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Enantiomeric and diastereomeric high-performance liquid chromatographic separation of cyclic β-substituted α-amino acids on a copper(II)-D-penicillamine chiral stationary phase

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Abstract

High-performance liquid chromatographic (HPLC) separation of stereoisomeric cyclic β-substituted α-quaternary α-amino acids was performed by ligand-exchange on a copper(II)-D-penicillamine chiral stationary phase. The investigated amino acids are the 1-amino-2-methylcyclohexanecarboxylic acids, the 1-amino-2-hydroxycyclohexanecarboxylic acids, the 1-amino-2-methylcyclopentanecarboxylic acids and the *trans*-configured 1,2-diaminocyclohexanecarboxylic acids. The effects of the mobile phase composition (copper(II) concentration, type and content of organic modifier, pH) and the temperature on the enantio- and diastereoselectivity were studied and the conditions were optimised to resolve the four stereoisomers of each of the said amino acids in single chromatographic runs. A reversal of the elution order occurred for enantiomers of some of the amino acids in dependence on the acetonitrile content of the eluent. This phenomenon is explained by at least two different copper(II) complexes of the tridentate ligand penicillamine.

Keywords: Chiral stationary phases; LC; Enantiomer separation; α-Amino acids; Copper(II)-D-penicillamine

1. Introduction

Cycloaliphatic α-quaternary α-amino acids offer a broad range of application in medicinal chemistry. They show affinity to both the ionotropic and the metabotropic glutamate receptors [1,2]. When incorporated into peptides, these constrained amino acids lead to modified biological properties and an enhanced stability towards hydrolysis [3,4].

Unsubstituted cycloaliphatic α-quaternary α-amino acids are prochiral molecules. By formal introduction of a substituent in the 2-position of the ring, they are converted into chiral compounds with two stereogenic centres which consequently exist 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 1-amino-2-methylcyclopentanecarboxylic acids (AMCP) **P1**–**P4** and the *trans*-configured 1,2-diaminocyclohexanecarboxylic acids (DACH) **D2**+**D4** (Fig. 1) was achieved by means of asymmetric Strecker synthesis [5–7]. Since the biological properties of amino acids, like most chiral drugs, are strongly related to their

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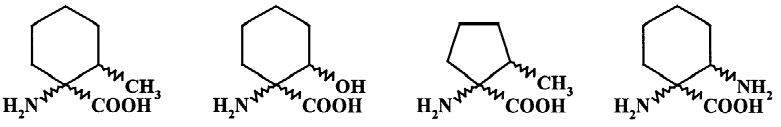


Fig. 1. Structures of the investigated compounds: (**M**): 1-amino-2-methylcyclohexanecarboxylic acids (AMCH), (**H**): 1-amino-2-hydroxycyclohexanecarboxylic acids (AHCH), (**P**): 1-amino-2-methylcyclopentanecarboxylic acids (AMCP), (**D**): 1,2-diaminocyclohexanecarboxylic acids (DACH). **1** *cis*-(1*R*,2*S*), **2** *trans*-(1*R*,2*R*), **3** *cis*-(1*S*,2*R*) and **4** *trans*-(1*S*,2*S*), respectively.

stereochemistry, a stereodifferentiating analytical tool allowing the simultaneous determination of *ee* and *de* values is quite indispensable.

A large variety of HPLC methods have been described for the resolution of enantiomeric amino acids. Among these, and based on the work of Cram and co-workers [8,9], Shinbo et al. [10] developed a chiral stationary phase (CSP) coated with a chiral crown ether which enables the formation of diastereomeric inclusion complexes with racemates bearing a primary amino group. However, the herein discussed β-substituted α-carbocyclic amino acids can not be separated in their enantiomeric forms with this CSP, most probably due to the steric hindrance of the amino function [11]. The group of Armstrong [12,13] introduced the glycopeptide antibiotic teicoplanin for chiral separation of amino acids. This CSP has proven to be a suitable tool for the analysis of AMCH and AHCH [14]. The basic amino acid DACH, however, failed to be eluted from this column even with an acidic mobile phase [11]. Another strategy is the formation of diastereomeric derivatives with a chiral reagent prior to chromatographic separation on an achiral column. For the analysis of amino acids, one of the derivatisating reagents is 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent, FDAA) [15]. Apart from the usual drawbacks of these methods concerning the enantiomeric purity of the reagent or the preferential derivatisation of one enantiomer, no reaction at all took place between FDAA and the herein discussed amino acids even after several hours at elevated temperature [11].

Another approach is the use of ligand-exchange columns. Based on the pioneering work of Davankov and co-workers [16,17], a variety of CSPs with different chiral selectors, such as proline [18,19], histidine [20] and tartaric acid derivatives [21], have been introduced. We have chosen a column developed by Ôi et al., where a copper(II)-*N*,*S*-dioctyl-D-penicillamine complex is embedded in reversed-phase (C18) silica [22,23]. Chiral discrimination on ligand-exchange columns is a result of the formation of diastereomeric complexes between the enantiomeric analytes and the chiral selector with Cu^{2+} as metal ion. The stability of these complexes is mainly governed by hydrophobic interactions between the analyte's moiety and the apolar surface of the packing material [24,25]. Penicillamine as tridentate ligand can interact with Cu^{2+} via its *N*-, *O*- and/or *S*-atom, and therefore complex formation is more manifold than with often used bidentate ligands like proline.

Apart from the broad enantioselectivity of ligand-exchange CSPs, they possess a further advantage with respect to more popular forms of chiral chromatography. The detection of underivatised, aliphatic amino acids is a key problem, since the direct UV detection between λ 195 and 210 nm strongly restricts the choice of the mobile phase and decreases the sensitivity of the determination. In ligand-exchange chromatography, Cu^{2+} ions are always present in the eluent forming dissolved complexes with the analytes which show a distinct absorption at λ 254 nm with enhanced ϵ-values and an overall improved sensitivity.

In this paper we will discuss the influence of the chromatographic conditions (copper(II) concentration in the eluent, type and content of organic modifier, pH of the mobile phase, temperature) on the enantio- and diastereomeric separation of the four stereoisomers of AMCH, AHCH and AMCP, respectively, as well as on the resolution of the *trans*-configured enantiomeric pair of DACH.

2. Experimental

2.1. Chemicals

The 1-amino-2-methylcyclohexanecarboxylic acids (AMCH) **M1**–**M4**, the 1-amino-2-hydroxycyclohexanecarboxylic acids (AHCH) **H1**–**H4**, the 1-amino-2-methylcyclopentanecarboxylic acids (AMCP) **P1**–**P4** and the *trans*-configured 1,2-diaminocyclohexanecarboxylic acids (DACH) **D2**+**D4** (Fig. 1) were synthesised in our laboratory according to earlier described methods [5–7]. 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 [26,27].

HPLC-grade methanol, 2-propanol and acetonitrile were obtained from Fluka Chemie AG (Buchs, Switzerland). Copper(II) sulfate pentahydrate (Merck KGaA, Darmstadt, Germany), copper(II) acetate monohydrate (Fluka Chemie AG, Buchs, Switzerland) and acetic acid 99–100% (Riedel-de Haën AG, Seelze, Germany) were of analytical grade.

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 Chirex (D)–Penicillamine chiral stationary phase, 250×4.6 mm I.D., (Phenomenex, Aschaffenburg, Germany).

The eluents were prepared by dissolving the indicated amount of copper salt in a mixture of organic modifier and freshly distilled water in the given ratio, followed by filtration through a 0.45 μm regenerated cellulose filter (Schleicher & Schuell GmbH, Dassel, Germany). For variations of the mobile phase pH, copper acetate was used as copper(II) source and pH was adjusted with acetic acid.

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

Unless otherwise stated, the injection volume was 5 μl, the mobile phase flow-rate was set at 0.8 ml/min and the detection wavelength was 254 nm. The hold-up time (t_M) was determined with a diluted aqueous solution of potassium chloride. 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 for asymmetric peaks [28].

3. Results and discussion

3.1. Influence of the chromatographic conditions

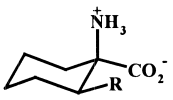
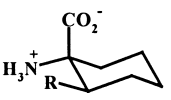
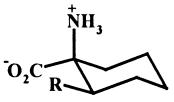
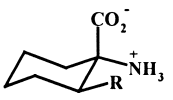
The initial mobile phase consisted of 2 m*M* $CuSO_4$ in methanol–water (15:85, v/v) at 20°C. Under these conditions, the four stereoisomers of AHCH are already well separated, but insufficient resolution or co-elution occurs in the three other amino acid series (Table 1). The retention times differ largely in these series: as expected, the less polar methyl-substituted amino acids are more strongly retained than the more hydrophilic hydroxy- or amino-substituted analytes. Starting from these conditions, the Cu^{2+} concentration (0.3–3 m*M*) and the methanol content (0–20%) as well as the temperature (10–50°C) were varied.

An increase of the copper(II) concentration results in drastically reduced retention times without alteration of the selectivities. However, at Cu^{2+} concentrations below 1 m*M* a partial resolution of the two *trans*-configured enantiomers of DACH is possible (retention times with 0.3 m*M* Cu^{2+} in methanol–water (15:85, v/v) at 20°C: **D-2**: 20.7 min; **D-4**: 22.9 min).

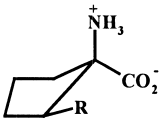
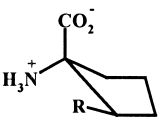
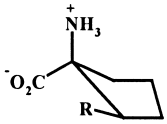
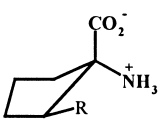
A similar effect is observed upon variation of the methanol content. A decrease in the modifier concentration results in a largely increased retention without influencing the separation factors again with the exception of the *trans*-configured DACH enantiomers which are partially resolved without any methanol (retention times with 1 m*M* Cu^{2+} in 100% water at 20°C: **D-2**: 32.9 min; **D-4**: 34.4 min).

The column temperature is a determining factor in

Table 1
Retention times of the investigated amino acids under the initial conditions[a]

Retention times [min]	*R*	**1** (1*R*,2*S*)	**2** (1*R*,2*R*)	**3** (1*S*,2*R*)	**4** (1*S*,2*S*)
AMCH	CH_3	57.5	87.9	51.0	92.5
AHCH	OH	21.0	16.8	27.5	34.1
DACH	NH_3^+	–	18.7	–	18.4
AMCP	CH_3	35.7	41.6	31.5	31.9

[a] Chromatographic conditions: 2 m*M* $CuSO_4$ in methanol–water (15:85, v/v), 20°C, 0.8 ml/min, UV detection λ 254 nm, t_M 2.6 min.

chiral ligand-exchange chromatography. Indeed, Davankov was the first to report an increased resolution at elevated temperatures [29]. Variations of the temperature on the copper(II)-penicillamine column with methanol–water mobile phases, however, do not exhibit large effects in the range from 10 to 50°C. The separation factors are influenced in a non-uniform way. The resolution of the *cis*-configured enantiomers of AHCH can be increased at elevated temperatures, the *trans*-configured enantiomers of AMCH, however, co-elute above 40°C.

The replacement of the organic modifier methanol by 2-propanol leads to similar results. The retention times are more strongly decreased with 2-propanol-enriched mobile phases compared with methanol due to the lower polarity of 2-propanol. The separation factors are hardly influenced by the 2-propanol content.

In contrast, when turning to acetonitrile as organic modifier, its concentration becomes a crucial parameter for the resolution of two of the amino acid enantiomeric pairs. Depending on the copper(II) concentration, defined acetonitrile contents lead to a co-elution of the enantiomers **H1/H3** and **P2/P4**, respectively, and the elution order even reverses at higher modifier concentrations shown in Figs. 2 and 3.

Some examples are given in the literature where an exchange between two modifiers [30–33] or variations in the mobile phase pH [34] led to a reversal of the enantiomeric elution order. In one case [35], this phenomenon was observed upon altering the concentration of the organic modifier analogously to the herein described results. It is generally admitted that changes in the enantiomeric elution order demand for at least two binding sites on the CSP which can be influenced in different ways by the mobile phase composition. Indeed, the columns described in literature are protein-based [31,33,34] or cellulose-derived CSPs [30,32,35]. These macromolecules comprise complex structures able to form diverse interactions with analytes. Therefore, such unusual retention behaviour is not surprising. The penicillamine molecule, however, has a simple structure. Different ''binding sites'' are only conceivable if penicillamine forms bidentate copper(II) complexes via its *N*/*O*-, *N*/*S*- and *O*/*S*-atoms. The stability of these three possible complex formations seems to be affected in different ways by the acetonitrile content. Additional investigations, especially thermodynamic studies, are in progress in order to better understand these observations.

Furthermore, acetonitrile as organic modifier has a beneficial effect on the resolution of the *trans*-configured enantiomers of AMCH **M2** and **M4** ($R_S > 3.5$) which are only partially resolved with methanol or 2-propanol as mobile phase modifiers.

Since the interactions between the column and the

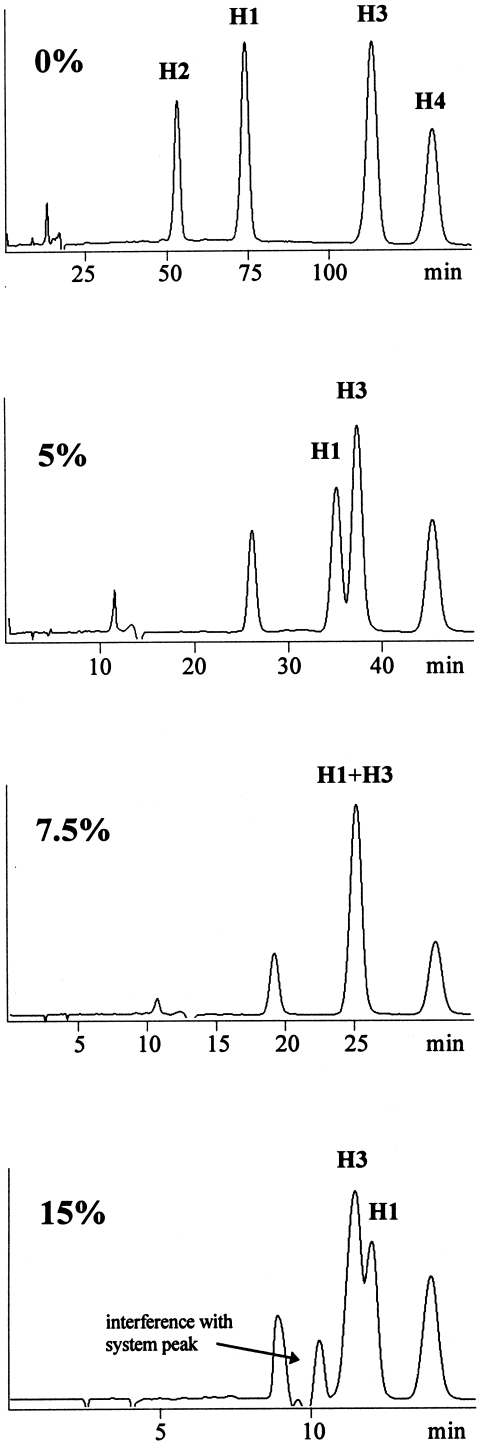


Fig. 2. Chromatograms of the four stereomers of *AHCH* on the copper(II)-D-penicillamine column with a 1 m*M* $CuSO_4$ mobile phase containing the indicated contents of acetonitrile at 20°C, 0.8 ml/min, UV detection λ 254 nm.

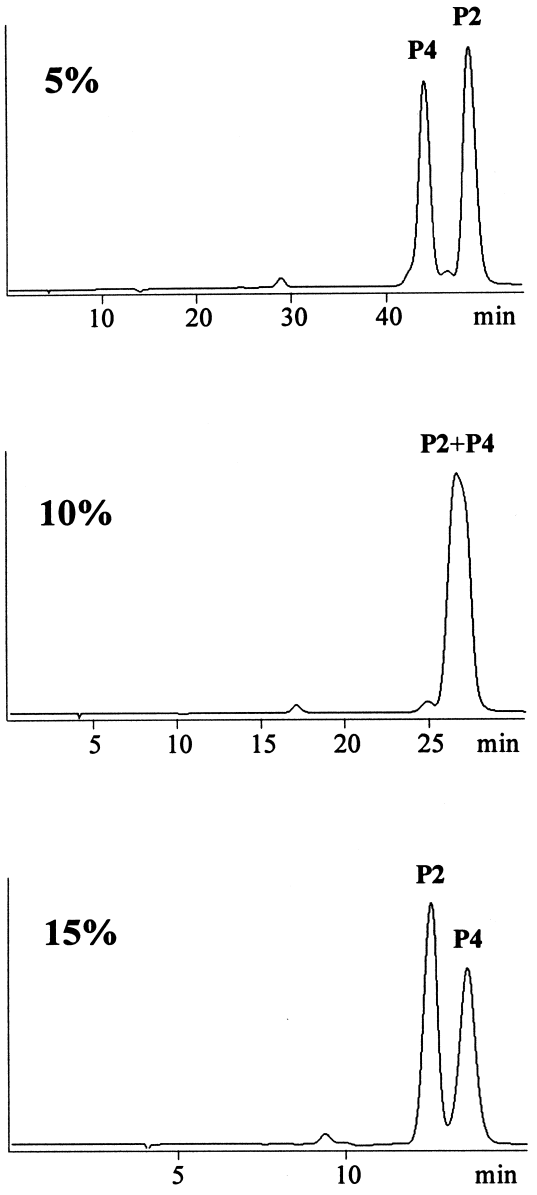


Fig. 3. Chromatograms of the *trans*-configured enantiomers **P2** and **P4** of AMCP on the copper(II)-D-penicillamine column with a 1 m*M* $CuSO_4$ mobile phase containing the indicated contents of acetonitrile at 20°C, 0.8 ml/min, UV detection λ 254 nm.

analytes comprise complex formation with a metal cation, the charges of the chelating functionalities are an important parameter. These charges can be controlled by the mobile phase pH. Indeed, by decreasing the pH from 6.2 to 4.5 a weaker retention of the

analytes is observed which can be explained by the increased protonation of the amino and the carboxylate functions resulting in a lower affinity to the metal cation. The separation factors, however, are hardly influenced by the pH on the analogy of the copper(II) and the methanol concentrations.

3.2. Optimised conditions

The aim of our work is to develop methods which allow the separation of all four stereoisomers of one amino acid in a single chromatographic run. The best chromatograms are shown in Fig. 4.

The separation of the AHCH stereomers is possible under various conditions. Best results are obtained with a 1 m*M* $CuSO_4$ in a 2-propanol–water (2.5:97.5, v/v) mobile phase at 20°C (Fig. 4A). The minimal resolution, the lowest value of resolution of any two compounds, is 3.45. This value largely exceeds the earlier obtained resolution of 1.88 on the teicoplanin CSP [14].

The AMCH stereomers, however, are more difficult to resolve. With a 1 m*M* $CuSO_4$ in an acetonitrile–water (5:95, v/v) mobile phase at 20°C a minimal resolution of 1.73 can be achieved (Fig. 4B), which is much lower than earlier results on the teicoplanin column (R_S: 2.65) [14]. Furthermore, the very long retention times are a drawback of this method.

The four stereoisomers of AMCP can not be baseline separated, since the diastereoisomers **P3** and **P4** are only partially resolved. A typical chromatogram is shown in Fig. 4C; it is noteworthy that the two *cis*-configured amino acids (**P1** and **P3**) have been injected in minor concentrations.

As described in the last chapter, the resolution of

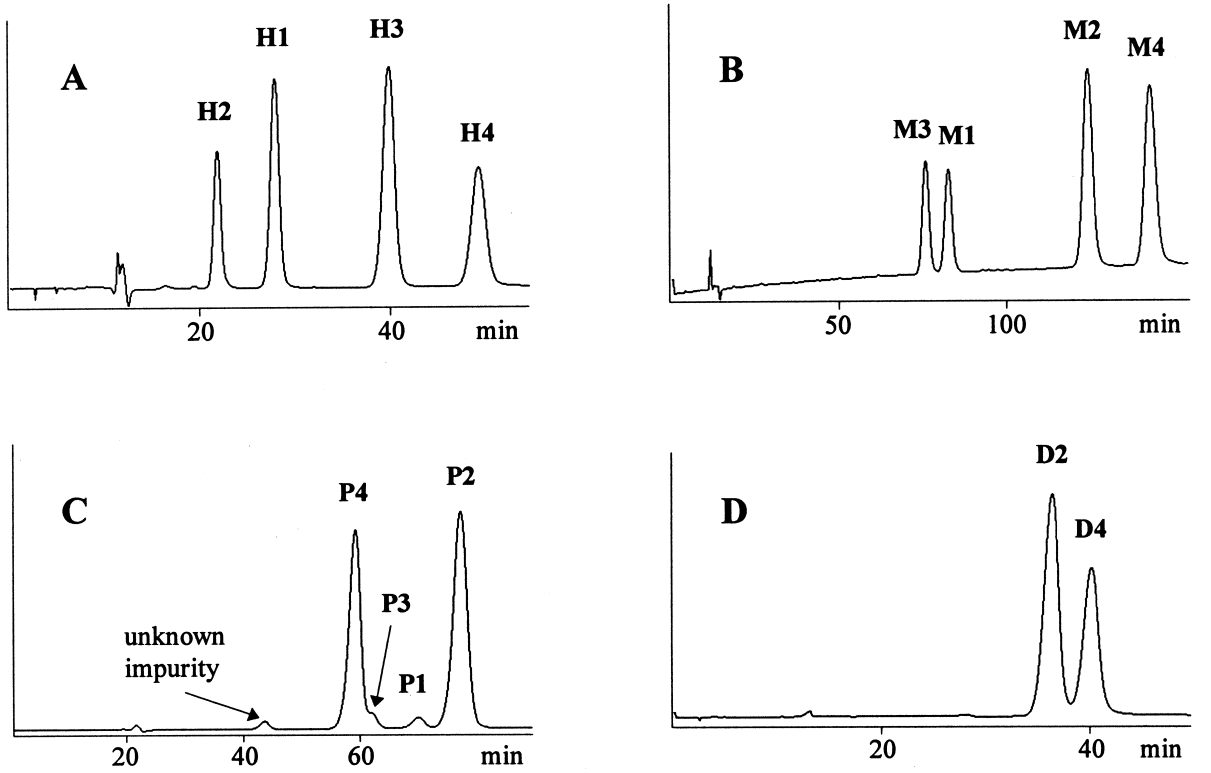


Fig. 4. Chromatograms of the four amino acid series (structures see Fig. 1) under optimised conditions on the copper(II)-D-penicillamine column, UV detection λ 254 nm: (A) AHCH: 1 m*M* $CuSO_4$ in 2-propanol–water (2.5:97.5, v/v) at 20°C, 0.8 ml/min, (B) AMCH: 1 m*M* $CuSO_4$ in acetonitrile–water (5:95, v/v) at 20°C, 0.8 ml/min, (C) AMCP: 0.3 m*M* $CuSO_4$ in methanol–water (15:85, v/v) at 20°C, 0.8 ml/min, (D) DACH: 0.3 m*M* $CuSO_4$ in 100% water at 20°C, 0.8 ml/min.

the *trans*-configured DACH can be improved by decreasing either the copper(II) concentration or the methanol content. By combination of these effects, a baseline separation (R_S: 1.4) is obtained with 0.3 m*M* $CuSO_4$ in 100% water at 20°C (Fig. 4D). Variations of the temperature do not further enhance the resolution.

3.3. Elution order of the amino acids

The D-configured amino acids are usually more retained on the copper(II)-D-penicillamine column than their L-enantiomers except for histidine [22], which is in accord with the general retention and separation mechanism of chiral ligand-exchange chromatography postulated by Davankov et al. [24]. The elution orders of the herein discussed amino acid enantiomers are listed in Table 2. On the assumption that the configuration at C-1 determines the retention behaviour, these amino acids show no regularity. The *cis*-configured compounds have mostly the normal elution order (*S* in front of *R*), whereas the *trans*-configured amino acids are preferentially eluted in the opposite order. AHCH and DACH can act as tridentate ligands and consequently untypical elution profiles are conceivable. No general rule about the retention behaviour of amino acid enantiomers can be deduced from these results.

The elution patterns (as shown in Fig. 4) of the four amino acid series differ largely despite their structural similarities. Comparing the methyl-substituted five- and six-membered rings (AMCP and AMCH, panel C and B), the two enantiomeric pairs of AMCH are well separated from each other whereas the *cis*-configured enantiomers of AMCP elute in-between the *trans* enantiomeric pair. The formal substitution of the methyl group by a hydroxyl function in the cyclohexane series (AMCH and AHCH, panel B and A) leads to a tremendous improvement in the separation of both the *cis*- and *trans*-configured enantiomeric pairs. The *trans* enantiomers are that highly resolved that they flank the *cis* enantiomeric pair. The two amino acid series with polar substituents (AHCH and DACH, panel A and D) differ largely with respect to the resolution of the *trans* enantiomeric pairs (AHCH: $R_S>12$, DACH: $R_S=1.4$). As a result, small changes in the molecular structure lead to completely different elution patterns of the amino acid stereoisomers on the copper(II)-D-penicillamine CSP, presumably because of different complex formation with its tridentate ligand moiety.

In an ongoing study, we are elucidating the chiral discrimination mechanism focusing on the importance of hydrophobic interactions between the analytes and the CSP, the results of which will be published in a forthcoming paper.

Table 2
Enantiomeric elution order of the investigated amino acids

		First	Second
AMCH	*cis*	(1*S*,2*R*)	(1*R*,2*S*)
	trans	(1*R*,2*R*)	(1*S*,2*S*)
AHCH	*cis*	(1*R*,2*S*)	(1*S*,2*R*)[a]
		(1*S*,2*R*)	(1*R*,2*S*)[b]
	trans	(1*R*,2*R*)	(1*S*,2*S*)
AMCP	*cis*	(1*S*,2*R*)	(1*R*,2*S*)
	trans	(1*S*,2*S*)	(1*R*,2*R*)[a]
		(1*R*,2*R*)	(1*S*,2*S*)[b]
DACH	*trans*	(1*R*,2*R*)	(1*S*,2*S*)

[a] with methanol, 2-propanol or acetonitrile (concentration<5%, depending on the copper(II) concentration) as organic modifier.
[b] with acetonitrile (concentration>10%) as organic modifier.

4. Conclusions

The copper(II)-D-penicillamine CSP is capable of separating the enantiomers and the diastereomers of carbocyclic, β-substituted α-amino acids with the exception of two diastereoisomers in the AMCP series. The obtained high resolutions enable the determination of *ee* and *de* values. The chromatographic parameters copper(II) concentration, pH and alcoholic modifier content have a strong influence on the retention times, but hardly affect the separation factors.

When using acetonitrile as organic modifier, its content is crucial for chiral discrimination governing even the elution order of two enantiomeric amino acid pairs (**H1/H3** and **P2/P4**). This scarcely observed phenomenon can only be explained by the multifunctionality of penicillamine in connection with the three and two functional groups of the

amino acids AHCH and AMCP, respectively, which leads to different combinations of chelating ligands for complex formation.

Concerning the elution order of the investigated amino acid enantiomers, no regularity can be derived. Therefore, it is not possible to conclude the absolute configuration of an amino acid from its retention behaviour on this column.

Acknowledgements

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