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High-performance liquid chromatographic enantioseparation of β-amino acids

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Abstract

Direct and indirect high-performance liquid chromatographic methods were developed for the enantioseparation of β-amino acids (β-substituted-β-alanines). Direct separation involved the application of chiral columns: Crownpak CR(+), Chirobiotic T and Chirobiotic R. Indirect separation was based on precolumn derivatization with 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate or *N*-α-(2,4-dinitro-5-fluorophenyl)-L-alanineamide (Marfey's reagent), with subsequent separation on an achiral column. The chromatographic conditions were varied to achieve optimum separation.

Keywords: Enantiomer separation; β-Amino acids

1. Introduction

β-Amino acids have increased in importance in the past decade [1,2]. They are important constituents of many natural products, and their free forms and derivatives exhibit interesting pharmacological effects [3–10]. β-Amino acids can be used as building blocks for the preparation of modified (unnatural) analogues of biologically active peptides. Oligomers of β-amino acids (β-peptides) fold into compact helices in solution [11–16]. In consequence of the wide-ranging utility of these compounds, much attention has been paid to their enantioselective synthesis [1,2,8,17], which requires analytical methods to control the enantiopurity of the final products.

For these purposes, chromatographic separations are widely used. Successful high-performance liquid chromatographic (HPLC) methods for the enantioresolutions include indirect and direct methods. The indirect methods involve precolumn derivatization with chiral derivatizing agents (CDAs), with subsequent separation of the diastereoisomers on an achiral column [18,19]. Direct methods are performed by ligand-exchange chromatography (LEC) [20,21], or by the application of chiral stationary phases (CSPs) [22,23].

The HPLC enantioseparation of β-amino acids started in the 1970s. Winnacker et al. [24] separated dipeptide diastereomers containing β-amino acids. Davankov et al. [25] reported a preparative LEC method, while Lindner and Hirshbock [26] found

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that the enantioseparation of β-amino acids by means of LEC was difficult. On a π-complex type column, Griffith et al. [27] separated aliphatic β-amino acids which were derivatized as *N*-3,5-dinitrobenzoyl alkyl esters prior to the separation. Yamazaki et al. [28] analysed three underivatized β-amino acids by LEC. Yamada et al. [29] used a tripeptide-oxysuccinimide as CDA. D'Acquarica et al. [30] separated different alicyclic and cyclic β-amino acids on a new type of CSP, containing a macrocyclic glycopeptide antibiotic as chiral selector.

In the present paper, direct and indirect HPLC methods are described for the enantioseparation of racemic β-substituted-β-amino acids (β-substituted-β-alanines). Direct methods were carried out by applying different CSPs: Crownpak CR(+), Chirobiotic T and Chirobiotic R columns. The Chirobiotic columns, containing macrocyclic antibiotics as chiral selector, were used in reversed-phase mode (RPM) or in a newly developed polar-organic mode (POM) [31–33]. The effects of the mobile-phase composition, temperature and flow-rate on the separation were investigated, and the conditions affording the best resolution were determined. The indirect chiral separations were performed on a C_{18} stationary phase working in the RPM, after precolumn derivatization with CDAs: 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) or *N*-α-(2,4-dinitro-5-fluorophenyl)-L-alanineamide (Marfey's reagent, FDAA).

2. Experimental

2.1. Apparatus

The HPLC system consisted of an L-6000 liquid chromatographic pump (Merck–Hitachi, Tokyo, Japan) equipped with an SPD-6AV variable-wavelength UV detector (Shimadzu, Tokyo, Japan) and an HP3395 integrator (Hewlett-Packard, Waldbronn, Germany). Some experiments were carried out on a Waters system, which consisted of an M-600 low-pressure gradient pump, an M-996 photodiode-array detector and a Millenium[32] Chromatography Manager data system (Waters Chromatography, Milford, MA, USA). The chromatographic systems were equipped with Rheodyne Model 7125 injectors (Cotati, CA, USA) with 20-μl loops.

The columns used for analytical separations were Vydac 218TP54 C_{18} (250×4.6 mm I.D.), 5 μm particle size (The Separations Group, Hesperia, CA, USA), Crownpak CR(+) CSP, covalently bonded with chiral crown ether (150×4 mm I.D.), 5 μm particle size (Daicel Chemical Industries, Tokyo, Japan); Chirobiotic T CSP, bonded with teicoplanin; and Chirobiotic R CSP, bonded with ristocetin A, both 250×4.6 mm I.D., 5 μm particle size (Astec, Whippany, NJ, USA). Generally, the analyses were carried out at 20°C, with detection 205 nm. Any other conditions are indicated.

The columns were thermostated in a water-bath. The temperature was regulated and controlled by a type MK-70 heating–cooling circulator system (Mechanik Prüfgeräte, Medlingen, Germany). The accuracy of temperature adjustment was ±1°C.

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 (Fig. 1) [34,35]. (*R*)-3-Aminobutanoic acid was prepared by this method, but (*R*)-(+)-α-methylbenzylamine was used in the addition step instead of benzylamine [36]. The other racemic β-amino acids, 3-amino-4-methylpentanoic acid (**3**), 3-amino-4,4-dimethylpentanoic acid (**4**), 3-amino-4-ethylhexanoic acid (**5**), 3-amino-3-cyclohexylpropanoic acid (**6**), 3-amino-3-(3-cyclohexen-1-yl)propanoic acid (**7**) and 3-amino-3-phenylpropanoic acid (**8**), were synthesized from the corresponding aldehydes by a modified Rodionov procedure [37]: the aldehydes were condensed with an equimolar amount of malonic acid in refluxing 96% ethanol in the presence of two equivalents of ammonium acetate [38,39] (Fig. 1). The hydrochlorides of (*S*)-3-amino-3-cyclohexylpropanoic acid and (*S*)-3-amino-3-phenylpropanoic acid were prepared by acidic hydrolysis of the corresponding ethyl (*S*)-3-propanoylamino-3-cyclohexylpropanoate [35] or ethyl (*S*)-3-amino-3-phenylpropanoate [40]. Hydro-

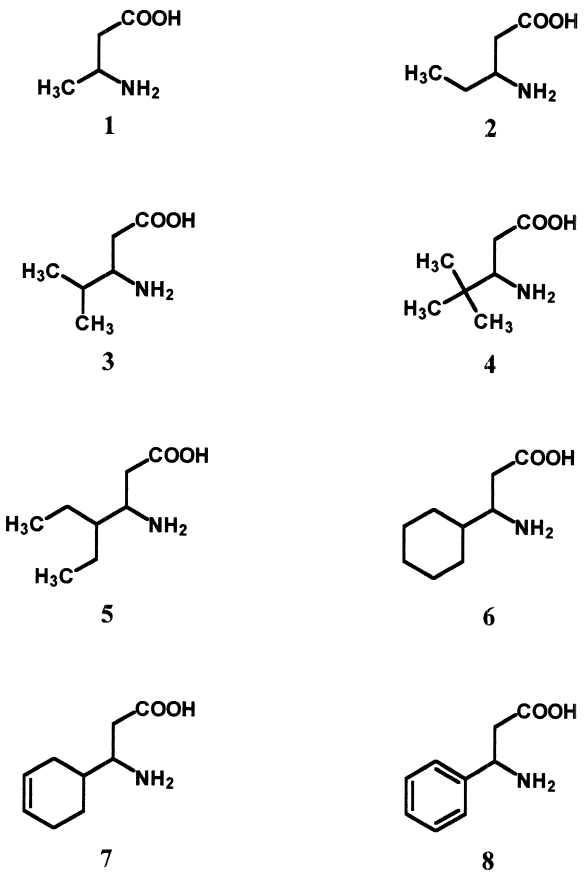

Fig. 1. Structures of β-amino acids investigated. 3-Aminobutanoic acid (**1**); 3-aminopentanoic acid (**2**); 3-amino-4-methylpentanoic acid (**3**); 3-amino-4,4-dimethylpentanoic acid (**4**); 3-amino-4-ethylhexanoic acid (**5**); 3-amino-3-cyclohexylpropanoic acid (**6**); 3-amino-3-(3-cyclohexene-1-yl)propanoic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

lyses were carried out in 10% hydrochloric acid by heating under reflux for 3 h.

FDAA was purchased from Sigma (St. Louis, MO, USA) and GITC from Aldrich (Steinheim, Germany). HPLC-grade acetonitrile (MeCN), methanol (MeOH) and ethanol (EtOH) were from Merck (Darmstadt, Germany). Triethylamine (TEA), glacial acetic acid (HOAc), perchloric acid, trifluoroacetic acid (TFA) and other reagents of analytical-reagent 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

For the direct separation, 1–10 m*M* solutions of the investigated β-amino acids were made in 0.01 *M* perchloric acid or in the mobile phase and injected after filtration on a 0.45-μm Millipore filter.

For the derivatization with FDAA by the modified method of Marfey [41,42], and with GITC by the method of Nimura et al. [43], 1 mg ml^{-1} solutions of the β-amino acids were used.

3. Results and discussion

3.1. Direct HPLC separation

In the first experiments for the enantioseparation of β-amino acids, a chiral crown ether-based column, Crownpak CR(+), was applied. This column can resolve compounds bearing a primary amino group near the chiral centre. Chiral recognition is achieved when a complex is formed between the ammonium ion derived from the analyte and the crown ether stationary phase. The isomers could be separated by influencing the stability of the complex formed through variation of the pH and the temperature.

The results relating to the separation of β-amino acid enantiomers on the Crownpak CR(+) column are to be seen in Table 1. The experiments were carried out at 20°C with perchloric acid, pH 2.0, as eluent, at a flow-rate of 0.5 ml min^{-1}. Under these conditions, the stereoisomers remained unresolved (data not shown). A decrease in the flow-rate led to partial separation of the analytes at 20°C. A decrease in the temperature of the separation on the crown ether column has been reported to result in improved enantioselectivity [44,45]. Table 1 reveals that a decrease in temperature with a parallel decrease of the flow-rate in some cases increased the enantioselectivity and resolution. R_S increased from 1.25 to 1.60 (**1**), from 1.15 to 1.88 (**2**), from 0 to 0.4 (**5**) and from 0.4 to 1.52 (**8**) upon decreasing the temperature from 20 to 5°C.

Decreasing the pH may influence the enantioseparation of amino acids through stronger complex formation under acidic conditions [44,46]. However, when the pH of the mobile phase is reduced from 2.0

Table 1
Chromatographic data, retention factors (k), separation factors (α) and resolutions (R_S) obtained on Crownpak CR(+) column

Compound	k_1	k_2	α	R_S	Temp. (°C)
1	0.33	0.46 (*R*)[b]	1.39	1.25	20
	0.62	0.92 (*R*)[b]	1.48	1.60	5
2	0.54	0.67	1.24	1.15	20
	0.83	1.08	1.30	1.88	5
3	1.26	1.26	100	0.00	5
4	2.60	2.60	1.00	0.00	5
5	10.22	10.52	1.03	<0.40	5
6	5.74 (*S*)[a]	6.17	1.07	<0.40	5
7	10.82	10.82	1.00	0.00	5
8	3.78(*S*)[a]	4.08	1.08	0.40	20
	7.98 (*S*)[a]	9.14	1.15	1.52	5

Chromatographic conditions: column, Crownpack CR(+); mobile phase, aqueous perchloric acid, pH 2.0; flow-rate, 0.25 ml min^{-1}; detection, 205 nm; void volume, 1.38 ml.
[a] Configuration of the first-eluting enantiomer.
[b] Configuration of the second-eluting enantiomer.

to 1.5 or 1.0, the separation of the enantiomers of **3–8** does not improve appreciably (data not shown).

The Crownpak CR(+) column effectively separated the enantiomers of **1**, **2** and **8**; and a partial separation was obtained for **5** and **6**. It seemed that, when the substituents on the β-carbon were more aliphatic in nature, the column produced decreased separation efficiency. The good resolution of analyte **8** could be due to the π–π interactions between the phenyl ring of the analyte and the binaphthyl ring of the selector or another explanation may be the better accessibility of the amino group for inclusion into the cavity of crown ether since the amino group and the aromatic ring do not lay in the same plane.

The literature data demonstrate that, with a Crownpak CR(+) column, the (+)-*R* enantiomers elute before the (−)-*S* enantiomers for α-amino acids [44]. For analytes **1**, **6** and **8**, the opposite elution sequence was observed: $S<R$. However, the same letters for **1**, **6** and **8** do not necessarily mean the same enantiomer: the steric arrangement of the substituents around the stereocentre is different for **1** from that for **6** or **8** (the C.I.P. priority sequence of the substituents for **1** changes with the structure of the β-substituent). This means that the general rule for the elution sequence observed for α-amino acids ($R<S$) on the Crownpak CR(+) column is not valid for β-amino acids.

The two other stationary phases applied for direct enantioseparation contained chiral antibiotics as chiral selectors: teicoplanin (Chirobiotic T column) and ristocetin A (Chirobiotic R column). The teicoplanin molecule has several characteristic features that make it suitable for amino acid analysis [47,48].

On the teicoplanin-containing stationary phase, the synthetic amino acids were analysed by working in the RPM or the POM. All the analytes were chromatographed and detected without pre- or post-column derivatization. The retention and selectivity can be controlled by altering the concentration and nature of the organic modifier, but variation of the temperature or flow-rate sometimes has a beneficial effect on the resolution. In the RPM, EtOH and MeOH were applied as organic modifiers, and change of the water–organic modifier ratio affected the retention. Increase of the water content in the mobile phase led to a decrease in the retention factor (k). It is unusual in RP-HPLC for a water-rich mobile phase to

produce a shorter retention time, but it is usual for a teicoplanin-containing stationary phase [47,48]. For α-amino acids, the more hydrophobic the solute, the more typical the reversed-phase retention behavior observed [47–49]. Here, with increasing hydrophobicity, i.e., with increase of the carbon number on the β-carbon of amino acid **1–6**, a decrease in the retention factor was observed (Table 2). At a mobile phase composition water–MeOH (10:90, v/v) and at 20°C, the retention factor decreased with a parallel increase in the selectivity factor and resolution (except **5**), and almost baseline resolutions were obtained for the enantiomers of analytes **3**, **4** and **6**. A further increase of the MeOH content of the mobile phase improved the resolution of the enantiomers of **5** to a great extent; for the other analytes, the improvements in α and R_S were not too great. A decrease in the temperature from 20 to 5°C improved the resolution to a slight extent.

The application of EtOH instead of MeOH in the mobile phase in the enantioseparation of α-amino acids led to an improved separation efficiency [50]. In the case of β-amino acids at a mobile phase composition water–EtOH (40:60, v/v) and at 5°C, almost baseline resolution was obtained for analytes **3**, **4** and **6**. For the other analytes, variation of the mobile phase composition did not improve the resolution.

For α-amino acids, the recently developed POM sometimes proved more efficient than the normal-phase mode or RPM [50]. At 20°C and at a mobile-phase composition MeOH–AcOH–TEA (100:0.4:0.1, v/v/v), the POM in all cases was less effective than the RPM, and variation of the buffer concentration

Table 2
Chromatographic data, retention factors (k), separation factors (α) and resolutions (R_S) obtained on Chirobiotic T column

Compound	Eluent composition: water–MeOH (v/v) or water–EtOH (v/v)	k_1	k_2	α	R_S	Method; temp. (°C)
1	10:90	2.51 (*R*)[a]	2.66	1.06	<0.40	RPM 1; 20
	10:90	3.15 (*R*)[a]	3.38	1.07	0.63	RPM 1; 5
	5:95	10.07 (*R*)[a]	10.76	1.07	0.58	RPM 1; 20
2	10:90	2.10	2.36	1.12	1.03	RPM 1; 20
	5:95	2.85	3.31	1.16	1.05	RPM 1; 20
3	10:90	1.91	2.35	1.23	1.55	RPM 1; 20
	10:90	2.17	2.54	1.17	1.25	RPM 1; 10
	40:60	0.93	1.13	1.22	1.38	RPM 2; 5
4	10:90	1.44	1.72	1.19	1.47	RPM 1; 20
	5:95	1.57	1.89	1.20	1.54	RPM 1; 20
	40:60	1.02	1.38	1.35	1.46	RPM 2; 5
5	10:90	1.48	1.57	1.06	<0.40	RPM 1; 20
	0:100	3.26	3.68	1,13	1.35	RPM 1; 20
6	10:90	1.60 (*S*)[a]	1.86	1.16	1.45	RPM 1; 20
	40:60	1.11 (*S*)[a]	1.35	1.22	1.38	RPM 2; 5
7	10:90	2.28	2.40	1.05	<0.40	RPM 1; 20
	10:90	2.52	2.66	1.06	<0.40	RPM 1; 15
8	10:90	1.95 (*S*)[a]	2.07	1.06	0.83	RPM 1; 20
	10:90	2.57 (*S*)[a]	2.74	1.07	0.97	RPM 1; 5

Chromatographic conditions: column, Chirobiotic T; mobile phase, water–MeOH (RPM 1), water–EtOH (RPM 2); flow-rate, 0.40 ml min^{-1}; detection, 205 nm; void volume, 3.46 ml.

[a] Configuration of the first-eluting enantiomer.

and ratio AcOH–TEA only slightly improved the separation. In the POM the R_S values for analytes **2, 3** and **6** were about 1.0; in the other cases, they were 0.4–0.6.

The elution sequence on the teicoplanin-containing stationary phase seems to follow a general rule. For **1**, the elution sequence was $R<S$; for analytes **6** and **8**, it was $S<R$. According to the C.I.P. rule, the steric arrangement of the substituents around the stereogenic centre is the same for **1**, **6** and **8**.

The third CSP applied for the enantioseparation of β-amino acids was the Chirobiotic R column, containing ristocetin A as the chiral selector. Ristocetin A is one of the more complex molecules evaluated as a CSP selector [51]. Separation of the enantiomers of β-amino acids was carried out in two different types

Table 3
Chromatographic data, retention factors (k), separation factors (α) and resolutions (R_S) obtained on Chirobiotic R column

Compound	Eluent composition: water–MeOH (v/v) or water–MeCN (v/v) or MeOH–AcOH–TEA (v/v/v)	k_1	k_2	α	R_S	Method; temp. (°C)
1	90:10	0.24	0.24	1.00	0.00	RPM 1; 20
	95:5	0.41	0.41	1.00	0.00	RPM 3; 5
	100:0.4:0.1	2.38	2.63 (*R*)[b]	1.11	0.98	POM; 20
	100:0.4:0.1	3.47	4.03 (*R*)[b]	1.16	1.48	POM; 5
2	90:10	0.35	0.38	1.08	<0.40	RPM 1; 20
	95:5	0.58	0.64	1.10	<0.40	RPM 3; 5
	100:0.4:0.1	2.05	2.40	1.17	1.05	POM; 20
	100:0.4:0.1	2.66	3.27	1.23	1.52	POM; 5
3	90:10	0.49	0.58	1.18	0.65	RPM 1; 20
	95:5	0.93	1.14	1.23	1.38	RPM 3; 5
	100:0.4:0.1	2.51	2.74	1.09	1.00	POM; 5
4	90:10	0.83	0.99	1.19	0.84	RPM 1; 20
	95:5	1.26	1.58	1.25	1.43	RPM 3; 5
5	90:10	1.17	1.17	1.00	0.00	RPM 1; 20
	95:5	1.8	1.89	1.00	0.00	RPM 3; 5
6	90:10	1.77	1.77	1.00	0.00	RPM 1; 20
	95:5	2.21	2.21	1.00	0.00	RPM 3; 5
	100:0.4:0.1	1.27 (*S*)[a]	1.36	1.07	<0.40	POM; 20
7	90:10	1.40	1.50	1.07	<0.40	RPM 1; 20
	95:5	1.93	2.15	1.11	0.63	RPM 3; 5
	100:0.4:0.1	1.18	1.25	1.06	<0.40	POM; 20
8	90:10	1.09	1.09	1.00	0.00	RPM 1; 20
	95:5	1.48	1.48	1.00	0.00	RPM 3; 5
	100:0.4:0.1	2.02	2.36 (*S*)[b]	1.17	1.35	POM; 20
	100:0.4:0.1	2.61	3.26 (*S*)[b]	1.25	1.54	POM; 5

Chromatographic conditions: column, Chirobiotic R; mobile phase, water–MeOH (RPM 1), water–MeCN (RPM 3), MeOH–AcOH–TEA (POM); flow-rate, 0.40 ml min^{-1}; detection, 205 nm; void volume, 3.06 ml.

[a] Configuration of the first-eluting enantiomer.

[b] Configuration of the second-eluting enantiomer.

of mobile-phase systems: the RPM and the POM. Table 3 presents results obtained with both separation modes. In the RPM, MeOH or MeCN-containing mobile-phase systems were applied: water–MeOH (90:10, v/v, or 95:5, v/v) (RPM 1) and water–MeCN (95:5, v/v, or 98:2, v/v) (RPM 3); the column temperature was varied between 20 and 5°C (Table 3). The ristocetin A stationary phase behaved as a real reversed-phase. Increase of the organic modifier content decreased the retention and, with increasing hydrophobicity of the molecule for analytes **1–6**, the retention factors increased. In the RPM, ristocetin A separated the enantiomers of analytes **2–4** and **7**, but the most bulky and hydrophobic analytes **5** and **6** underwent no separation on this CSP. However, almost baseline separation was achieved for these compounds on teicoplanin CSP. One explanation of this phenomenon may be that teicoplanin has a hydrophobic side-chain, whereas ristocetin A does not, and in the latter case there is no possibility for hydrophobic interactions.

In the POM, a mobile-phase composition MeOH–AcOH–TEA (100:0.4:0.1, v/v/v) was applied in most cases and the concentrations or ratio of the buffer components (AcOH–TEA) were varied together with the column temperature. Baseline separation was achieved for the enantiomers of **1**, **2** and **8** in the POM on ristocetin A. The enantiomers of analyte **8**, which were also separable on Crownpak CR(+) but not on Chirobiotic T, which revealed a

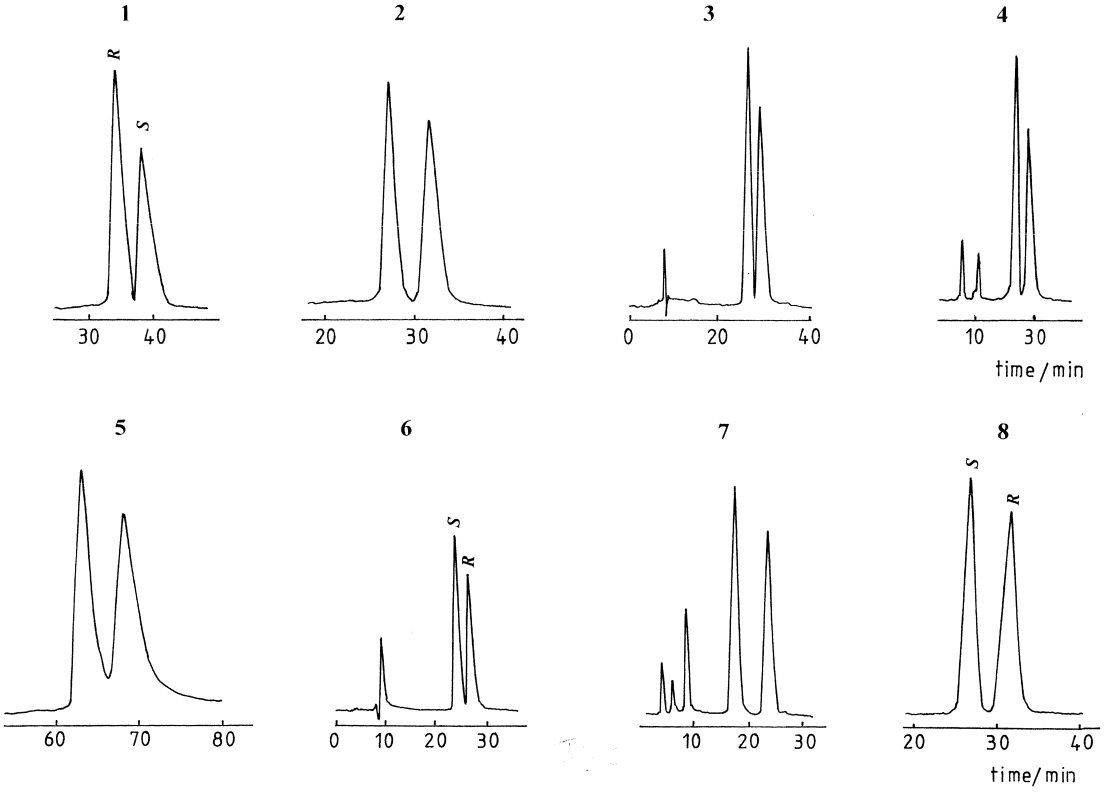

Fig. 2. Selected chromatograms of β-amino acids investigated, Column: for compounds **1**, **2**, **8**: Chirobiotic R; for compounds **3–6**: Chirobiotic T; for compound **7**: Vydac 218TP54. Flow rate: for compounds **1–4**, **6**, **8**: 0.4 ml min^{-1}; for compound **5**: 0.2 ml min^{-1}; for compound **7**: 0.8 ml min^{-1}. Detection: 205 nm (**7**: 250 nm, GITC derivative). Temperature: for compounds **1**, **2**, **4**, **5**, **8**: 5°C; for compounds **3**, **6**: 15°C; for compound **7**: 25°C. Mobile phase: for compounds **1**, **2**, **8**: MeOH–HOAc–TEA (100:0.4:0.1, v/v/v); for compounds **3**, **6**: water–MeOH (10:90, v/v); for compound **4**: water–EtOH (40:60, v/v); for compound **5**: water–MeOH (5:95, v/v); for compound **7**: 0.1% aqueous TFA–MeOH (50:50, v/v); *R* denotes the configuration of the peak *R*; *S* denotes the configuration of the peak *S*.

baseline separation in the POM. As far as the elution sequence, no general rule was observed, e.g., analytes **6** and **8**, which have the same arrangement of substituents around the stereogenic centre exhibited opposite elution sequences (Table 3).

Selected chromatograms characteristic of the enantioseparations are depicted in Fig. 2.

3.2. Indirect HPLC separations

The enantiomers were separated as GITC or FDAA derivatives; some of the results are presented in Table 4. The mobile phase included 0.1% aqueous TFA and MeOH as organic modifier. Since diastereomers formed with CDA contain an ionizable carboxy group, 0.1% aqueous TFA was applied in the mobile phase to keep the ionization at a constant level along the column, and to ensure the reproducibility of retention. The organic modifier was MeOH; this seemed more efficient than MeCN (data not shown). The optimum mobile phase contained around 45–50% (v/v) MeOH. At these mobile phase compositions, the retention factors for both derivatives (GITC and FDAA) increased with increasing hydrophobicity of the molecules and a parallel increase in the separation factor and resolution was

Table 4
Chromatographic data, retention factors (k), separation factors (α) and resolutions (R_S) obtained by indirect method

Compound	Eluent composition TFA–MeOH (v/v)	k_1	k_2	α	R_S	Method
1	50:50	0.65	0.65	1.00	0.00	GITC
	55:45	1.10	1.27 (*R*)[a]	1.15	<0.40	GITC
	50:50	1.20	1.81 (*R*)[a]	1.51	4.25	FDAA
2	50:50	0.94	1.16	1.23	0.75	GITC
	55:45	1.70	2.23	1.32	1.47	GITC
	50:50	1.83	3.51	1.92	9.85	FDAA
3	50:50	1.45	1.99	1.37	2.02	GITC
	55:45	2.68	3.99	1.49	2.30	GITC
	50:50	2.74	6.05	2.21	14.16	FDAA
4	50:50	1.88	2.73	1.45	2.14	GITC
	55:45	3.73	5.83	1.55	4.29	GITC
	50:50	5.13	12.65	2.46	9.54	FDAA
5	50:50	4.71	6.92	1.47	3.04	GITC
	55:45	10.53	17.75	1.69	5.86	GITC
	50:50	5.34	11.84	2.21	16.17	FDAA
6	50:50	5.95	8.63 (*S*)[a]	1.45	3.14	GITC
	55:45	13.10	21.25 (*S*)[a]	1.62	7.14	GITC
	50:50	11.90	29.00 (*S*)[a]	2.43	12.76	FDAA
7	50:50	4.01	5.72	1.43	2.25	GITC
	55:45	8.66	13.13	1.52	3.56	GITC
	50:50	6.93	17.64	2.54	11.38	FDAA
8	50:50	1.62	2.10 (*S*)[a]	1.30	1.77	GITC
	55:45	3.47	4.75 (*S*)[a]	1.37	2.40	GITC
	50:50	2.21	6.15 (*S*)[a]	2.78	17.14	FDAA

Chromatographic conditions: column, Vydac 218TP54; mobile phase, 0.1% aqueous TFA–MeOH; flow-rate, 0.80 ml min^{-1}; detection, 250 nm (GITC), 340 nm (FDAA); void volume, 2.80 ml.

[a] Configuration of the second-eluting diastereomer. Method: GITC, derivatization with GITC; FDAA, derivatization with Marfey's reagent.

observed. The diastereomers of analyte **1** were not separable as GITC derivatives, but all the other analytes displayed baseline separations. To compare the two CDAs, FDAA seemed more efficient than GITC in the separation of the β-amino acid stereoisomers. At the same eluent composition, TFA–MeOH (50:50, v/v), the *k* values for the GITC and FDAA derivatives exhibited the following tendencies: for the FDAA derivatives, the *k* values for the first-eluting and the second-eluting components were 2 and 3 times higher, respectively, than those for the GITC derivatives. This difference resulted in much higher α and R_S values for the FDAA derivatives. The stereoisomers of analyte **7**, which were not separable by direct methods, could be separated well after precolumn derivatization (Fig. 2).

The sequence of elution of the investigated analytes with both CDAs seemed to follow a general rule. Analytes **6** and **8** exhibited the opposite elution sequence ($R<S$) to that for analyte **1** ($S<R$), in accordance with the real steric arrangement of the substituents around the stereogenic centre.

4. Conclusions

Direct and indirect HPLC methods have been developed and optimized for the enantioseparation of β-substituted-β-amino acids (β-substituted-β-alanines). Of three CSPs, Crownpak CR(+) proved efficient in the separation of the enantiomers of analytes **1**, **2** and **8**, Chirobiotic T separated the stereoisomers of **2–6** and **8**, while Chirobiotic R ensured baseline separation for the stereoisomers of **1**, **2**, **4** and **8**. The chirobiotic phases, and especially Chirobiotic T, revealed a good separation capability for most of the β-amino acids investigated.

In indirect separation, of the two CDAs, Marfey's reagent seemed to be more efficient than GITC in the enantioseparation of these β-amino acids.

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