ELSEVIER

Journal of Chromatography A, 868 (2000) 197–207

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Enantiomeric and diastereomeric high-performance liquid chromatographic separation of cyclic β-substituted α-amino acids on a teicoplanin chiral stationary phase

Michael Schlauch, August W. Frahm*

Department of Pharmaceutical Chemistry, University of Freiburg, Hermann-Herder-Str. 9, 79104 Freiburg, Germany

Received 20 August 1999; received in revised form 1 November 1999; accepted 19 November 1999

Abstract

High-performance liquid chromatographic (HPLC) separation of stereomeric cyclic β-substituted α-quaternary α-amino acids was performed on a chiral stationary phase based on the glycopeptide antibiotic teicoplanin. The investigated amino acids are the 1-amino-2-methylcyclohexanecarboxylic acids, the 1-amino-2-hydroxycyclohexanecarboxylic acids, Ala, Cha, Phe and Tle. The effects of the mobile phase composition (type and content of organic modifier, pH) and of the temperature on the enantio- and diastereoselectivity were studied and the conditions were optimised to resolve the four stereomers of one amino acid in a single chromatographic run. The influence of the modifier concentration and the pH of the mobile phase reveal two enantiomeric and diastereomeric discrimination mechanisms based on different interactions with the stationary phase. For optimal separation of diastereomers the column has to be conditioned with an acidic eluent.

Keywords: Chiral stationary phases, LC; Enantiomer separation; α-Amino acids, β-substituted; 1-Amino-2-methylcyclohexanecarboxylic acids; 1-Amino-2-hydroxycyclohexanecarboxylic acids

1. Introduction

In recent years carbocyclic α-amino acids attracted increasing interest due to their diverse biological effects. They are reported to exhibit agonistic and/or antagonistic properties at both the ionotropic and the metabotropic glutamate receptors [1–5]. Furthermore, the incorporation of these constrained amino acids as building blocks into peptides and proteins results in modified biological activities of peptidomimetics and enhances their stability towards hydrolysis [6,7].

Since the biological properties of these amino acids, like most chiral drugs, strongly depend on their stereochemistry, it is necessary to establish effective analytical tools for the determination of the *ee* values of these compounds. The amino acids discussed herein have two chiral centres and consequently exist as four stereoisomers (Fig. 1(I,II)); thus, we focused on the development of a chromatographic method which allows the simultaneous separation of both the enantiomers and the diastereomers.

HPLC is a widely used technique in the chiral analysis of amino acids. Enantiomeric amino acids

*Corresponding author. Tel.: +49-761-203-6335 fax: +49-761-203-6351.

E-mail address: awfrahm@ruf.uni-freiburg.de (A.W. Frahm)

0021-9673/00/$ – see front matter
PII: S0021-9673(99)01222-4

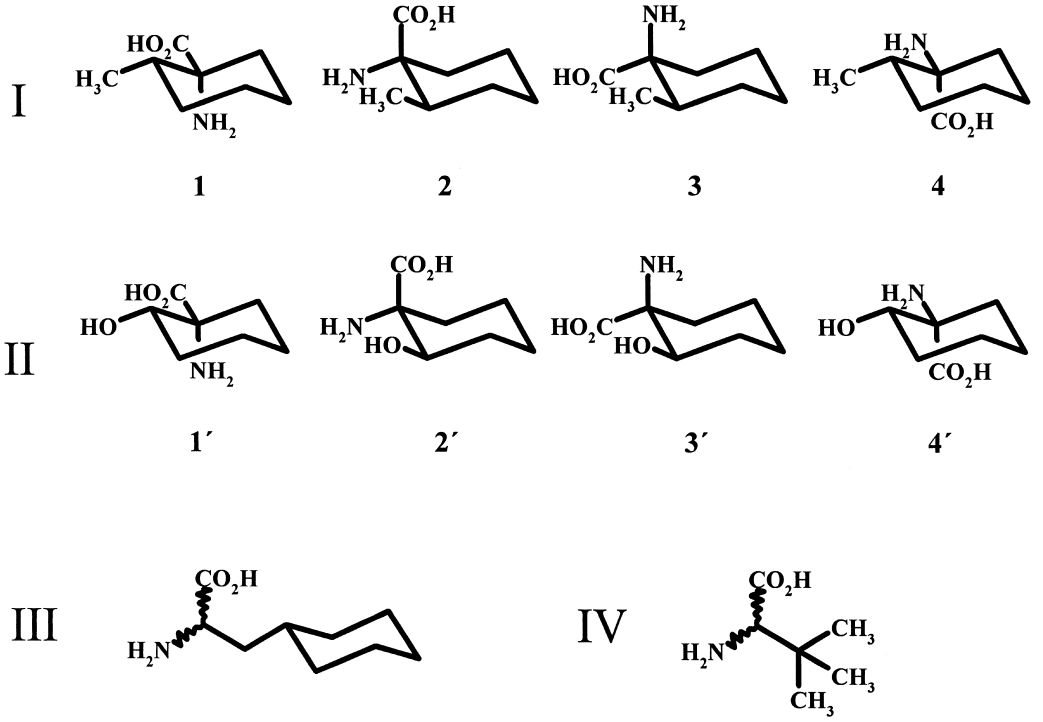

Fig. 1. Structures of the investigated compounds. (I) 1-Amino-2-methylcyclohexanecarboxylic acids (AMCH) **1**–**4**: **(1)** *cis*-(1*R*,2*S*); **(2)** *trans*-(1*R*,2*R*); **(3)** *cis*-(1*S*,2*R*); **(4)** *trans*-(1*S*,2*S*). (II) 1-Amino-2-hydroxycyclohexanecarboxylic acids (AHCH) **1**′–**4**′: **(1′)** *cis*-(1*R*,2*S*); **(2′)** *trans*-(1*R*,2*R*); **(3′)** *cis*-(1*S*,2*R*); **(4′)** *trans*-(1*S*,2*S*). (III) DL-Cyclohexylalanine (DL-Cha). (IV) DL-*tert*.-Leucine (DL-Tle).

can be separated directly on chiral stationary phases (CSP) based on, e.g., ligand-exchange [8,9] and interactions with a chiral crown ether [10]. In contrast, separation on achiral columns requires derivatisation with a chiral reagent to form diastereomeric entities. Among these chiral reagents, the most common are 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) [11] or *o*-phthaldialdehyde combined with a chiral thiol [12,13]. The major drawback of the latter method originates from the inadequate enantiopurity of the reagents causing incorrect *ee* determinations.

A new approach, first described by the group of Armstrong, uses macrocyclic antibiotics for chiral discrimination in liquid chromatography [14]. Among these, teicoplanin proved to be the most potent stationary phase for the resolution of amino acid enantiomers [15,16]. Recently, Péter et al. published the separation of a large variety of unusual amino acids on Chirobiotic T, the commercially available teicoplanin-based CSP [17]. They have also investigated the temperature dependence on the retention of some β-methyl amino acids [18].

Teicoplanin with four fused macrocyclic rings forming a semi-rigid basket comprises several structural features susceptible to influence the chiral recognition: a carboxylic acid approximately 12 Å away from an amino group, both responsible for the charge of the molecule, additional polar groups (sugar moieties and phenolic fragments) and an apolar alkyl side-chain [16]. As a result of earlier studies [16], the teicoplanin ammonium group is the most probable binding site for the analyte. Near this initial docking site, additional interactions (hydrogen bonding, dipole stacking, $\pi-\pi$ aromatic stacking, hydrophobic and steric interactions) with the teicoplanin molecule occur which are essential for stereoselective recognition. In contrast to this hypothesis for the teicoplanin CSP, the fundamental work of Williams and co-workers, who examined the binding mechanism of small peptides to teicoplanin in solution, led to the conclusion that various

H-bondings are the preliminary interactions between peptide and antibiotic [19–21].

The behaviour of amino acid enantiomers on Chirobiotic T is well-examined. However, only little information exists about the separation of diastereomers. In this paper, we describe the influence of the different parameters (nature and content of organic modifier, temperature, pH) on both the enantio- and the diastereoselectivity of β-substituted α-quaternary α-amino acids (Fig. 1(I,II)). Furthermore, other amino acids (Fig. 1(III,IV)) exhibiting close structural features with regard to the steric hindrance of the functional groups and the space-filling properties, are discussed, alongside with alanine and phenylalanine as 'standard amino acids'.

2. Experimental

2.1. Chemicals

The 1-amino-2-methylcyclohexanecarboxylic acids (AMCH) **1**–**4** and the 1-amino-2-hydroxycyclohexanecarboxylic acids (AHCH) **1′**–**4′** (Fig. 1(I,II)) were synthesised in our laboratory according to earlier described methods [22,23]. The structures were proven by ^{1}H and ^{13}C NMR spectroscopy, and the absolute configurations were determined by X-ray analysis and spectral correlation studies [24,25].

Cyclohexylalanine (Cha) and *tert*.-leucine (Tle) were generous gifts from the Degussa (Hanau, Germany). Phenylalanine (Phe) was purchased from Merck (Darmstadt, Germany) and alanine (Ala) from Riedel-de Haën (Seelze, Germany). HPLC-grade methanol, 2-propanol and acetonitrile were obtained from Fluka Chemie (Buchs, Switzerland) and ethanol from Riedel-de Haën. Two other chemicals were of analytical grade: acetic acid 99–100% (Riedel-de Haën) and ammonium nitrate (Merck).

2.2. Apparatus and chromatography

The HPLC system consisted of a Waters 515 HPLC Pump, a Waters 717 plus Autosampler, and a Waters 2487 Dual λ Absorbance Detector. The software used for recording the chromatograms was ChromStar light Version 4.05 (SCPA, Stuhr, Germany). Eluents were degassed by passing an in-line degasser, the column temperature was controlled by a Jetstream 2 plus Peltier-Column-Thermostat (Waters, Milford, MA, USA).

The column used was a Chirobiotic T chiral stationary phase, 250×4.6 mm I.D., 5 μm particle size, combined with a Chirobiotic T guard column, 20×4 mm I.D., 5 μm particle size (Astec, Whippany, NJ, USA).

The eluents were prepared by mixing the indicated volumes of organic modifier and freshly distilled water, followed by filtration through a 0.45-μm cellulose acetate filter (Schleicher & Schuell, Dassel, Germany). Acetic acid was used for adjusting the mobile phase to a given pH.

All amino acids were analysed without derivatisation. Sample solutions (1 mg/ml) were prepared by dissolving the analytes in water and filtered through a 0.45-μm filter.

Unless otherwise indicated, the injection volume was 5 μl, the mobile phase flow-rate was set at 0.8 ml/min, and the detection wavelength was 205 nm. The hold-up time (t_M) was determined with an aqueous solution of ammonium nitrate (0.02 mg/ml). Sample solutions of the single stereomers were injected to establish the elution order of their stereomeric mixtures.

Resolution was calculated according to the special method of Schoenmakers et al. for asymmetric peaks [26].

3. Results and discussion

The Chirobiotic T column, the commercially available teicoplanin CSP, can be generally used in the normal-phase mode, the reversed-phase mode and the polar-organic mode [15]. However, amino acids as ionic compounds cannot be analysed in the normal-phase mode, since they are not soluble in apolar normal-phase eluents. In the polar-organic mode the selectivity and the retention are often controlled by addition of acid and base, for example acetic acid and triethylamine, which shift the UV cutoff of the eluent to approximately 230 nm, whereas the investigated amino acids (Fig. 1) bearing no pronounced chromophoric group show very poor absorption above 210 nm. Owing to these detection problems the polar-organic mode is not

useful for the separation of underivatised amino acids, in contrast to the reversed-phase mode which is the first choice.

The amino acids (Fig. 1) treated in this paper are the following. AMCH **1**–**4**: **(1)** *cis*-(1*R*,2*S*); **(2)** *trans*-(1*R*,2*R*); **(3)** *cis*-(1*S*,2*R*); and **(4)** *trans*-(1*S*,2*S*). AHCH **1′**–**4′**: **(1′)** *cis*-(1*R*,2*S*); **(2′)** *trans*-(1*R*,2*R*); **(3′)** *cis*-(1*S*,2*R*); and **(4′)** *trans*-(1*S*,2*S*)). DL-Cha, DL-cyclohexylalanine; DL-Tle, DL-*tert*.-leucine; DL-Ala, DL-alanine; and DL-Phe, DL-phenylalanine.

3.1. Influence of the chromatographic conditions

3.1.1. Effect of the nature of the organic modifier

The organic modifiers, methanol, ethanol, 2-propanol and acetonitrile, were tested with regard to the separation of enantiomers (Table 1) and diastereomers (Table 2) of the selected amino acids.

The enantioselectivity strongly depends on the nature of the organic modifier. Best results in the chiral separation of all investigated amino acids can be achieved with ethanol and 2-propanol, followed by methanol, whereas the amino acid enantiomers are poorly resolved with acetonitrile. However, the diastereomeric discrimination is not significantly influenced by the organic modifiers with the acetonitrile giving slightly better results.

The enantioseparation factors of the amino acids vary largely: Cha is best resolved, while the sterically hindered substances like Tle, bearing a *tert*.-butyl group at the α-carbon atom, and the two β-substi-

Table 1
Influence of different organic modifiers on the chromatographic parameters k_1, α_e and R_S of the enantioseparation of the selected amino acids[a]

Compound		Methanol	Ethanol	2-Propanol[b]	Acetonitrile
Ala	k_1[c]	2.60	2.82	2.88	3.23
	α_e[d]	1.47	1.62	1.64	1.11
	R_S[d]	6.45	6.83	6.92	1.98
Phe	k_1	3.06	3.20	3.34	3.00
	α_e	1.35	1.66	1.71	1.16
	R_S	4.93	6.79	7.36	2.73
Tle	k_1	2.58	2.70	2.80	3.52
	α_e	1.19	1.30	1.35	1.10
	R_S	2.86	3.59	4.29	1.77
Cha	k_1	3.24	3.08	2.96	3.63
	α_e	1.87	2.16	2.14	1.26
	R_S	11.29	11.58	11.48	4.79
cis-AMCH	k_1	3.10	3.39	3.74	3.70
(**3**+**1**)	α_e	1.18	1.23	1.22	1.06
	R_S	2.41	2.65	2.47	0.89
trans-AMCH	k_1	5.06	5.49	6.06	6.29
(**4**+**2**)	α_e	1.13	1.19	1.19	1.04
	R_S	2.01	2.70	2.80	0.62
cis-AHCH	k_1	2.69	2.74	2.81	3.12
(**1′**+**3′**)	α_e	1.14	1.21	1.27	1.04
	R_S	1.73	2.28	3.11	0.55
trans-AHCH	k_1	3.73	3.60	3.72	4.47
(**2′**+**4′**)	α_e	1.10	1.18	1.25	1.02
	R_S	1.36	2.43	3.30	<0.5

[a] These data were generated with a modifier–water mixture (60:40, v/v), pH 4, mobile phase, 0.8 ml/min, 20°C and 205 nm UV detection, unless otherwise indicated.
[b] The flow-rate was set at 0.5 ml/min to avoid a too high back pressure produced by the viscosity of the 2-propanol–water mobile phase.
[c] k_1 is the retention factor of the first eluting enantiomer.
[d] α_e and R_S are the separation factor and the resolution of the enantioseparation, respectively.

Table 2
Influence of different organic modifiers on the chromatographic parameters k_1, α_d and R_S of the separation of the diastereomers of AMCH and AHCH, respectively[a]

Compound		Methanol	Ethanol	2-Propanol[b]	Acetonitrile
1*R*-AMCH	k_1[c]	3.64	4.18	4.56	3.91
(**1**+**2**)	α_d[d]	1.57	1.56	1.58	1.67
	R_S[d]	6.57	5.76	6.15	10.31
1*S*-AMCH	k_1	3.10	3.39	3.74	3.70
(**3**+**4**)	α_d	1.63	1.62	1.62	1.70
	R_S	6.83	5.80	6.46	10.66
1*R*-AHCH	k_1	2.69	2.74	2.81	3.12
(**1′**+**2′**)	α_d	1.39	1.31	1.33	1.43
	R_S	3.23	3.16	3.30	5.53
1*S*-AHCH	k_1	3.05	3.30	3.57	3.24
(**3′**+**4′**)	α_d	1.35	1.29	1.30	1.41
	R_S	3.50	2.97	3.18	6.37

[a] For chromatographic conditions, see Table 1.
[b] Flow-rate, 0.5 ml/min.
[c] k_1 is the retention factor of the first eluting diastereomer.
[d] α_d and R_S are the separation factor and the resolution of the diastereoseparation, respectively.

tuted α-quaternary AMCH and AHCH show rather small α and R_S values. When comparing Cha with Phe and Ala, the better separation of the former amino acid can be explained by the higher hydrophobicity and bulkiness of the structure without hindering the functional groups. In accord with the observations of Péter et al. [17] the chiral recognition is deteriorated, if the carboxylate group of the amino acid is sterically hindered, and increased with the hydrophobicity of the molecule.

With regard to the structural features improving the separation of diastereomers, it can be stated that an apolar methyl group (AMCH) is more favourable than a polar hydroxyl group (AHCH) as substituent in the β-position of the cyclic α-amino acids. However, these data are not sufficient to draw further conclusions, in order to establish structure–diastereoselectivity relationships. These will be based on results of ongoing studies with different types of substituents.

3.1.2. Effect of the concentration of the organic modifier

The concentration of the organic modifier is another important parameter. Fig. 2 shows the retention factors of compounds **1′**–**4′** as a function of the methanol content in the mobile phase. The results with the two modifiers ethanol and acetonitrile, both at concentrations of 30 and 60%, are listed in Tables 3 and 4. The retention increases at higher concentrations of organic modifiers. This untypical behaviour of RP-HPLC was earlier reported by Berthod et al. [16], who explained this phenomenon by the minor solubility of amino acids in modifier-enriched eluents. We confirm this hypothesis by the fact that the retention of apolar amino acids like Cha and Phe is much less influenced by the modifier content than the retention of the more hydrophilic Ala.

Among the three alcoholic modifiers (methanol, ethanol and 2-propanol), the most polar one, methanol, is best capable to dissolve the analytes and thus causes less increase in retention at high concentrations. However, the solvent polarity does not seem to be the only important factor for this retention behaviour, as the more polar solvent acetonitrile (polarity according to Snyder [27]) causes a much stronger increase in retention compared to all three alcohols (Table 3). Studies on the interaction of a small peptide with glycopeptide antibiotics have shown that the organic modifier acetonitrile (in the range of 40–60%) increased the rate constant of association in comparison with methanol [28].This phenomenon was explained by the greater ease of desolvation of the peptides because of the weak H-bonding properties of acetonitrile.

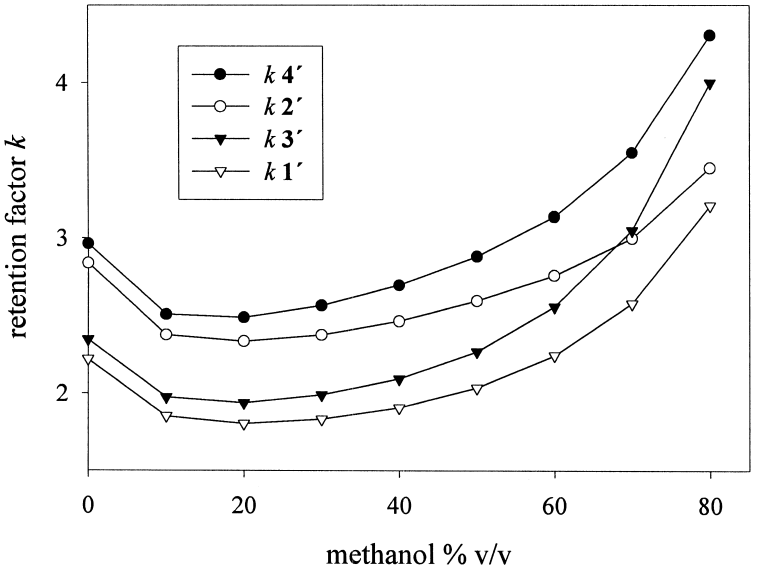

Fig. 2. Effect of the methanol content of the mobile phase on the retention of the four stereomers of AHCH **1′**–**4′**. Conditions: methanol–water mobile phase, 0.8 ml/min, 20°C.

The influence of the methanol content on the separation factors of AHCH is shown in Fig. 3. The enantioselectivity continuously rises with increasing modifier concentrations, whereas the diastereoselectivity strongly decreases with modifier-enriched eluents. Consequently, the discrimination mechanisms of enantiomers on the one hand and of diastereomers on the other hand are based on different interactions with the CSP. The increasing enantioselectivity together with the decreasing diastereoselectivity result in a reversal of the elution order of compound **3′** and **2′** (Fig. 2). Comparing the four modifiers with regard to the influence of their concentrations on the separation factors, the three alcohols do not differ significantly, whereas acetonitrile causes a rather poor augmentation with rising content (Table 3).

To summarise, acetonitrile in high concentrations causes a stronger increase in retention and a weaker increase in enantioselectivity compared to alcohol-enriched eluents. The modifiers differ in their physico-chemical properties: the dipole moment of acetonitrile exceeds the corresponding values of the investigated alcohols. This applies also to the dielectric constant with the exception of the methanol value. As a result, ionic interactions and dipole stacking between the amino acid and the CSP are reduced in acetonitrile-enriched eluents compared to those in alcohol-enriched mobile phases. On the other hand, acetonitrile poorly forms hydrogen bonding and, therefore, promotes these interactions between the analyte and the CSP. This leads to the conclusion that hydrogen bondings are important for the retention, whereas ionic interactions and dipole stacking are responsible for chiral discrimination. A large number of H-bonding groups occur on the sugar moieties which are not near the amino acid binding site, and thus provide additional interactions without differentiation between the enantiomers of amino acids.

On the other hand it might be possible that the modifiers have different influences on the conformation of teicoplanin. Especially the sugar moieties are free to rotate leading to a better or worse access to the amino acid binding site. Another indication for a conformational change of teicoplanin in dependence on the mobile phase, is the decrease of the diastereoselectivity in modifier-enriched eluents.

3.1.3. Effect of the temperature

Chiral separations are often very sensitive to

Table 3
Influence of the modifier concentration on the chromatographic parameters k_1, α_e and R_S of the enantioseparation of the selected amino acids[a]

Compound		Ethanol			Acetonitrile		
		30%	60%	Change[b] (%)	30%	60%	Change (%)
Ala	k_1[c]	1.95	2.82	+45	1.67	3.23	+93
	α_e[d]	1.27	1.62	+130	1.060	1.111	+85
	R_S[d]	3.31	6.83	+106	0.89	1.98	+126
Phe	k_1	3.22	3.20	−1	2.18	3.00	+38
	α_e	1.28	1.66	+136	1.109	1.158	+45
	R_S	3.60	6.79	+89	1.71	2.73	+60
Tle	k_1	2.32	2.70	+16	2.11	3.52	+69
	α_e	1.11	1.30	+173	1.057	1.096	+68
	R_S	1.44	3.59	+149	0.93	1.77	+90
Cha	k_1	3.87	3.08	−20	2.95	3.63	+23
	α_e	1.80	2.16	+45	1.222	1.259	+17
	R_S	9.53	11.58	+22	3.80	4.79	+26
cis-AMCH	k_1	2.92	3.39	+16	2.32	3.70	+59
(**3**+**1**)	α_e	1.12	1.23	+92	1.038	1.056	+47
	R_S	1.56	2.65	+70	0.52	0.89	+71
trans-AMCH	k_1	5.22	5.49	+5	4.16	6.29	+51
(**4**+**2**)	α_e	1.09	1.19	+111	1.028	1.039	+39
	R_S	1.25	2.70	+116	<0.5	0.62	–
cis-AHCH	k_1	2.28	2.74	+20	1.85	3.12	+69
(**1′**+**3′**)	α_e	1.09	1.21	+133	1.018	1.038	+111
	R_S	0.95	2.28	+140	<0.5	0.55	–
trans-AHCH	k_1	3.62	3.60	−1	2.92	4.47	+53
(**2′**+**4′**)	α_e	1.07	1.18	+157	1.00	1.02	–
	R_S	1.07	2.43	+117	<0.5	<0.5	–

[a] These data were generated with a mobile phase containing the given amount of organic modifier at pH 4, 0.8 ml/min, 20°C and 205 nm UV detection.
[b] This value gives the percentage difference between the chromatographic parameters at a modifier concentration of 30 and 60%, respectively.
[c] k_1 is the retention factor of the first eluting enantiomer.
[d] α_e and R_S are the separation factor and the resolution of the enantioseparation, respectively.

temperature changes with either improved or worse results achieved at low temperature. The investigated cyclic amino acids show an unspectacular increase in retention and selectivity (Table 5). However, the resolution is slightly influenced because of the compensation of the gain in selectivity by the loss in efficiency. A prolonged time of analysis at low temperature can sometimes be even more perceptible if the flow-rate has to be reduced because of the higher back pressure produced by the more viscous eluent. Therefore, runs at reduced temperature should be limited to low-resolution separations.

3.1.4. Optimisation of the chromatographic conditions

The aim of our work is to develop a method which allows the separation of the four stereomers of AMCH and AHCH, respectively, in a single chromatographic run. As will be shown later, a low pH mobile phase is required for optimal resolution. Buffer systems, except phosphate buffers (not recommended by the manufacturer) [29], show strong absorbance in the UV spectral range of the analytes (<210 nm) causing a dramatically reduced sensitivity. Nevertheless, two of the buffer systems (am-

Table 4
Influence of the modifier concentration on the chromatographic parameters k_1, α_d and R_S of the separation of the diastereomers of AMCH and AHCH, respectively[a]

Compound		Ethanol			Acetonitrile		
		30%	60%	Change[b] (%)	30%	60%	Change (%)
1*R*-AMCH	k_1[c]	3.28	4.18	+27	2.41	3.91	+62
(**1**+**2**)	α_d[d]	1.73	1.56	−23	1.78	1.67	−14
	R_S[d]	7.48	5.76	−23	10.43	10.31	−1
1*S*-AMCH	k_1	2.92	3.39	+16	2.32	3.70	+59
(**3**+**4**)	α_d	1.79	1.62	−22	1.79	1.70	−11
	R_S	8.94	5.80	−35	10.65	10.66	+0
1*R*-AHCH	k_1	2.28	2.74	+20	1.85	3.12	+69
(**1′**+**2′**)	α_d	1.58	1.31	−47	1.58	1.43	−26
	R_S	5.61	3.16	−44	6.82	5.53	−19
1*S*-AHCH	k_1	2.48	3.30	+33	1.88	3.24	+72
(**3′**+**4′**)	α_d	1.55	1.29	−47	1.55	1.41	−25
	R_S	5.66	2.97	−48	7.62	6.37	−16

[a] For chromatographic conditions, see Table 3.
[b] This value gives the percentage difference between the chromatographic parameters at a modifier concentration of 30 and 60%, respectively.
[c] k_1 is the retention factor of the first eluting diastereomer.
[d] α_d and R_S are the separation factor and the resolution of the diastereoseparation, respectively.

monium acetate, triethylamine acetate) have been examined in the pH range 4–7 and led furthermore to a poor diastereomeric resolution. In contrast, suitable mobile phases could be generated just by adding acetic acid omitting any buffer salts.

The investigations really have shown that the

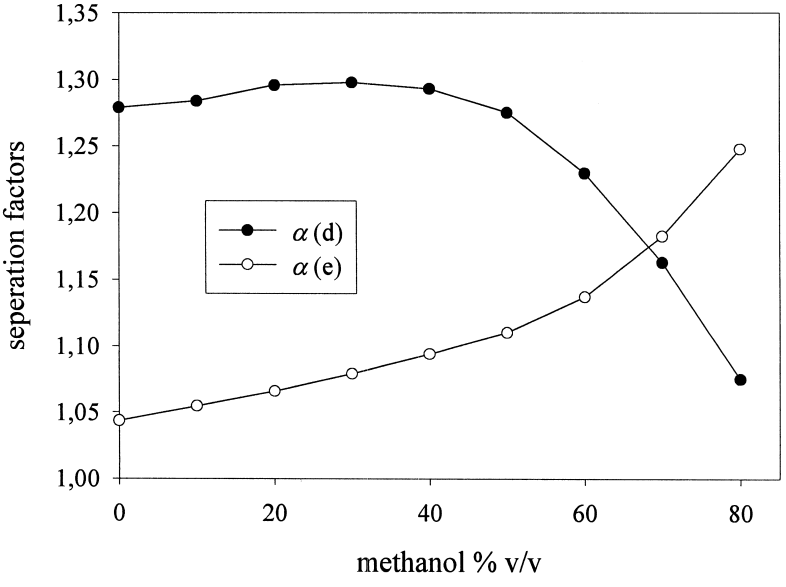

Fig. 3. Effect of the methanol content of the mobile phase on the enantioselectivity (α(e), separation of **2′**–**4′**) and diastereoselectivity (α(d), separation of **1′**–**2′**) of AHCH. Conditions: methanol–water mobile phase, 0.8 ml/min, 20°C.

Table 5
Influence of the temperature on the chromatographic parameters k, α and R_S of the separation of the AMCH stereomers[a]

	5°C	30°C
k (**3**)[b]	2.73	2.24
α (*cis*)[c]	1.24	1.15
R_S (*cis*)[c]	2.28	1.95
α (1*S*)[d]	1.46	1.36
R_S (1*S*)[d]	4.48	4.58

[a] Mobile phase: methanol–water (60:40, v/v), 0.8 ml/min, 205 nm UV detection.

[b] Retention factor of compound **3** (*cis*-(1*S*,2*R*)).

[c] α (*cis*) and R_S (*cis*) are the separation factor and the resolution of the enantioseparation of *cis*-AMCH (**3**+**1**), respectively.

[d] α (1*S*) and R_S (1*S*) are the separation factor and the resolution of the diastereoseparation of the 1*S*-configured stereomers of AMCH (**3**+**4**), respectively.

Chirobiotic T column is potent to separate enantiomers and diastereomers of the investigated cyclic amino acids with identical configuration at C-1 (Tables 1 and 2), whereas the diastereomers with identical configuration at C-2 are often badly resolved. Hence, the chromatographic conditions must be chosen rather carefully to receive a balanced separation of all four stereomers.

The stereomers of AMCH can be resolved under the following conditions: ethanol–water (60:40, v/v), pH 4, 0.8 ml/min, 20°C within 16 min. The minimal resolution, the lowest value in resolution of any two compounds, of 2.65 ($n=4$, SD=0.024) obtained is sufficient for the determination of the stereochemical composition of synthetic products. Best results for the separation of AHCH stereomers can be achieved with ethanol–water (50:50, v/v), at pH 4, with 0.6 ml/min and 15°C within 15 min. The minimal resolution of 1.88 ($n=5$, SD=0.032) is significantly lower than that for the AMCH stereomers. The corresponding chromatograms are shown in Fig. 4.

3.2. Elution order of the four stereomers

As the carboxylate group of the amino acid is involved in the chiral discrimination mechanism, the configuration at the carbon atom adjacent to this key group is supposed to control the elution order. On Chirobiotic T, a stronger retention of the (*R*)-configured amino acid is usually observed corresponding to the antibiotic property of teicoplanin selectively binding at the D-Ala–D-Ala unit of the muramyl pentapeptide during the bacterial cell wall synthesis [30]. To date, there are only three amino acids described, the (*R*)-configured enantiomers of which are less retained on Chirobiotic T [17].

The chromatograms in Fig. 4 illustrate the *cis*-isomers of both amino acids, AMCH and AHCH being eluted before the corresponding *trans*-isomers, but the two amino acids differ in the elution order of their enantiomers. In the case of AMCH, as expected, the (1*S*)-configured enantiomers are eluted first, which in contrast applies for the (1*R*)-configured enantiomers of AHCH. Obviously, the interactions with the CSP are strongly influenced by the type of substituent at C-2 leading even to a reversal of the elution order if the methyl group is replaced

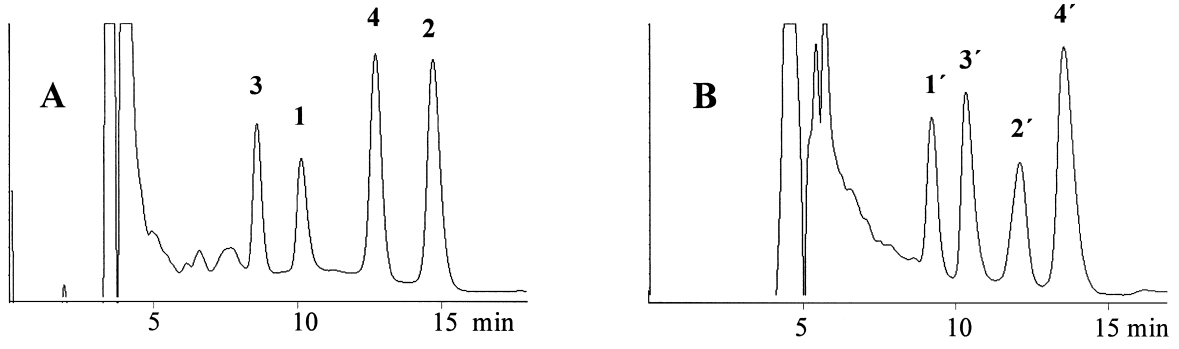

Fig. 4. Separation of the stereomers of AMCH (A) and AHCH (B), respectively. For peak numbering, see Fig. 1. Chromatographic conditions: (A) ethanol–water (60:40, v/v), pH 4, 0.8 ml/min, 20°C; (B) ethanol–water (50:50, v/v), pH 4, 0.6 ml/min, 15°C; UV detection 205 nm.

by the hydroxyl function. Further investigations are planned in order to elucidate a relationship between the elution sequence and the type of substituent.

3.3. Differences between the enantiomeric and diastereomeric separation

As shown by the influence of the modifier content, the mechanism of the enantiomeric discrimination differs from that of the diastereomeric separation. This conclusion is supported by the effect of the mobile phase pH. The addition of acetic acid, up to pH 4, causes a tremendous improvement of the diastereomeric separation, whereas the enantiomeric discrimination remains unchanged or even becomes slightly worse (Fig. 5). Interestingly, the chromatograms are unchanged, if the same mobile phase at pH 7 is used consecutively. Obviously, the CSP remains conditioned after treatment with acid. Repetitive runs at neutral pH cause a gradual loss of diastereoselectivity, as the protons are 'rinsed out' of the column. Reproducible results in the diastereomeric separation of amino acids on the Chirobiotic T column are, therefore, only guaranteed at a low pH value of 4.

The only possible binding site on the teicoplanin skeleton, which is pH-sensitive in the working range from pH 4 to 7, is the carboxyl group with a pK_a value of 5.0 [31]. As a consequence, this functional group is anionic at pH 7 and mainly protonated at pH 4. The influence of the protonation of the carboxylate group on the diastereomeric separation can be explained in two different ways: either, the carboxylate group of teicoplanin is directly involved in interactions with the analyte or, the conformation of teicoplanin changes if the carboxylate group is protonated resulting in a stronger retention of the *trans*-isomers.

4. Conclusions

The Chirobiotic T column is capable of achieving a baseline separation of the four stereomers of AMCH and AHCH, respectively, in a single chromatographic run. As shown by the influence of the organic modifier content, and especially of the pH of the mobile phase, the discrimination mechanism for the enantiomers and the diastereomers differ, most probably due to different effects caused by changes of the conformation of teicoplanin.

We have found that the reproducibility of the diastereomer separation of amino acids on Chirobiotic T strongly depends on the conditioning of the column. Indeed, the results are only reproducible on columns pretreated with an acidic mobile phase. This is the first report on this phenomenon since the previous studies focused on the separation of enantiomers which are not, or only slightly, affected by the mode of preconditioning.

Since the AHCH enantiomers show the opposite elution order in comparison to the generally observed behaviour of amino acids, it is not possible, in accord with the findings of Péter et al. [17], to predict the absolute configuration of an amino acid by its retention behaviour on the Chirobiotic T column.

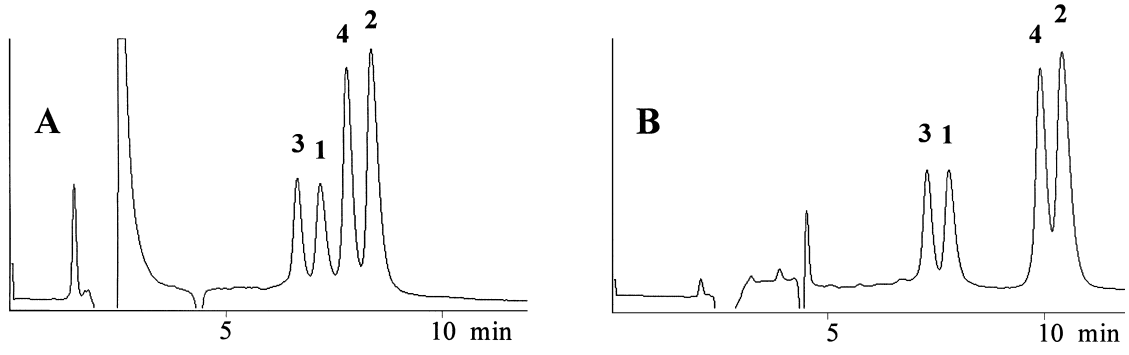

Fig. 5. Influence of the addition of acid to the mobile phase on the separation of AMCH: (A) without acid, (B) with acid (pH 4). Conditions: methanol–water (30:70, v/v), 0.8 ml/min, 20°C.

Acknowledgements

The authors wish to thank Franz-Josef Volk and Kamalesh Pai Fondekar for the provision of the cyclic amino acids, Degussa AG (Hanau, Germany) for the generous gifts of amino acids and the 'Fonds der Chemischen Industrie' (Frankfurt am Main, Germany) for financial support. We are grateful to the 'Landesgraduiertenförderung' (Bundesland Baden-Württemberg, Germany) for a doctoral fellowship.

References

[1] R.D. Allan, J.R. Hanrahan, T.W. Hambley, G.A.R. Johnston, K.N. Mewett, A.D. Mitrovic, J. Med. Chem. 33 (1990) 2905.
[2] Y. Gaoni, A.G. Chapman, N. Parvez, P.C.-K. Pook, D.E. Jane, J.C. Watkins, J. Med. Chem. 37 (1994) 4288.
[3] T. Knöpfel, R. Kuhn, H. Allgeier, J. Med. Chem. 38 (1995) 1417.
[4] N.J. Toms, P.J. Roberts, T.E. Salt, P.C. Straton, Trends Pharmacol. Sci. 17 (1996) 429.
[5] F.C. Acher, F.J. Tellier, R. Azerad, I.N. Brabet, L. Fagni, J.-P. Pin, J. Med. Chem. 40 (1997) 3119.
[6] A. Breveglieri, R. Guerrini, S. Salvadori, C. Bianchi, S.D. Bryant, M. Attila, L.H. Lazarus, J. Med. Chem. 39 (1996) 773.
[7] E. Gershonov, R. Granoth, E. Tzehoval, Y. Gaoni, M. Fridkin, J. Med. Chem. 39 (1996) 4833.
[8] V.A. Davankov, in: J.C. Giddings, J. Cazes, P.R. Brown (Eds.), Advances in Chromatography, Vol. 22, Marcel Dekker, New York, 1983, pp. 71–103.
[9] V.A. Davankov, in: A.M. Krstulovic (Ed.), Chiral Separations by HPLC, Ellis Horwood, Chichester, 1989, pp. 446–475, Chapter 15.
[10] T. Shinbo, T. Yamaguchi, K. Nishimura, M. Sugiura, J. Chromatogr. 405 (1987) 145.
[11] P. Marfey, Carlsberg Res. Commun. 49 (1984) 591.
[12] D.W. Aswad, Anal. Biochem. 137 (1984) 405.
[13] R.H. Buck, K. Krummen, J. Chromatogr. 315 (1984) 279.
[14] D.W. Armstrong, Y. Tang, S. Chen, Y. Zhou, C. Bagwill, J.-R. Chen, Anal. Chem. 66 (1994) 1473.
[15] D.W. Armstrong, Y. Liu, K.H. Ekborgott, Chirality 7 (1995) 474.
[16] A. Berthod, Y. Liu, C. Bagwill, D.W. Armstrong, J. Chromatogr. A 731 (1996) 123.
[17] A. Péter, G. Török, D.W. Armstrong, J. Chromatogr. A 793 (1998) 283.
[18] A. Péter, G. Török, D.W. Armstrong, G. Tóth, D. Tourwé, J. Chromatogr. A 828 (1998) 177.
[19] J.C.J. Barna, D.H. Williams, M.P. Williamson, J. Chem. Soc. Chem. Commun. 5 (1985) 254.
[20] M.S. Westwell, U. Gerhard, D.H. Williams, J. Antibiot. 48 (1995) 1292.
[21] D.H. Williams, Nat. Prod. Rep. 13 (1996) 469.
[22] F.-J. Volk, A.W. Frahm, Liebigs Ann. Chem. (1996) 1893
[23] K.P. Fondekar, F.-J. Volk, A.W. Frahm, Tetrahedron: Asymmetry 10 (1999) 727.
[24] E. Weckert, G. Mattern, F.-J. Volk, A.W. Frahm, Acta Crystallogr. C 54 (1998) 387.
[25] K.P. Fondekar, F.-J. Volk, E. Weckert, A.W. Frahm, Acta Crystallogr. C 55 (1999) 1167.
[26] P.J. Schoenmakers, J.K. Strasters, Á. Bartha, J. Chromatogr. 458 (1988) 355.
[27] L.R. Snyder, J. Chromatogr. Sci. 16 (1978) 223.
[28] P.H. Popieniek, R.F. Pratt, J. Am. Chem. Soc. 113 (1991) 2264.
[29] Chirobiotic Handbook, Advanced Separation Technologies Inc., 2nd Edition, 1997.
[30] F.P. Meyer, H. Walther, in: F. von Bruchhausen, G. Dannhardt, S. Ebel, A.W. Frahm, E. Hackenthal, U. Holzgrabe (Eds.), Hagers Handbuch der Pharmazeutischen Praxis, Vol. 9, Springer-Verlag, Berlin, 1994, p. 789.
[31] Standard information for clinical pharmacists from Hoechst Marion Roussel, Bad Soden, Germany.