE

The electrophoretic measurements were carried out using a Hewlett-Packard 3D CE apparatus (Hewlett-Packard, Waldbronn, Germany), equipped with an in-line variable-wavelength detector. The data were collected at three different λ values: 214, 254 and 275 nm. A HP ChemStation (Hewlett-Packard) was used to control the instrument and to process the data.

An untreated silica capillary 50 μm I.D., total length 33.4 cm, length to the detector 25 cm, was used at an applied potential of 10 kV and a temperature of 25°C. The samples were injected hydrodynamically at a pressure of 10 mbar for 4 s. The samples were dissolved in water and their concentration was the same as in HPLC.

The background electrolytes contained 0.06 *M* phosphate buffer, pH 4.30, 5.30 or 6.30. The buffers of required pH values were prepared by mixing various volumes of 0.06 *M* Na_2HPO_4 with 0.06 *M* H_3PO_4. Then teicoplanin was dissolved at concentrations ranging from 0.1 to 2.0 m*M*. The same BGE containing 10% acetonitrile was used for compari-

Table 1
Effect of the triethylamine concentration in the mobile phase on HPLC enantioresolution (R) of *N*-*t*-Boc–L/D-Aas using the teicoplanin-based chiral stationary phase[a]

N-*t*-Boc-Aa	TEAA (%)		
	1.0	0.1	0.0
Tyr	0.57	0.00	0.00
Phe	0.98	0.53	0.00
Trp	2.47	0.28	0.00
Arg	1.76	1.13	0.00

[a] Mobile phase: 40% MeOH in TEAA buffer, pH 4.1 (or water). For other details see Section 2.

son. (In the latter case the teicoplanin concentrations were calculated in the total volume of the BGE with ACN.)

3. Results and discussion

Two separation systems were tested using teicoplanin as the chiral selector for enantioseparation of *N*-*tert*.-butyloxycarbonyl amino acids. The teicoplanin-based CSP was used in reversed-phase separation mode in HPLC, and teicoplanin CS was added to the BGE in HPCE. The original idea was to compare identical (or at least very similar) separation systems in HPLC and HPCE in order to reveal differences in the analyte–teicoplanin interactions in the two systems, i.e., when the CS is bonded to the silica gel surface and when it is free in solution. Unfortunately, it was impossible to use the most suitable buffer from HPLC in the CE system, as triethylamine interacts with the capillary inner surface and reduces (or even eliminates) the EOF that is needed for the enantioseparation of *N*-*t*-Boc-Aas. Therefore, the 1% (~0.06 *M*) TEAA solution was replaced by a 0.06 *M* phosphate buffer in the HPCE measurements.

3.1. Buffer concentration

The effect of the buffer concentration on the HPLC enantioseparation of *N*-*t*-Boc-Aas can be seen in Table 1. In contrast to the enantiomers of native amino acids that can be separated in an aqueous-methanolic mobile phase alone [10,22,23], their blocked analogues require a buffer to obtain a

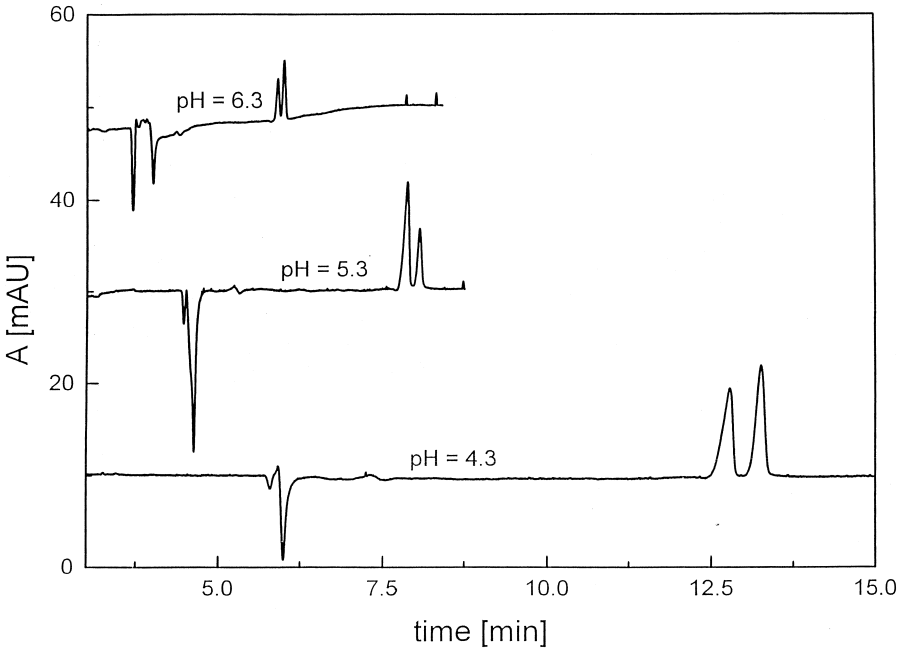

Fig. 2. Effect of the background electrolyte pH on HPCE enantioseparation of *N*-*t*-Boc–L/D-Arg-Tos. The BGE composition: 0.1 m*M* teicoplanin in 0.06 *M* phosphate buffer, pH 6.3, 5.3, and 4.3; for other conditions see Section 2.

separation [23]. Higher concentrations of TEAA are advantageous.

Two concentrations of the phosphate buffer (0.06 *M* and 0.006 *M*) were tested in our preliminary HPCE experiments. At the lower BGE concentration the EOF was about twice as rapid. Therefore, the time available for the interaction between the CS and *N*-*t*-Boc-Aas (which migrate in the opposite direction to EOF and CS) in the dilute buffer solution is shorter and does not suffice for the enantioseparation. The higher concentration of the phosphate buffer was thus preferred in further experiments.

The buffer in the mobile phase or BGE can either compete with the analytes for interactions with teicoplanin [37] or can enhance them. A higher ionic strength causes a decrease in the intensity of electrostatic interactions between the analytes and the CS while hydrophobic interactions are enhanced. Changes in the ionic strength of the buffer also influence the formation of micelles in the BGE (see below).

3.2. Buffer pH

In general, the buffer pH does not much affect the retention and resolution of enantiomers of acids in the pH range from 4.0 to 7.0 that is recommended for the use of CSP-TE [2,23]. A lower pH value is preferable because of slightly lower retention times.

The dependence of the electrophoretic mobility on the BGE pH shows that teicoplanin migrates at a rate almost identical with the EOF up to a pH of 6.5, depending on the buffer used [14]. The increased negative electrophoretic mobility that was observed at higher pH values can be attributed to a change in or partial destruction of the teicoplanin molecule. For this reason we only worked in the pH range from 4.3 to 6.3 in HPCE. *N*-*t*-Boc-Aas are slightly negatively charged; they migrate away from the detector window if a positive voltage is applied. This arrangement, in which the analytes and the CS migrate in the opposite directions, is advantageous for enantioseparation [5,6]. HPCE enantioseparations of *N*-*t*-Boc-Arg at three different pH values are compared in Fig. 2. It is obvious that both the migration times and the resolution increase with decreasing pH. The improvement in the enantioseparation of *N*-*t*-Boc-Aas at lower pH values can be explained by a combination of several effects, namely, enhanced electrostatic interactions between teicoplanin and the blocked amino acids, reduced EOF, and a decreased critical micelle concentration (CMC) of the chiral selector at lower pH values [14].

3.3. Teicoplanin concentration

The amount of teicoplanin bonded to the silica surface is specified by the manufacturer of a commercial HPLC column. A higher coverage of the carrier surface with a CS offers, of course, more possibilities for stereoselective interaction with the analytes [38–40]

The CS concentration in the BGE is the most important variable controlling the chiral recognition in CE. The effect of the teicoplanin concentration on the enantioseparation of *N*-*t*-Boc-Arg is shown in

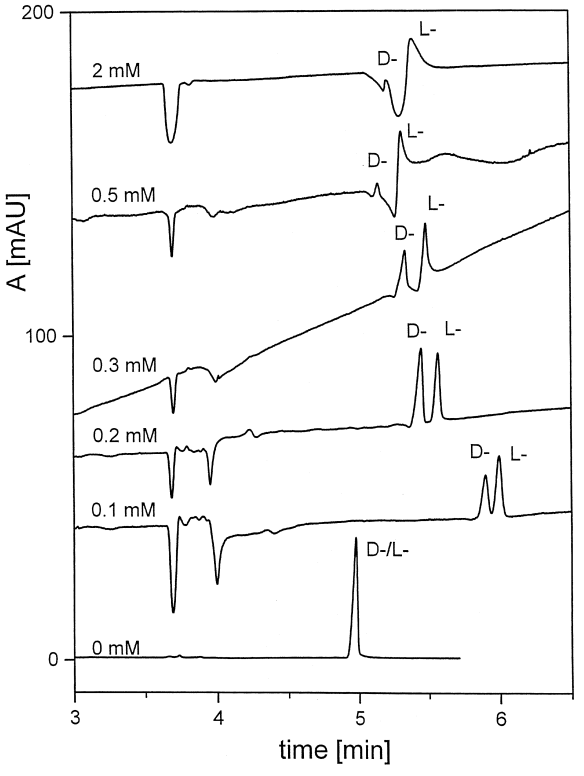

Fig. 3. Effect of the teicoplanin concentration in the BGE on HPCE enantioseparation of *N*-*t*-Boc–L/D-Arg-Tos. The BGE composition: 0.06 *M* phosphate buffer, pH 6.3, with different concentrations of teicoplanin.

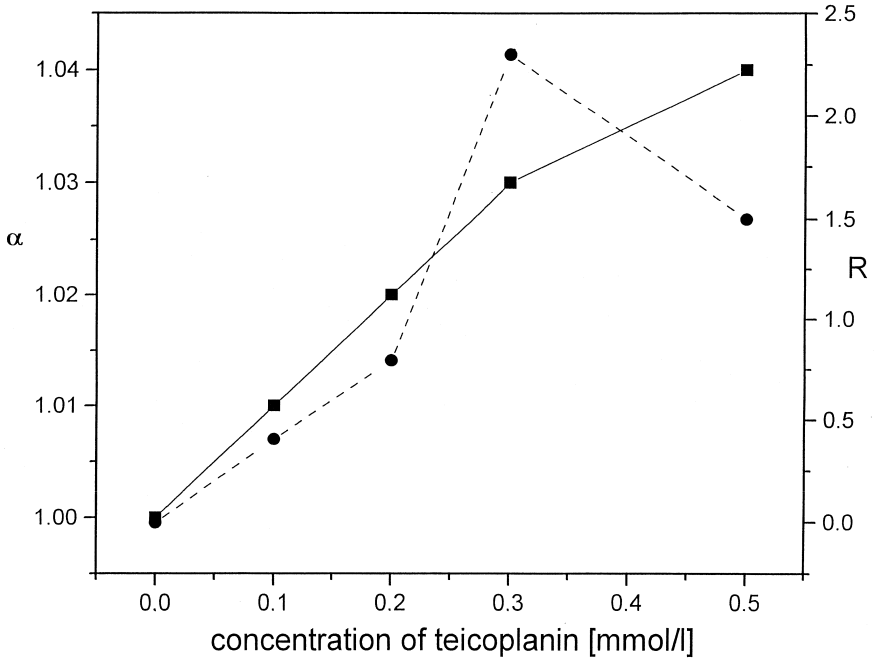

Fig. 4. Effect of the concentration of teicoplanin on enantioselectivity (■, solid line) and enantioresolution (●, dashed line) of *N*-*t*-Boc–L/D-Trp in HPCE. The BGE composition: 0.06 *M* phosphate buffer, pH 5.3, with given concentrations of teicoplanin.

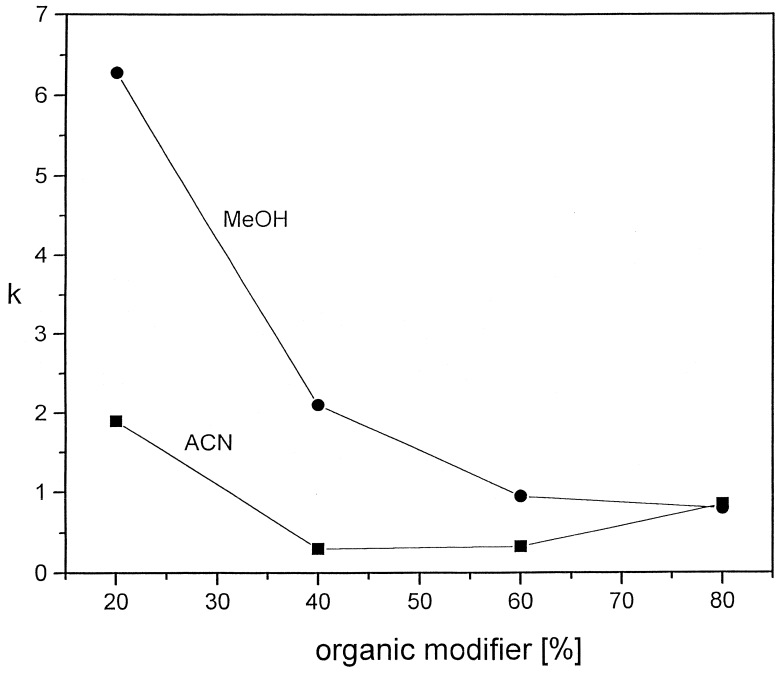

Fig. 5. Influence of organic modifiers (methanol and acetonitrile) on the retention of *N*-*t*-Boc–L/D-Trp in HPLC. Mobile phase composition: % OM in 1% TEAA buffer, pH 4.1, for other conditions see Section 2. Note: *k*, retention factor of the first eluted enantiomer.

Fig. 3. A very low teicoplanin concentration (sometimes even less than 0.1 m*M*) is sufficient for baseline enantioseparation of *N*-*t*-Boc-Aas. The elution order of the individual enantiomers was the D-isomer followed by the L-isomer over the studied concentration range of teicoplanin. The migration order was verified by spiking with the pure enantiomers. As can be seen from Fig. 3, the best enantioseparation of *N*-*t*-Boc-Arg was obtained at the 0.2 m*M* teicoplanin concentration. The dependences in Fig. 4 demonstrate an improvement in the enantioselectivity with increasing concentration of teicoplanin within the measured range, while the resolution reaches a maximum and then slightly decreases. The concentration of teicoplanin that yields the optimum selectivity of enantioseparation differs from that at which the best enantioseparation is achieved. This is due to peak broadening and a poorer peak shape in the system containing a greater fraction of teicoplanin in the micellar form.

3.4. Organic modifier

The type and concentration of the organic modifier can influence the retention and resolution of enantiomers in HPLC. If enantioselective interactions play a predominant role in the interaction mechanism, an increase in the retention is accompanied by improved enantioseparation (if the peak broadening does not negatively influence the retention value at a high retention time). The retention of *N*-*t*-Boc-Aas decreases with increasing MeOH content in the mobile phase (1% TEAA/OM) within the measured range [23]. A similar trend was observed with ACN, but only within a narrower concentration range (see Fig. 5). Due to decreased solubility of *N*-*t*-Boc-Aas in

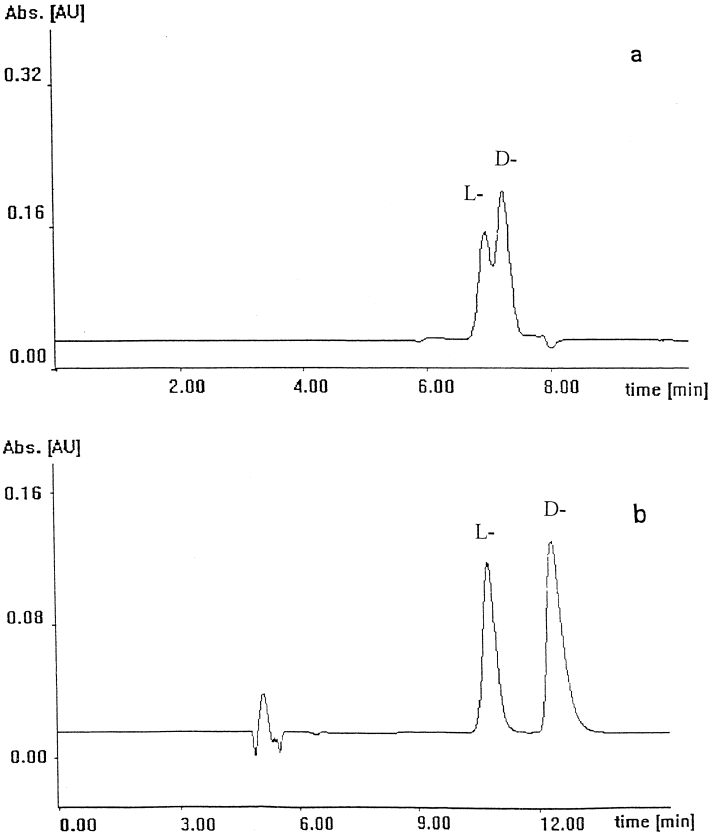

Fig. 6. Comparison of enantioseparation of *N*-*t*-Boc–L/D-Trp in mobile phases with different ACN contents in HPLC: (a) 60% (v/v) ACN in 1% TEAA, pH 4.1; (b) 20% (v/v) ACN in 1% TEAA, pH 4.1.

acetonitrile, the retention time increased in a mobile phase containing 80% ACN. The mobile phases with acetonitrile yielded lower retention of the analytes compared to those containing methanol. The improvement in the enantioseparation for lower organic modifier contents is also connected with the effect of a higher buffer concentration (see above) in the whole mobile phase volume at the same time (Fig. 6).

A small amount of ACN in the BGE containing the teicoplanin CS was reported to enhance enantioselectivity in HPCE systems [16]. The effect of addition of 10% ACN to the BGE is evident from the dependences of the enantioseparation on the teicoplanin concentration (Fig. 7). For the attaining enantioseparation of *N*-*t*-Boc-Aas in the BGE containing ACN, a higher initial concentration of teicoplanin is necessary compared to that in the BGE without the organic modifier, in order that micelles are formed in the presence of acetonitrile. A certain concentration of aggregates present along with the teicoplanin monomers seems to be required for chiral separation of *N*-*t*-Boc-Aas. The micelle/monomer ratio differs for different derivatives.

Organic modifiers enhance electrostatic interaction forces, which are required for chiral recognition. On the other hand, OMs can reduce both the enantioselective and non-stereoselective interactions between the CS and the analytes. MeOH (H-donor), as well as ACN (H-acceptor), can compete with the solutes for H-bonding with the CS or with other interaction sites. MeOH mostly exhibits a better separation performance in HPLC with the CSP-TE, while ACN is advantageous in HPCE with teicoplanin in the BGE.

3.5. Correlation between the HPLC and HPCE data

The elution order of the studied *N*-*t*-Boc-Aas in the HPLC system, consisting of CSP-TE/20% MeOH in 0.06 *M* buffer, was *N*-*t*-Boc-Tyr, *N*-*t*-Boc-Phe, *N*-*t*-Boc-Trp, *N*-*t*-Boc-Arg. In the HPCE separation system containing 10% ACN in 0.06 *M* BGE (with 0.5 m*M* teicoplanin), the migration order was almost opposite to that obtained with HPLC measurements (see Table 2).

The elution order of D/L-enantiomers of the *N*-*t*-Boc-Aas was as follows: the L-isomer eluted before the D-enantiomer in HPLC due to a higher affinity of

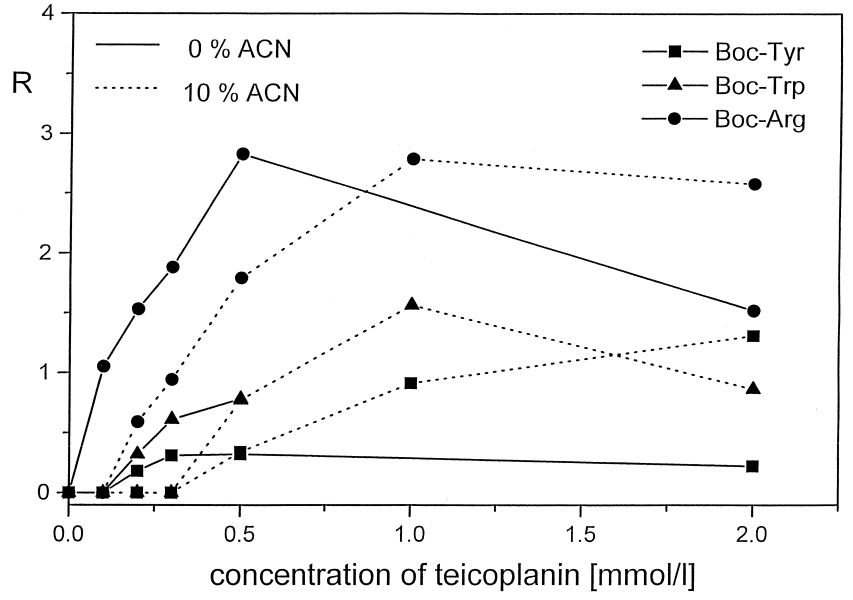

Fig. 7. Dependences of HPCE enantioseparation of *N*-*t*-Boc-Aas on the teicoplanin concentration in BGEs containing (full lines) and not containing (dashed lines) ACN.

Table 2
Capacity factors and electrophoretic mobilities of *N-t*-Boc-Aas in HPLC and HPCE[a]

N-t-Boc-Aas	HPLC, k_1	HPCE, μ_2^{eff} (10^{-8} m^2 V^{-1} s^{-1})
Tyr	1.33	−1.61
Phe	1.71	−1.66
Trp	6.18	−1.49
Arg	6.85	−1.31

[a] HPLC–CSP-TE/20% MeOH in 1% aqueous TEAA, pH 4.1; k_1, capacity factor of the first eluted enantiomer (L-enantiomer). HPCE–BGE 10% ACN in 0.06 *M* phosphate buffer, pH 4.3 with 0.5 m*M* teicoplanin; μ_2^{eff}, effective mobility of the second enantiomer (L-enantiomer).

the latter to the CSP-TE. The opposite migration order was found in HPCE where D-blocked amino acids reached the detector first under the conditions used in this work. Teicoplanin migrates at almost the same rate as the EOF and free *N-t*-Boc-Aas migrate in the opposite direction. The more stable teicoplanin–enantiomer complex thus passes along the detector first. A higher affinity of D-enantiomers of all the blocked amino acids to teicoplanin in both the studied separation systems caused this behavior.

4. Conclusion

Teicoplanin was shown to be a suitable chiral selector for enantioseparation of *N-tert.*-butyloxycarbonyl amino acids in both liquid chromatography and capillary electrophoresis.

The complexation between teicoplanin and the individual *N-t*-Boc-Aas exhibited similar trends in HPLC and HPCE. These results indicate an identical (or rather similar) interaction mechanism occurring in the two separation systems.

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