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Short communication

Direct high-performance liquid chromatographic enantioseparation of apolar β-amino acids on a quinine-derived chiral anion-exchanger stationary phase

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Abstract

A quinine-derived chiral anion-exchanger stationary phase was applied for the direct high-performance liquid chromatographic separation of the enantiomers of *N*-protected unusual β-amino acids, i.e. 3-aminobutanoic acid, 3-aminopentanoic acid, 3-amino-4-methylpentanoic acid, 3-amino-4,4-dimethylpentanoic acid, 3-amino-4-methylhexanoic acid, 3-amino-4-ethylhexanoic acid, 3-amino-3-cyclohexylpropanoic acid, 3-amino-3-(3-cyclohexen-1-yl)propanoic acid and 3-amino-3-phenylpropanoic acid. The readily prepared *N*-2,4-dinitrophenyl derivatives were well separable, with good efficiency and high resolution. The chromatographic conditions (eluent composition, pH and buffer concentration) were varied to achieve optimal separation. In some cases, the elution sequences of the enantiomers of the derivatives were determined.

Keywords: Enantiomer separation; Chiral stationary phases, LC; Amino acids

1. Introduction

The past decade has seen growing interest in β-amino acids [1] which are relevant intermediates for the synthesis of compounds of pharmaceutical potential and are important constituents of natural products such as alkaloids, peptides and β-lactam antibiotics [2,3]. β-Amino acids can be used as building blocks for the preparation of modified (unnatural) analogs of biologically active peptides. Oligomers of β-amino acids (β-peptides) fold into compact helices in solution [4–6]. As a result of the wide-ranging utility of these compounds, much attention has been paid to their enantioselective synthesis [1,2,7], which requires analytical methods for control of the enantiopurity of the final products.

High-performance liquid chromatography (HPLC) is one of the most useful techniques for the recognition and/or separation of stereoisomers, including enantiomers. A number of review articles and books deal with the methods and results of the direct enantioseparation of various compounds on chiral stationary phases (CSPs) and many attempts have been made to interpret how these CSPs operate with respect to molecular recognition [8,9]. Of the many CSPs described, those involving cinchona alkaloid derivatives, and in particular quinine carbamates, immobilized on porous silica have been successfully applied as CSPs with an anion-exchange character.

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The racemic acids that have been resolved range from chiral aryl-, aryloxy- and arylthiocarboxylic acids to *N*-derivatized amino acids and many other chiral acids, including sulfonic, phosphonic and phosphoric acids [10–13].

The HPLC enantioseparation of β-amino acids may involve either direct or indirect methods. Winnacker et al. [14] separated the diastereomeric dipeptide pair of L-glutamil-D,L-β-amino-*n*-butyric acid on a C_{18} column. Davankov et al. [15] reported a preparative ligand-exchange chromatographic (LEC) method using proline analogues (L-Pro, L-hydroxyproline, L-allo-hydroxyproline, L-azetidincarboxylic acid)+Cu^{2+} as chiral selector. Lindner and Hirshbock [16], applying (*R,R*)-tartaric acid mono-*n*-octylamide (TAMOA)+Cu^{2+} or Ni^{2+} system, found that the enantioseparation of β-amino acids by means of LEC was difficult. On a π-complex-type column, containing *N*-acetylated α-arylalkylamines as chiral selector, Griffith et al. [17] separated aliphatic β-amino acids which were derivatized as *N*-3,5-dinitrobenzoyl alkyl esters prior to the separation. Yamazaki et al. [18] analyzed three underivatized β-amino acids on octadecylsilanized silica coated with *N*-*n*-dodecyl-L-hydroxyproline+Cu^{2+} in mobile phase. Yamada [19] used a tripeptide-oxysuccinimide (*Z*-L-Val-Aib-Gly-ONSu) as CDA in reversed-phase mode. Péter et al. applied different CDAs [20–22], while D'Acquarica et al. [23] and Péter et al. [24] separated different alicyclic and cyclic β-amino acids on a new type of CSP, containing a macrocyclic glycopeptide antibiotic (teicoplanin) as chiral selector.

In the present paper, a direct HPLC method is described for the enantioseparation of racemic β-substituted-β-amino acids (β-substituted-β-alanines, Fig. 1). This HPLC method relies on the use of a unique quinine-derived weak anion-exchange (WAX) CSP (Fig. 2). The amino acids analyzed were used in *N*-2,4-dinitrophenyl (*N*-2,4-DNP)- or *N*-3,5-dinitrobenzoyl (*N*-3,5-DNB)-derivatized form. The effects of pH, mobile phase additives (buffer and organic modifier) and temperature on the separations were investigated and optimized. In some cases, the sequence of elution of the enantiomers was determined by the co-chromatography of racemic analytes with enantiomers with known absolute configurations.

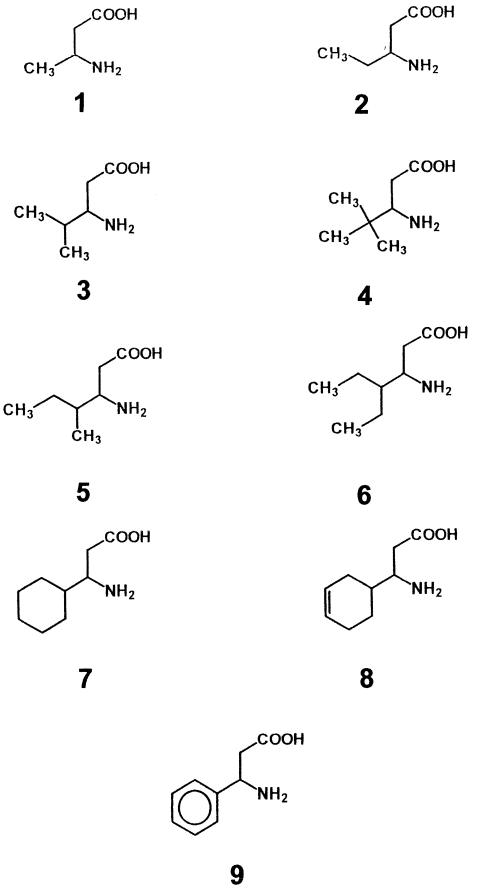
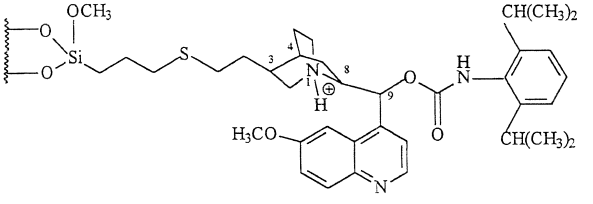


Fig. 1. Structures of β-amino acids investigated. 3-Aminobutanoic acid (**1**); 3-aminopentanoic acid (**2**); 3-amino-4-methylpentanoic acid (**3**); 3-amino-4,4-dimethylpentanoic acid (**4**); 3-amino-4-methylhexanoic acid (**5**); 3-amino-4-ethylhexanoic acid (**6**); 3-amino-3-cyclohexylpropanoic acid (**7**); 3-amino-3-(3-cyclohexen-1-yl)propanoic acid (**8**); and 3-amino-3-phenylpropanoic acid (**9**).



(1*S*,3*R*,4*S*,8*S*,9*R*)

Fig. 2. Structure of the quinine-derived chiral stationary phase.

2. Experimental

2.1. Apparatus

The HPLC measurements were carried out on two Waters systems. One of them consisted of an M-600 low-pressure gradient pump, an M-996 photodiode-array detector and a Millenium^{32} Chromatography Manager data system; the Waters Breeze system consisted of a 1525 binary pump, a 487 dual channel absorbance detector, a 717 plus autosampler and Breeze data manager software (both systems from Waters Chromatography, Milford, MA, USA). Both chromatographic systems were equipped with Rheodyne Model 7125 injectors (Cotati, CA, USA) with 20-μl loops.

The quinine-derived WAX CSP column was a generous gift from Professor W. Lindner (University of Vienna) and the dimensions of the column were 150×4.0 mm I.D. 5-μm particle size. The column was thermostated with a water-bath thermostat MK 70 (Mechanik Prüfgeräte, Medlingen, Germany). The column temperature was usually set at 40±0.1 °C.

The starting operating conditions were as follows: the mobile phase consisted of aqueous NaH_2PO_4 buffer or aqueous NH_4OAc buffer, which was mixed with the organic modifier, i.e. MeOH or MeCN. After the mixing of the aqueous and organic phases, the pH of the mobile phase was re-adjusted with phosphoric acid (phosphate buffer) or acetic acid (acetate buffer) to apparent pH_a values between 4.5 and 6.5. The concentration of the buffer was also varied. The pH was measured with an OP 208/1 precision pH-meter (Radelkis, Budapest, Hungary).

The flow-rate was 0.8 ml min^{-1}. The derivatized β-amino acids were detected at 360 nm (*N*-2,4-DNP) or 250 nm (*N*-3,5-DNB).

2.2. Chemicals and reagents

Racemic 3-aminobutanoic acid (**1**) and 3-aminopentanoic acid (**2**) were prepared from the corresponding α,β-unsaturated acids by benzylamine addition and subsequent debenzylation of the products with a 20% palladium hydroxide on carbon catalyst in a hydrogen atmosphere [25] (the structures of investigated amino acids are depicted in Fig. 1). (*R*)-3-Aminobutanoic acid was prepared by the same method, but (*R*)-(+)-α-methylbenzylamine was used in the addition step instead of benzylamine [26]. The other racemic β-amino acids, 3-amino-4-methylpentanoic acid (**3**), 3-amino-4,4-dimethylpentanoic acid (**4**), 3-amino-4-methylhexanoic acid (**5**), 3-amino-4-ethylhexanoic acid (**6**), 3-amino-3-cyclohexylpropanoic acid (**7**), 3-amino-3-(3-cyclohexen-1-yl)-propanoic acid (**8**) and 3-amino-3-phenylpropanoic acid (**9**), were synthesized from the corresponding aldehydes by a modified Rodionov procedure [27]: the aldehydes were condensed with an equimolar amount of malonic acid in refluxing 96% ethanol in the presence of two equivalents of ammonium acetate [28]. 3-Amino-4-methylhexanoic acid (**5**) and 3-amino-3-(3-cyclohexen-1-yl)propanoic acid (**8**) possess two chiral centers, and thus two diastereomers (two pairs of enantiomers, *S,S* and *R,R* or *S,R* and *R,S*) are possible. Their synthesis led to mixtures of the two diastereomers. The hydrochlorides of (*S*)-3-amino-3-cyclohexylpropanoic acid (**7**) and (*S*)-3-amino-3-phenylpropanoic acid (**9**) were prepared by acidic hydrolysis of the corresponding ethyl (*S*)-3-propanoylamino-3-cyclohexylpropanoate [25] or ethyl (*S*)-3-amino-3-phenylpropanoate [25,29]. Hydrolyses were carried out in 10% hydrochloric acid by heating under reflux for 3 h.

The reagents for derivatization, 2,4-dinitrofluorobenzene (2,4-DNFB, Sanger's reagent) and 3,5-dinitrobenzoyl chloride (3,5-DNB-Cl) were purchased from Aldrich (Steinheim, Germany).

Acetonitrile (MeCN) and methanol (MeOH) of HPLC grade were purchased from Merck (Darmstadt, Germany). Sodium dihydrogenphosphate (NaH_2PO_4), sodium carbonate, ammonium acetate (NH_4OAc), acetic acid, phosphoric acid, triethylamine (TEA) and other reagents of analytical grade were also from Merck. The Milli-Q water was further purified by filtering it on a 0.45-μm filter, type HV, Millipore (Molsheim, France).

2.3. Sample preparation and derivatization procedure

Stock solutions of amino acids (1 mg ml^{-1}) were prepared by dissolving them in water or buffers. *N*-2,4-DNP derivatives were prepared by common methods [12]. For the preparation of *N*-3,5-DNB

derivatives, 10 μl TEA and 20 μl 3,5-DNB-Cl (12% in MeCN (w/v)) were added to 1 ml of an aqueous stock solution of the amino acid. The mixture was agitated at room temperature for 10 min. Before injection, reaction mixtures were generally acidified by addition of a few μl of 2 *M* HCl, diluted 5- to 10-fold with the mobile phase and filtered on a 0.45-μm Millipore filter.

3. Results and discussion

3.1. Optimization of enantioseparation

The β-amino acids were analyzed mainly as their *N*-2,4-DNP derivatives. The chromogenic label of DNP derivatives provides a much better detection sensitivity and an additional π-acidic aromatic site, which may interact favorably with the complementary π-basic quinoline group of the chiral selector, thereby supporting the overall chiral recognition mechanism. In this context, it should be mentioned that the hydrolyzate of Sanger's reagent (2,4-dinitrophenol) in a few cases partially coeluted with the analytes; this is indicated in Table 1.

The experimental conditions, such as the pH of the mobile phase, the buffer type and concentration, the organic modifier type and the temperature, were first optimized using *N*-2,4-DNP-protected racemic 3-amino-4-methylhexanoic acid (**5**) and 3-amino-4,4-dimethylpentanoic acid (**4**) as model compounds. The main results are summarized in Table 1 and Fig. 3. For *N*-2,4-DNP-protected racemic 3-amino-4-methylhexanoic acid (**5**), a decrease in the pH_a of the 0.05 *M* NH_4OAc buffer/MeOH=35:65 (v/v) system from pH_a 6.5 to 5.0 considerably increased the retention factor, while the selectivity and the resolution of the enantiomers increased to a slight extent (Fig. 3). The results relating to the pH dependence supported the proposed separation mechanism [10–13]. The retention and separation of all the acidic analytes on the quinine-derived CSP are primarily based on an anion-exchange retention mechanism, as reflected by the decrease in the retention time on increase in the ionic strength (data not shown). In the pH_a range 5.0–6.5, the chiral selector is largely positively charged (the tertiary amine of the quinuclidine ring is protonated) and the carboxy groups of the amino acids are negatively charged, leading to optimal coulombic attraction. The pK_a values for the carboxy group of β-amino acids do not differ significantly from those for the corresponding α-amino acids [30]. In addition to the long-range and non-directed coulombic attractions, the enantioseparation is facilitated by the controlled intermolecular interaction between the analyte and the chiral selector, via hydrogen-bonding, hydrophobic, π–π and steric interactions, thereby supporting or triggering the overall stereodiscrimination process [10].

The buffer concentration influences the retention on the basis of the underlying ion-exchange principles. In the case of *N*-2,4-DNP-3-amino-4-methylhexanoic acid (**5**), increase in the acetate buffer concentration decreased the retention, whereas the selectivity did not change significantly. A plot of ln *k* versus buffer concentration exhibited a linear decrease with increasing buffer concentration. The resolution changed by up to 15% (R_S increased from 6.69 to 6.81 by changing buffer concentration from 0.01 to 0.1 *M*) and the best value (R_S 7.37) was obtained at a buffer concentration of 0.025 *M*, though this value did not differ significantly from that observed at 0.05 *M* buffer content. As a result of the limited solubility of the buffer in the aqueous–organic mobile phase with high organic modifier content, a buffer concentration of 50 m*M* or less is recommended.

As concerns the type of organic modifier, for the *N*-2,4-DNP derivatives, MeOH led to a higher resolution and a much better peak shape than those achieved with MeCN (the results obtained with MeCN are not presented in Table 1). Application of phosphate buffer instead of acetate buffer at the same concentration and buffer/MeOH ratio resulted in a shorter retention time, whereas the selectivity and R_S did not differ significantly.

The organic modifier content of the mobile phase strongly influences retention. For *N*-2,4-DNP-3-amino-4,4-dimethylpentanoic acid (**4**) in aqueous phosphate buffer/MeOH mobile phase system at constant ionic strength, keeping the total NaH_2PO_4 buffer concentration at 0.01 *M* in the final volume, the ln *k* linearly increased with decreasing MeOH concentration. R_S values also increased with decreasing MeOH content, but this was not accompanied by

Table 1
Chromatographic data, retention factors (k), separation factors (α) and resolutions (R_S) of *N*-2,4-DNP derivatives of β-amino acids

Compound	Eluent composition NaH_2PO_4/MeOH (v/v)	k_1	k_2	α	R_S
1	2:98	5.50	7.71	1.40	4.64
	5:95	5.76	8.16	1.42	4.64
	15:85	6.52	8.48	1.39	4.60
	25:75	10.94	14.86[c](15.84)	1.35	3.66
2	2:98	4.75	7.57	1.59	6.00
	5:95	4.97	8.10	1.63	5.90
	15:85	6.67	10.53[a](9.79)	1.58	5.65
	25:75	11.80	19.23	1.63	6.18
3	2:98	4.11	8.20	1.99	7.27
	5:95	4.37	9.03	2.07	9.64
	15:85	6.26	12.69	2.03	9.08
	25:75	11.76	24.19	2.06	8.24
4	2:98	3.36	9.05	2.69	11.78
	5:95	3.57	10.05[c](9.21)	2.81	11.80
	15:85	5.21	14.44	2.77	12.44
	25:75	10.98	30.05	2.63	14.01
5	5:95[a]	4.42	9.31	2.10	7.25
	5:95[b]	4.42	10.79	2.44	8.02
	15:85[a]	6.47	13.47	2.08	10.22
	15:85[b]	6.58	14.26	2.16	8.60
	NH_4OAc/MeOH				
	15:85[a]	8.58	18.51	2.15	9.0
6	2:98	4.03	8.22	2.04	7.73
	5:95	4.37	9.23	2.11	8.30
	15:85	6.80	14.14	2.08	8.87
	25:75	13.91	29.42	2.11	9.00
7	2:98	5.16	11.11	1.96	8.50
	5:95	5.88	11.48[c](9.21)	1.95	8.72
	15:85	9.11[c](9.79)	17.97	1.97	9.33
	25:75	20.26	38.32	1.90	8.12
8	2:98	5.33	10.42[c](8.21)	1.96	8.03
	5:95	5.92	11.57	1.95	7.88
	15:85	9.78	17.58	1.80	7.73
	25:75	19.52	37.61	1.93	7.83
9	2:98	6.74	11.32	1.68	6.38
	5:95	8.96	15.31	1.71	6.34
	15:85	11.69	19.80	1.69	6.59
	25:75	22.44	38.15	1.70	6.62

Chromatographic conditions: column, quinine-derived CSP; flow-rate, 0.8 ml min^{-1}; detection, 360 nm; temperature 40 °C; mobile phase, 0.05 *M* NaH_2PO_4/MeOH or 0.05 *M* NH_4OAc/MeOH; pH_a of mobile phase, pH_a 5.5; k_1, retention factor of first-eluting enantiomer; k_2, retention factor of second-eluting enantiomer.

[a] Chromatographic data on first pair of diastereomers.

[b] Chromatographic data on second pair of diastereomers.

[c] Partial coelution with reagent hydrolyzate (in parenthesis: retention factor of 2,4-dinitrophenol); $t_M = 1.9$ min.

significant increases in α. As concerns the change in k for other analytes, similar tendencies were obtained (Table 1). An increase in R_S with decreasing MeOH content did not always occur: for analytes **1, 2, 3, 7** and **8** lower R_S values were observed at lower MeOH concentration than at higher MeOH content (Table 1). To increase the speed of analysis from a practical point of view, a mobile phase with higher MeOH content is recommended.

To shorten the analysis time and also to sharpen

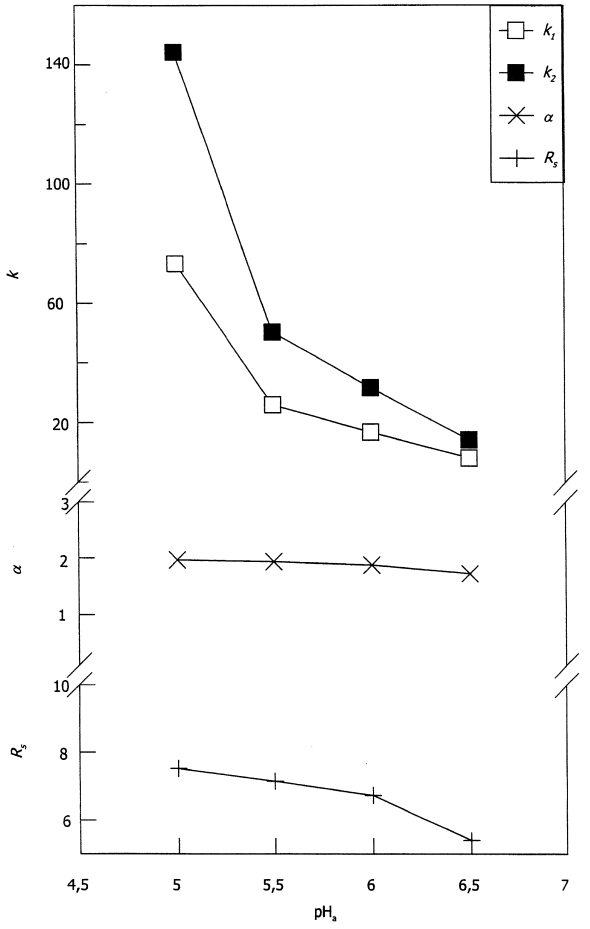


Fig. 3. Influence of pH on retention factor (k), selectivity factor (α) and resolution (R_S) of *N*-2,4-dinitrophenyl-3-amino-4-methylhexanoic acid (**5**) on quinine-derived chiral stationary phase. Chromatographic conditions: mobile phase, 0.05 *M* $NH_4OAc/MeOH = 35:65$ (v/v); pH adjusted to the respective value by the addition acetic acid; flow-rate, 0.8 ml min^{-1}; detection, 360 nm; temperature, 40 °C; retention factor, □ k_1, first-eluting stereoisomer; ■ k_2, second-eluting stereoisomer; × separation factor, $\alpha = k_2/k_1$; + resolution, R_S.

the peaks, the column temperature was raised from ambient to 40 °C, although the enantioselectivity decreases on elevation of the temperature (data not shown). The working conditions optimized for the enantioseparation of *N*-2,4-DNP-3-amino-4-methylhexanoic and *N*-2,4-DNP-3-amino-4,4-dimethylpentanoic acid in this way were used for the other analytes, with slight modifications of pH and eluent composition in order to obtain the best resolution within a reasonable analysis time.

3.2. Separation of enantiomers of N-2,4-DNP-β-amino acids

Data on the enantioseparations of *N*-2,4-DNP-β-amino acids are given in Table 1. The experimental findings reveal that the retentions of the different analytes on this CSP depend on the hydrophobicity, the bulkiness and the aromaticity of the molecules for the given coulombic attraction mechanism, which is considered to be the primary interaction. At the same eluent composition and pH$_a$ (0.05 *M* $NaH_2PO_4/MeOH = 15:85$, pH$_a$ 5.5), the least hydrophobic *N*-2,4-DNP-3-aminobutanoic acid (**1**) displayed the smallest retention, while the most hydrophobic *N*-2,4-DNP-3-amino-3-cyclohexylpropanoic acid (**7**) had one of the largest (Table 1).

Table 1 reveals that the retention factors (especially for the second-eluting stereoisomer) increased with increasing number of carbon atoms, i.e. with increasing hydrophobicity (analytes **1–7**). This phenomenon lends support to the view that, besides coulombic attraction, the hydrophobic interaction is one of the driving forces in the retention. All these analytes had aliphatic side-chains in position 3, and with increasing carbon number, not only the hydrophobicity, but also the bulkiness and the steric effect changed. The enantiomers of the least hydrophobic analytes **1** and **2** exhibited the lowest separation factors and resolutions, while for analytes **3–7**, the α-values were around 2 and the R_S values were around 8–9 (except for analyte **4**). The very similar α or R_S values obtained for solutes **3** and **5–7** demonstrated that the increasing hydrophobicity contributed to the retention, but not to the chiral discrimination. Analyte **4** excelled among the compounds investigated having the highest α and R_S values. The more symmetric, bulky *t*-butyl group in analyte **4** is probably a favorable structure for steric interaction with the stationary phase and resulted in the highest α and R_S.

In analytes **7–9,** the number of carbon atoms was the same, which resulted in almost the same retention factors. The slightly higher k-value for

analyte **9** can be attributed to the π–π interactions between the analyte and the stationary phase, but this interaction was the same for the two enantiomers. Therefore, the α and R_S values did not improve relative to analytes **7** and **8.**

The retention behavior of the molecules as a function of MeOH content confirmed the importance of the hydrophobic interactions in the retention. The increase in the retention factors with decrease in the MeOH content was much higher for those analytes which exhibited higher hydrophobicity (i.e. a higher carbon number on the β-carbon of amino acid **1–7**) or increased aromaticity (Table 1). This behavior is typical for a retention mechanism based on a hydrophobic interaction between the solute and the stationary phase.

In some cases, the sequence of elution of the enantiomers was identified by co-chromatography of racemic mixtures with an enantiomer with known configuration. The elution sequence for *N*-2,4-DNP derivatives of β-amino acids on the quinine-derived CSP seems to follow a general rule. For **1**, the elution sequence was $S < R$; for analytes **3, 7** and **9**, it was $R < S$. According to the Cahn–Ingold–Prelog rule, the steric arrangement of the substituents around the stereogenic center is the same for (*S*)-**1,** (*R*)-**3,** (*R*)-**7** and (*R*)-**9**.

Selected chromatograms relating to the separation of the stereoisomers of *N*-2,4-DNP-β-amino acids are depicted in Fig. 4.

3.3. Diastereoselectivity of the column

The optimization of the separation of the enantiomers of *N*-2,4-DNP-3-amino-4-methylhexanoic acid (**5**) was discussed above. However, the separation of all four enantiomers (two pairs of diastereomers) in one chromatographic run appeared to be difficult. The same holds true for the separation of 3-amino-3-(3-cyclohexen-1-yl)propanoic acid (**8**), which also contains two chiral centers.

In an earlier investigation, different types of protecting groups were found to contribute to different degrees to the enantioselectivity and retention under constant mobile phase conditions [13]. The introduction of an aryl group, and especially a π-acidic 3,5-dinitroaryl group, resulted in pronounced increases in enantioselectivity; the α-values were highest for the *N*-3,5-DNB-derivatives. The separation of *N*-3,5-DNB-3-amino-4-methylhexanoic acid (**5**) exhibited higher enantioselectivity, similarly as observed for α-amino acids, and the diastereoselectivity also increased. Fig. 5A depicts the separation of four stereoisomers of *N*-3,5-DNB-3-amino-4-methylhexanoic acid (**5**) in phosphate buffer and as it is seen almost baseline separation could be achieved for the first eluting diastereomers (**a–c**), while the second eluting diastereomers (**b–d**) separated well as *N*-3,5-DNB-derivatives.

For 3-amino-3-(3-cyclohexen-1-yl)propanoic acid (**8**), the *N*-3,5-DNB derivatives displayed poorer diastereoselectivity than that for the *N*-2,4-DNP derivatives (data not shown). Fig. 5B shows that for the *N*-2,4-DNP-3-amino-3-(3-cyclohexen-1-yl)propanoic acid (**8**) the first eluting diastereomers (**a–c**) underwent a poor resolution, whereas the second eluting diastereomers (**b–d**) were almost baseline separated. The enantioresolution was very high for both derivatives.

4. Conclusions

The direct analyses of unusual apolar β-amino acids have clearly demonstrated that the quinine-derived CSP used here permits the separation of enantiomeric pairs of β-substituted β-amino acids. In addition to the long-range and non-directed coulombic attraction, *N*-2,4-DNP substitution in amino acids ensures controlled intermolecular interactions between the analyte and the chiral selector, via hydrogen-bonding, π–π, steric, hydrophobic, etc. interactions, thereby supporting or triggering the overall stereodiscrimination process. The separations were carried out with high selectivity and resolution, and the method was therefore suitable for monitoring of the enantiomeric excess after chiral synthesis or enzymatic resolution.

A more sensitive point seems to be the limited diastereoselectivity of this CSP for the amino acid derivatives with two chiral centers. In other words, the diastereo- and enantioselectivity of this type of CSP must be assessed separately, as with all other chromatographic systems.

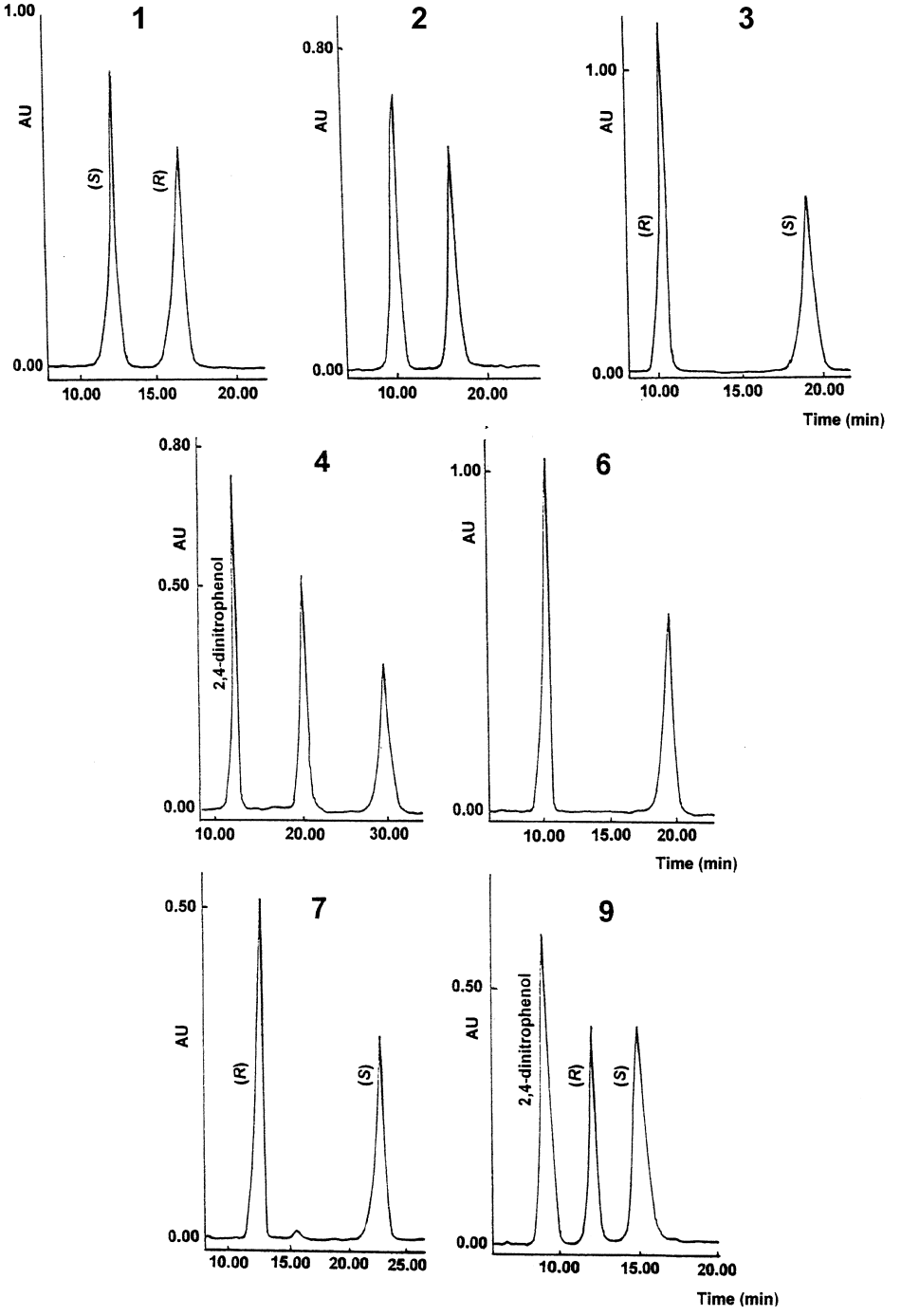


Fig. 4. Selected chromatograms of investigated amino acids as *N*-2,4-DNP derivatives. **1**, 3-Aminobutanoic acid; **2**, 3-aminopentanoic acid; **3**, 3-amino-4-methylpentanoic acid; **4,** 3-amino-4,4-dimethylpentanoic acid; **6**, 3-amino-4-ethylhexanoic acid; **7**, 3-amino-3-cyclohexylpropanoic acid and **9**, 3-amino-3-phenylpropanoic acid. Conditions of analysis: column, quinine-derived chiral stationary phase; flow-rate, 0.8 ml min^{-1}; detection, 360 nm; temperature, 40 °C; mobile phase, **1**, **2**, **7** and **9**, 0.05 *M* NaH_2PO_4/MeOH = 2:98 (v/v), pH_a 5.5; **3** and **6**, 0.05 *M* NaH_2PO_4/MeOH = 5:95 (v/v), pH_a 5.5; **4,** 0.025 *M* NaH_2PO_4/MeOH = 15:85 (v/v), pH_a 5.5; (*S*) or (*R*) denotes the peak configuration.

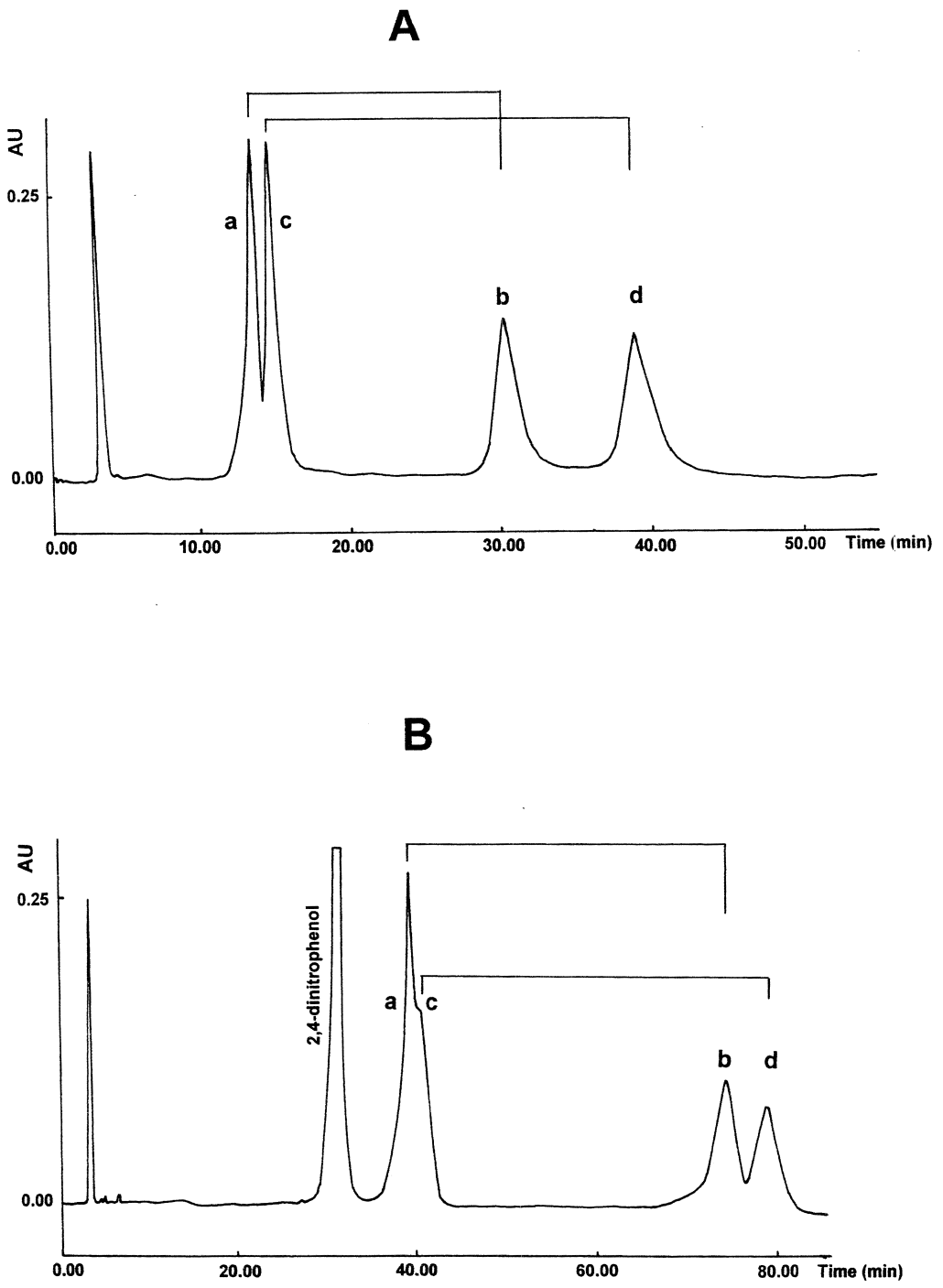


Fig. 5. Chromatograms of *N*-3,5-DNB-3-amino-4-methylhexanoic acid (**5**) and *N*-2,4-DNP-3-amino-3-(3-cyclohexen-1-yl)propanoic acid (**8**). Conditions of analysis: column, quinine-derived chiral stationary phase; flow-rate, 0.8 ml min^{-1}; detection, **A**, 250 and **B**, 360 nm; temperature, 40 °C; mobile phase, **A**, 0.025 *M* NaH_2PO_4/MeOH = 30:70 (v/v), pH_a 4.5; **B**, 0.05 *M* NaH_2PO_4/MeOH = 30:70 (v/v), pH_a 5.5; peaks, **a** and **b**, first pair of stereoisomers; **c** and **d**, second pair of stereoisomers.

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