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Comparison of three chiral stationary phases with respect to their enantio- and diastereoselectivity for cyclic β-substituted α-amino acids

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Dedicated to Professor Dr Gottfried Blaschke on the occasion of his 65th birthday.

Abstract

Three chiral stationary phases were examined for the enantio- and diastereoseparation of cycloaliphatic β-substituted α-quaternary α-amino acids. Resolution of diastereomeric analytes is feasible with a chiral crown ether based column, whereas the separation of enantiomers, except for one pair of amino acids, could not be achieved. The two chiral stationary phases with the glycopeptide antibiotic teicoplanin and with the copper(II)-D-penicillamine complex, respectively, are, however, both very potent in the separation of the enantiomeric, as well as of the diastereomeric amino acids. A baseline separation of all four stereoisomeric forms in one chromatographic run was possible with the exception of one type of amino acid. The results of the method development are presented in this paper.

Keywords: Chiral stationary phases; LC; Enantiomer separation; α-Amino acids; Cyclic; β-Substituted; Chiral crown ether; Teicoplanin; Copper(II)-D-penicillamine

1. Introduction

The development of new chiral drugs always demands exact knowledge of their stereoisomeric purity due to the frequently observed diversity of biological activities of the stereoisomeric forms. In particular, in the field of amino acid drugs, some known examples exist indicating significant differences between the pharmacological and toxicological properties of drug enantiomers: Levodopa is an important substance in the treatment of Parkinson's disease, whereas its enantiomer is toxic [1]. Dextrothyroxine, the enantiomer of the thyroid hormone levothyroxine, is used as a lipid-lowering agent and consequently, must be applied in enantiomerically pure form to avoid side-effects on the thyroid gland [2].

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Cycloaliphatic α-quaternary α-amino acids are promising compounds in medicinal chemistry. They are reported to show affinity to both the ionotropic and the metabotropic glutamate receptors [3,4]. When incorporated into peptides, these constrained amino acids lead to modified biological properties and an enhanced stability towards hydrolysis [5,6]. Our group focused on the investigation of β-substituted cycloaliphatic α-quaternary α-amino acids, bearing two chiral centres and consequently existing as four stereoisomers. The synthesis of the 1-amino-2-methylcyclohexanecarboxylic acids (AMCH) **M1**–**M4**, the 1-amino-2-hydroxycyclohexanecarboxylic acids (AHCH) **H1**–**H4**, the *trans*-configured 1,2-diaminocyclohexanecarboxylic acids (DACH) **D2** + **D4**, the 1-amino-2-methylcyclopentanecarboxylic acids (AMCP) **P1**–**P4** and the 1-amino-2-ethylcyclopentanecarboxylic acids (AECP) **E1**–**E4** (Fig. 1) was achieved by means of asymmetric Strecker synthesis [7–9]. Because of the above-mentioned dependence of the physiological activities on the stereochemistry, one of our main concerns remained the enantiomeric and the diastereomeric analysis of these compounds.

High-performance liquid chromatography (HPLC) is a widely used technique in the chiral separation of amino acids. There are two strategies feasible: the analytes can be either separated directly on chiral stationary phases (CSP) or they can be transformed to diastereomeric entities by derivatization with chiral reagents allowing the discrimination on achiral columns. For the analysis of amino acids, one of these derivatizating reagents is 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent, FDAA) [10]. Apart from the usual drawbacks of these methods concerning the enantiomeric purity of the reagent or the preferential derivatization of one enantiomer, no reaction at all took place between FDAA and the herein discussed amino acids, even after several hours at an elevated temperature [11].

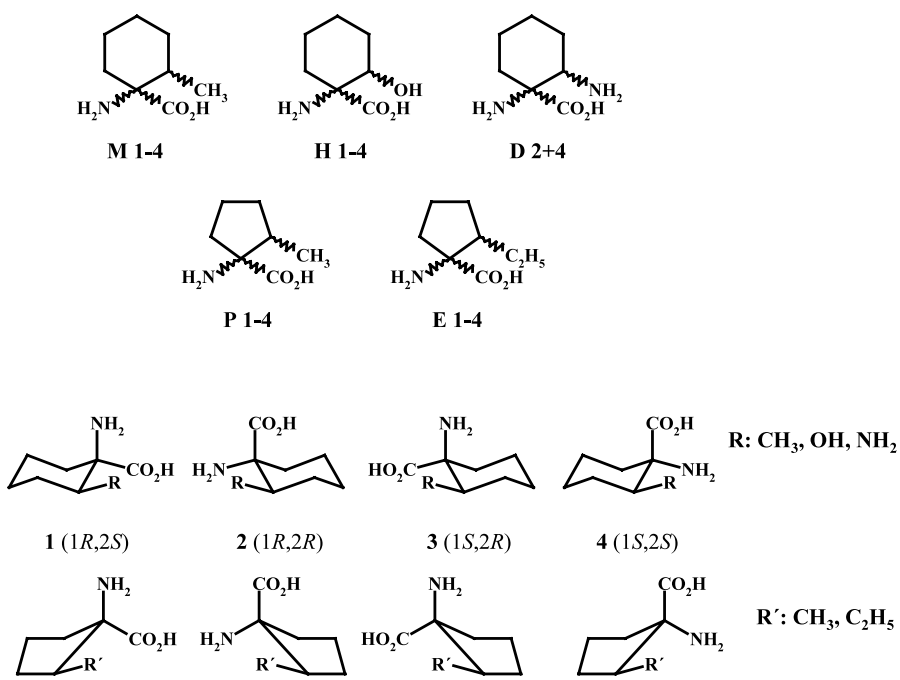

Fig. 1. Structures of the investigated compounds: **M**: 1-amino-2-methylcyclohexanecarboxylic acids (A**M**CH); **H**: 1-amino-2-hydroxycyclohexanecarboxylic acids (A**H**CH); **D**: 1,2-diaminocyclohexanecarboxylic acids (**D**ACH); **P**: 1-amino-2-methylcyclopentanecarboxylic acids (AMC**P**); **E**: 1-amino-2-ethylcyclopentanecarboxylic acids (A**E**CP). **1**: *cis*-(1*R*,2*S*); **2**: *trans*-(1*R*,2*R*); **3**: *cis*-(1*S*,2*R*); **4**: *trans*-(1*S*,2*S*).

Three different types of CSPs have been used for the direct separation of amino acid enantiomers via interactions with a chiral crown ether, with the glycopeptide antibiotic teicoplanin and with a copper(II)-amino acid complex (ligand-exchange type), respectively.

Cram et al. found that chiral crown ethers are able to form diastereomeric inclusion complexes with racemic compounds bearing a primary amino group [12,13]. Shinbo et al. packed a reversed-phase silica column with such a chiral crown ether resulting in the Crownpak CSP [14]. The glycopeptide antibiotic teicoplanin was introduced by Armstrong et al. for the chiral separation of amino acids due to its highly stereospecific affinity to D-configured amino acids explaining its antimicrobiotic activity [15,16]. Based on the pioneering works of Davankov et al. on ligand-exchange columns [17–20], Ôi et al. developed a CSP with a copper(II)-*N*,*S*-dioctyl-D-penicillamine complex embedded in reversed-phase (C18) silica [21,22].

Preliminary work showed that the teicoplanin column is suitable for the resolution of AMCH and AHCH [23]. Further, the copper(II)-D-penicillamine CSP allowed the separation of the *trans*-configured enantiomers of DACH [24]. On this column, an unusual reversal of the enantiomeric elution order of two amino acid pairs in dependence on the temperature was observed [25]. In this paper, these three CSPs are compared with respect to their enantio- and diastereoselectivity for the five cycloaliphatic β-substituted α-quaternary α-amino acids shown in Fig. 1.

2. Experimental

2.1. Chemicals

The 1-amino-2-methylcyclohexanecarboxylic acids (AMCH) **M1**–**M4**, the 1-amino-2-hydroxycyclohexanecarboxylic acids (AHCH) **H1**–**H4**, the *trans*-configured 1,2-diaminocyclohexanecarboxylic acids (DACH) **D2** + **D4,** the 1-amino-2-methylcyclopentanecarboxylic acids (AMCP) **P1**–**P4** and the 1-amino-2-ethylcyclopentanecarboxylic acids (AECP) **E1**–**E4** (Fig. 1) were synthesized in our laboratory according to earlier described methods [7–9]. The structures were proven by ^{1}H- and ^{13}C-NMR spectroscopy and the absolute configurations were determined by X-ray analysis and CD correlation studies [26,27]. 3-Cyclohexylalanine and *tert*-leucine were generous gifts from Degussa AG (Hanau, Germany).

HPLC-grade methanol, 2-propanol and acetonitrile were obtained from Fluka Chemie AG (Buchs, Switzerland) and ethanol from Riedel-de Haën AG (Seelze, Germany). Perchloric acid (70–72%), sodium hydroxide solution (1 M), ammonium nitrate, potassium chloride, copper(II) sulfate pentahydrate (all Merck KGaA, Darmstadt, Germany) and acetic acid 99–100% (Riedel-de Haën AG) were of analytical grade.

2.2. Reagents

The mobile phases for the Crownpak column consisted of a 0.1 N perchloric acid, pH 1.0 (16.30 g/l $HClO_4$) and of corresponding dilutions thereof with water. 0.1 N $HClO_4$, pH 1.0, 10% methanol was prepared by mixing 16.30 g $HClO_4$, 900 ml water and 100 ml methanol; 0.1 M $HClO_4$, pH 3.0 was obtained by adjusting a solution of 16.30 g $HClO_4$ in ~850 ml water with ~100 ml 1 M NaOH to pH 3.0 and by making the resulting mixture up to 1 l with water.

The eluents for the Chirobiotic T column were prepared by stirring the indicated volumes of organic modifier and water; acetic acid was used to adjust the mobile phase to a given pH.

The eluents for the Chirex (D) Penicillamine CSP were obtained by dissolving the indicated amount of copper salt in a mixture of organic modifier and water in the given ratio.

Prior to chromatography, all mobile phases were filtered through a 0.45 μm regenerated cellulose filter (Schleicher and Schuell GmbH, Dassel, Germany).

All amino acids were analyzed without derivatization. Sample solutions (~1 mg/ml) were pre-

pared by dissolving the analytes in water and filtering through a 0.45 μm filter.

2.3. 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 Ver. 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).

The columns used were a Crownpak CR(+) chiral stationary phase (CSP), 150 × 4 mm ID, 5 μm particle size (Daicel Chem. Ind. Ltd., Tokyo, Japan), a Chirobiotic T CSP, 250 × 4.6 mm ID, 5 μm particle size, combined with a Chirobiotic T guard column, 20 × 4 mm ID, 5 μm particle size (Astec, Whippany, NJ) and a Chirex (D) Penicillamine CSP, 250 × 4.6 mm ID (Phenomenex, Aschaffenburg, Germany).

2.4. Chromatographic conditions

Unless otherwise stated, the injection volume was 10 μl for the Crownpak column and 5 μl for the two other CSPs. The mobile phase flow-rate was set at 0.5 ml/min for the Crownpak CSP and 0.8 ml/min for the two other columns. The detection wavelength was 205 nm for the Crownpak and the Chirobiotic T columns and 254 nm for the Chirex (D) Penicillamine CSP. The hold-up time (t_M) was determined with water (Crownpak, negative peak signal), with an aqueous solution of ammonium nitrate (0.02 mg/ml) in case of the Chirobiotic T column and with a diluted aqueous solution of potassium chloride (Chirex (D) Penicillamine, negative peak signal). Sample solutions of the single stereoisomers were injected to establish the elution order of their stereoisomeric mixtures.

Resolution was calculated according to the special method of Schoenmakers et al. for asymmetric peaks [28].

3. Results and discussion

3.1. Crownpak CSP

The retention on the Crownpak CSP can be attributed to two equilibria [14], where the protonated analyte forms (1) an ion pair with the anion present in the mobile phase and (2) a complex with both the chiral crown ether and the anion. The formation of simple ion pairs affects the two enantiomers in the same way ('achiral retention'), whereas the ternary complex is responsible for enantioselectivity ('chiral retention'). The knowledge of these interaction mechanisms is helpful in the interpretation of the results gained during the method development.

The choice of mobile phases for the Crownpak CSP is rather limited. The eluent has to be acidic since a protonated amino function is necessary for the interaction with the crown ether. When analyzing aliphatic amino acids, perchloric acid is the only suitable inorganic acid because it does not absorb in the low UV range required for detection. As the chiral crown ether is not covalently bound to the reversed-phase silica support, the addition of an organic modifier is strictly limited. The manufacturer recommends exclusively the use of methanol to a maximum of 15% [29]. Temperature is an additional parameter to be varied with promising results [30].

The influence of these parameters on the enantio- and diastereoselectivity was investigated with the example of AMCH. Whereas the separation of the enantiomers could not be achieved under any of the investigated conditions, resolution of the diastereomers was feasible.

The concentration of perchloric acid was varied from 0.1 to 0.001 M, resulting in a pH change from 1 to 3. The retention of the amino acids strongly decreased with lower $HClO_4$ content in accordance with a weaker ion pair as well as with a weaker complex formation. In parallel, the selectivity of the diastereoseparation increased in contrast to a loss in selectivity observed for the separation of enantiomers [31]. This implies the complex formation with the crown ether, which is by far, not the determining factor for the retention of these amino acids. The resolution, how-

Table 1
Separation results (k, α, R_S) of the investigated amino acids on the Crownpak CSP with a 0.1 M $HClO_4$ mobile phase at pH 1, at 5 °C and 0.5 ml/min

	k (*cis*)[a]	k (*trans*)[a]	α (*cis*/*trans*)[b]	R_S (*cis*/*trans*)[b]
AMCH	6.43	9.32	1.45	3.44
AHCH[c]	1.60	1.96 **H4**	1.23 **H4**	1.19 **H4**
		2.33 **H2**	1.45 **H2**	2.24 **H2**
DACH	–	0.96	–	–
AMCP	2.76	3.16	1.14	1.08
AECP	7.30	8.91	1.22	1.60

[a] k is the retention factor of the *cis*- and *trans*-configured amino acids, respectively.
[b] α and R_S are the separation factor and the resolution, respectively, for the separation of the *cis*- from the *trans*-configured amino acids.
[c] For the *trans*-configured AHCH, k, α and R_S of both enantiomers are given.

ever, did not rise with the selectivity, since the peaks showed increasing tailing at low $HClO_4$ concentrations. This can be explained by the worse solubility of the analyte in the mobile phase due to an enhanced deprotonation of the carboxyl functionality leading to zwitterionic compounds. To distinguish between the influence of the perchlorate concentration and the pH value, a mobile phase with 0.1 M $HClO_4$, where the pH is adjusted to 3 with sodium hydroxide was prepared. The retention times under these conditions were more similar to the results gained with a 0.001 M $HClO_4$ eluent at pH 3 than to those obtained with a 0.1 M $HClO_4$ eluent at pH 1. Hence, the pH value is the dominating factor over the perchlorate concentration for the retention.

The addition of an organic modifier reduces the achiral hydrophobic interactions with the CSP without influencing the complex formation with the chiral crown ether. By application of a mobile phase with 10% methanol, the retention times of the AMCH stereoisomers were nearly halved with unchanged selectivity, whereas the retention times of a well separated racemic compound, like DL-alanine, were hardly affected on addition of methanol. This indicates that the retention of AMCH is based on hydrophobic interactions with the reversed-phase support.

The effect of temperature was investigated in the range from 5 to 30 °C. The retention and the selectivity both slightly increased with decreasing temperature, but not as significantly as described in the literature for the separation of a large variety of amino acid enantiomers [14,30–33].

The enantiomers of AMCH could not be resolved with the Crownpak CSP. For comparison, two further amino acids (cyclohexylalanine and *tert*-leucine) with similar polarity and space-filling properties were investigated. They only differ in the sterical hindrance of their amino group. The enantiomers of cyclohexylalanine with a cyclohexane ring at the β-carbon were very well separated, whereas DL-*tert*-leucine, bearing a *tert*-butyl group at the α-carbon, was only partially resolved. In the case of AMCH, the quaternary α-carbon and the presence of an additional substituent in the β-position caused too strong shielding of the amino group for effective interactions with the crown ether. This is in accordance with the observations of Esquivel et al., who found that the sterical hindrance of the amino group should be kept to a minimum for optimal resolution [34].

The results for all five cyclic β-substituted amino acids are listed in Table 1. Surprisingly the *trans*-configured enantiomers of AHCH could be separated with a selectivity factor of 1.19 and a resolution of 1.23. In all other cases, only a discrimination of the diastereomers was feasible. As expected, the amino acids with polar substituents, AHCH and DACH, were less retained on the column than the alkyl substituted amino acids. This is another hint for the importance of hydrophobic interactions for the retention of these amino acids on the Crownpak CSP.

3.2. Teicoplanin CSP

The Chirobiotic T column containing the glycopeptide antibiotic teicoplanin can be generally used in the normal-phase mode, the reversed-phase mode and the polar-organic mode, respectively [15]. Since amino acids are ionic compounds, they are not soluble in apolar normal-phase eluents. In the polar-organic mode, the mobile phases consist of methanol or acetonitrile. To control the retention and selectivity of the analytes, various amounts of triethylamine and acetic acid are added. These additives raise the UV cutoff of the mobile phase, so that this mode is also unsuitable for the analysis of underivatized aliphatic amino acids. Hence, the reversed-phase mode is the first choice.

The aim of our work was the simultaneous separation of both the enantiomers and the diastereomers of one amino acid in a single chromatographic run. For rapid method optimisation, initially several organic modifiers were investigated, then the content of the most promising modifier was varied and finally, the temperature was optimized. All eluents had to be adjusted to pH 4 in order to achieve reproducible results, as we found earlier [23].

The separation results for four amino acids (AMCH, AHCH, AMCP and AECP) with the four organic modifiers methanol, ethanol, 2-propanol and acetonitrile, are listed in Table 2. The basic compound DACH did not elute from the teicoplanin CSP. Berthod et al. found that basic amino acids are very strongly retained due to interactions with anionic sites on the column [16].

Table 2
Separation results (k, R_{min}) of the investigated amino acids with four organic modifiers on the teicoplanin CSP

Compound		Methanol	Ethanol	2-Propanol[a]	Acetonitrile
AMCH	k (**M1**)[b,c]	3.64	4.18	4.56	3.91
	k (**M3**)[c]	3.10	3.39	3.74	3.70
	k (**M2**)	5.71	6.52	7.19	6.53
	k (**M4**)[c]	5.06	5.49	6.06	6.29
	R_{min}[d]	2.01	2.65	2.47	<0.5
AHCH	k (**H1**)[c]	2.69	2.74	2.81	3.12
	k (**H3**)[c]	3.05	3.30	3.57	3.24
	k (**H2**)[c]	3.73	3.60	3.72	4.47
	k (**H4**)	4.12	4.26	4.64	4.56
	R_{min}	1.36	0.89	<0.5	<0.5
AMCP	k (**P1**)	3.85	4.71	5.13	4.02
	k (**P3**)	3.38	3.95	4.33	3.84
	k (**P2**)	4.03	4.80	5.44	4.74
	k (**P4**)	3.64	4.15	4.67	4.53
	R_{min}	<0.5	<0.5	<0.5	<0.5
AECP	k (**E1**)	5.29	6.73	7.07	4.80
	k (**E3**)	3.35	3.79	4.17	4.11
	k (**E2**)	4.56	5.65	6.44	5.48
	k (**E4**)	3.88	4.50	5.12	5.12
	R_{min}	2.33	2.32	1.42	<0.5

[a] The flow-rate was set at 0.5 ml/min to avoid too high a back pressure produced by the viscosity of the 2-propanol-water mobile phase.

[b] k is the retention factor of a given stereoisomer.

[c] See Ref. [23].

[d] R_{min} is the lowest value of resolution of any two stereoisomers.

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.

The best separation of enantiomers (**3**/**1** and **4**/**2**) could be achieved with ethanol or 2-propanol, followed by methanol, whereas the amino acid enantiomers were poorly resolved with acetonitrile. On the other hand, the diastereomeric discrimination (**1**/**2** and **3**/**4**) was favored with acetonitrile as organic modifier, but the three alcohols as well allowed a satisfactory discrimination between the amino acid diastereoisomers.

The retention of the four amino acids differed largely. The four stereoisomers of AMCH and AECP eluted well separated from the column with methanol or ethanol as modifier, whereas the elution window of the four stereoisomeric forms of AMCP was rather narrow. Small structural differences in the aliphatic ring (five- versus six-membered) or the substituent in the β-position (methyl versus ethyl) have a strong effect on the resolution of the corresponding amino acids.

For AMCH and AECP, no further method development was necessary, since very good results could be achieved under the initial conditions. The separation of AMCH stereoisomers was feasible with an ethanol–water (60:40, v/v) mobile phase at 20 °C with 0.8 ml/min within 16 min. The minimal resolution R_{min}, the lowest value in resolution of any two compounds of 2.65 obtained is sufficient for the determination of the stereochemical composition of synthetic products. For the separation of the AECP stereoisomers, methanol and ethanol were equipotent, the first modifier however was preferred due to the shorter analysis times of 14 min with a minimal resolution of 2.33.

The separation of AHCH and AMCP had to be further improved by variation of the organic modifier content. As earlier reported [23], the retention increases at higher concentrations of the organic modifier. This untypical behaviour of RP-HPLC was also observed by Berthod et al. [16], who explained this phenomenon by the minor solubility of amino acids in modifier-enriched eluents. The enantioselectivity continuously rises with increasing modifier concentrations, whereas the diastereoselectivity strongly decreases with high modifier contents [23]. Although, according to Table 2, methanol appeared to be the most suitable modifier for the separation of AHCH, the best resolution was obtained with ethanol as co-solvent by decreasing its content to 50% and simultaneously lowering the temperature to 15 °C (R_{min}: 1.88). AMCP was the only amino acid that could be best resolved with acetonitrile, in contrast to the insufficient diastereomeric discrimination with the alcoholic modifiers. By increasing the acetonitrile content to 80%, a minimal resolution of 1.14 could be achieved which is, however, far from a baseline separation. The chromatograms of the four amino acids under optimized conditions are shown in Fig. 2.

The *cis*-configured amino acids were less retained than their *trans*-configured epimers and the enantiomeric elution order was (1*S*) in front of (1*R*) corresponding to the antibiotic property of teicoplanin, which selectively binds at the D-Ala-D-Ala unit of the muramyl pentapeptide during the bacterial cell wall synthesis. However, there were two exceptions: The enantiomeric pairs of AHCH were eluted in the opposite order. To date, there are only three other amino acids described, the (*R*)-configured enantiomers of which are less retained on Chirobiotic T [35]. Hence, it is not possible to predict the absolute configuration of an amino acid by its retention behaviour on this CSP. The second exception was the elution profile of AECP, with the *cis*-(1*R*,2*S*) stereoisomer strongly retained on the column leading to an elution even after the *trans*-stereoisomers. Apparently, this stereoisomeric form fits very well in the binding pocket of teicoplanin.

3.3. Copper(II)-D-penicillamine CSP

Chiral discrimination on the copper(II)-D-penicillamine CSP takes place by formation of diastereomeric complexes between the enantiomeric analytes and the chiral selector D-penicillamine with Cu^{2+} as metal ion. The stability of these complexes is mainly governed by hydrophobic interactions between the analyte and the apolar surface of the packing material [20,25,36]. Due to the presence of copper ions in the eluent, the amino acids form soluble complexes which show a distinct absorption at λ 254 nm with enhanced ε-values and an overall improved sensitivity com-

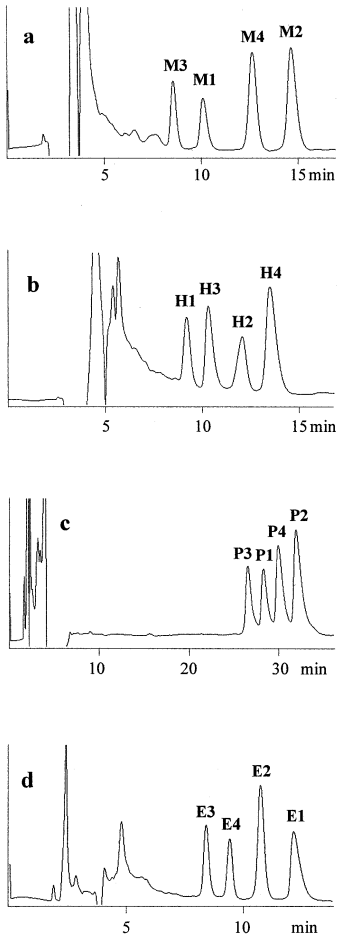

Fig. 2. Chromatograms of the four stereoisomers of AMCH (**a**), AHCH (**b**), AMCP (**c**) and AECP (**d**) on the teicoplanin CSP. Chromatographic conditions: (**a**) ethanol–water (60:40, v/v), pH 4, 0.8 ml/min, 20 °C [23]; (**b**) ethanol–water (50:50, v/v), pH 4, 0.6 ml/min, 15 °C [23]; (**c**) methanol–water (60:40, v/v), pH 4, 0.8 ml/min, 20 °C; and (**d**) acetonitrile–water (80:20, v/v), pH 4, 0.8 ml/min, 20 °C; UV detection 205 nm.

pared to the direct detection at λ 205 nm on the teicoplanin CSP. The chromatographic parameters to be varied are the concentration of Cu^{2+} in the eluent (0.3–3 mM), the type (methanol, 2-propanol and acetonitrile) and content of organic modifier (0–20%), the mobile phase pH (4.5–6.2) and the temperature (5–50 °C). Details of this method development for the separation of the β-substituted amino acids are reported elsewhere [24].

The separation of all four stereoisomers was possible for AMCH (R_{min}: 1.73), AHCH (R_{min}: 3.45) and AECP (R_{min}: 2.68). For the first time, the *trans*-enantiomeric pair of *DACH* could be resolved (R_S: 1.4). The chromatograms of these amino acids under optimized conditions are shown in Fig. 3. The four stereoisomers of AMCP, however, could not be baseline separated,

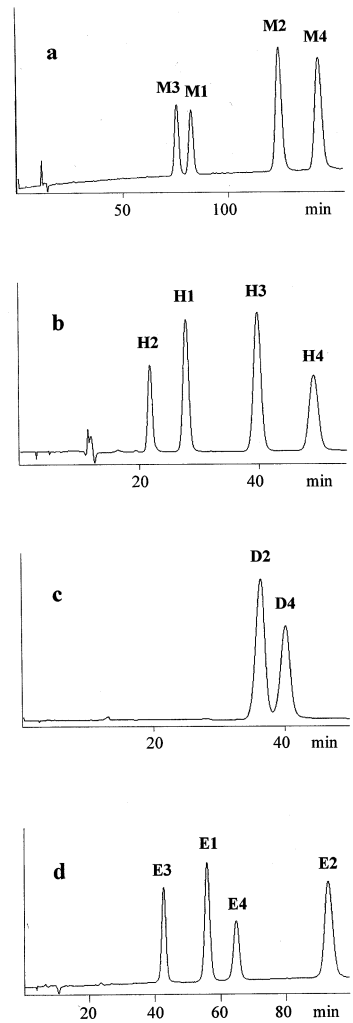

Fig. 3. Chromatograms of the stereoisomers of AMCH (**a**), AHCH (**b**), DACH (**c**) and AECP (**d**) on the copper(II)-D-penicillamine CSP at 20 °C, 0.8 ml/min, UV detection 254 nm. Mobile phases: (**a**) 1 mM $CuSO_4$ in acetonitrile–water (5:95, v/v) [24]; (**b**) 1 mM $CuSO_4$ in 2-propanol–water (2.5:97.5, v/v) [24]; (**c**) 0.3 mM $CuSO_4$ in 100% water [24]; and (**d**) 2 mM $CuSO_4$ in methanol–water (15:85, v/v).

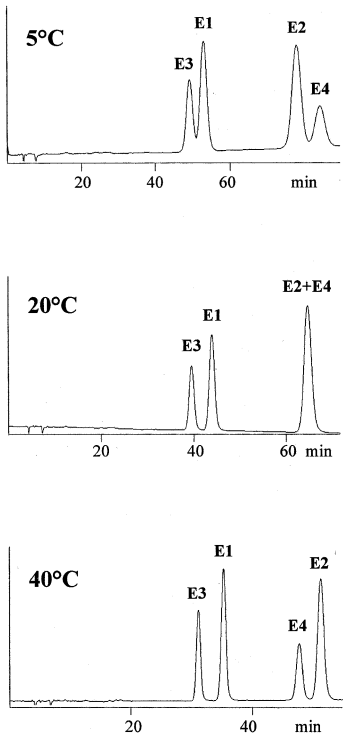

Fig. 4. Chromatograms of the four stereoisomers of AECP on the copper(II)-D-penicillamine CSP with 2 mM $CuSO_4$ in acetonitrile–water (10:90, v/v) mobile phase, 0.8 ml/min at the indicated temperature.

since the diastereomers **P3** and **P4** were only partially resolved.

Earlier we observed a reversal of the enantiomeric elution order of the *cis*-configured AHCH and the *trans*-configured AMCP by varying the temperature [25]. This applies also for the *trans*-enantiomers of the AECP series, as shown in Fig. 4. This phenomenon can be explained by the co-existence of different binding sites on the CSP originating in the formation of different complexes with the tridentate ligand, penicillamine [24,25].

3.4. Comparison of the chromatographic separation behaviour of the differently substituted amino acids

The separation results of the investigated amino acids can be compared with respect to the polarity of the substituent at the cyclohexane skeleton, with respect to the ring size in the case of methyl substitution and with respect to the type of the aliphatic substituent in the cyclopentane series.

Since the three columns were used uniquely in the reversed-phase mode, the more polar analyte was eluted first, disregarding the teicoplanin CSP where cationic compounds are more strongly retained due to interactions with anionic sites on the column. The selectivity for the polar substituted amino acids was poorer on the teicoplanin column, whereas they were better separated than the methyl-substituted amino acids on the copper(II)-D-penicillamine CSP because of additional complex formation equilibria between the polar substituent and this special CSP.

On all three CSPs, the methyl-substituted amino acids with a six-membered ring were better resolved than the corresponding cyclopentane series. Obviously, the chiral discrimination is facilitated by the chair conformation of the cyclohexane ring compared to the envelope conformation of the cyclopentane ring.

Concerning the alkyl substituents in the cyclopentane series, an ethyl group in comparison with a methyl substituent improved, as expected, the enantiomeric as well as the diastereomeric separation on all three CSPs. In an ongoing study, the corresponding isopropyl- and *tert*-butyl-substituted amino acids are investigated. It is expected that steric effects will play an increasing role leading to a loss in selectivity.

4. Conclusions

In this paper, three chiral stationary phases were compared with respect to their ability in the enantio- and diastereoseparation of cyclic β-substituted α-amino acids (Table 3). The separation of enantiomeric amino acids was not possible on the Crownpak CSP with one exception. The teicoplanin and the copper(II)-D-penicillamine CSPs, however, were both very potent in the resolution of the investigated amino acid stereoisomers with slight differences in selectivity. Amino acids bearing aliphatic substituents were better separated on the teicoplanin column, whereas the copper(II)-D-

Table 3
Comparison of the three investigated CSPs

	Crownpak CSP	Teicoplanin CSP	Copper(II)-D-penicillamine CSP
Enantioselectivity for cyclic amino acids	Poor	Good	Very good
Diastereoselectivity for cyclic amino acids	Good	Very good	Good
Enantiocapability besides amino acids	Restricted to analytes with primary amino group [14]	Broad applicability for pharmaceuticals of different structures [15]	Restricted to amino alcohols, hydroxy acids [21] and diamines [37]
Sensitivity	Fair	Fair	Very good
Analyses times	Short	Short	Long
Column stability	Good, but limitation for certain organic modifiers	Good	Good, but limitation for certain organic modifiers
Cost	~1300 Euro	~1500 Euro	~1000 Euro

penicillamine CSP was more suitable for polar-substituted compounds. The analysis times were much shorter on the teicoplanin phase, but sensitivity was increased with the copper(II)-D-penicillamine column. With these two chiral stationary phases, powerful tools for the determination of *ee* and *de* values of synthetic cycloaliphatic amino acids are available. The choice of the column depends on the structure type of the analyte and on the requirements concerning duration and sensitivity of the analysis.

When turning from analytical to preparative scale, the teicoplanin CSP is advantageous since the mobile phases can easily be removed. The evaporation of perchloric acid containing eluents needed with the crown ether column comprises the danger of explosion in the presence of an organic cosolvent. In the case of the copper(II)-D-penicillamine CSP, the copper(II) ions required for the ligand exchange process need to be eliminated in preparative chromatography. The summarizing comparison of the three CSPs is found in Table 3.

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