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Direct and indirect high-performance liquid chromatographic enantioseparation of β-amino acids

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Abstract

Direct and indirect reversed-phase (RP) high-performance liquid chromatographic methods were developed for the separation of enantiomers of 18 unnatural β-amino acids, including several β-3-homo amino acids. The direct separations of the underivatized analytes were performed on chiral stationary phases (CSPs) containing macrocyclic glycopeptide antibiotic teicoplanin (Chirobiotic T column) and teicoplanin aglycone (Chirobiotic TAG column). The indirect method involved pre-column derivatization with a new chiral derivatizing agent (CDA), (*S*)-*N*-(4-nitrophenoxycarbonyl)phenylalanine methoxyethyl ester ((*S*)-NIFE), and subsequent separation of diastereomers on Discovery C_{18} and Hyperpep 300 C_{18} columns. The different methods were compared in systematic chromatographic examinations. The effects of organic modifier, mobile phase composition, pH and flow rate on the separation were investigated.

Keywords: Enantiomer separation; Stationary phases, LC; Derivatization, LC; Amino acids; Nitrophenoxycarbonyl phenylalanine methoxyethyl ester

1. Introduction

The past decade has seen a growing interest in β-amino acids [1], which are important intermediates for the synthesis of compounds of pharmaceutical interest [2–4] and are important constituents of natural products such as alkaloids, peptides and β-lactam antibiotics [5,6]. β-Amino acids can be used as building blocks for peptidomimetics [7,8]. Oligomers of β-amino acids (β-peptides) fold into compact helices in solution [9,10]. Recently, a novel class of β-peptide analogues adopting predictable and reproducible folding patterns was evaluated as a potential source of new drugs and catalysts [11].

Studies on synthetic or natural β-amino acids can be facilitated by versatile and robust methods for determining the enantiomeric purity of starting materials and products. High-performance liquid chromatography (HPLC) is one of the most useful techniques for the recognition and/or separation of stereoisomers including enantiomers. The HPLC enantioseparation of β-amino acids include both direct and indirect methods. Winnacker et al. [12], Yamada et al. [13] and Péter and co-workers [14] applied different chiral derivatizing agents (CDAs). Davankov et al. [15], Lindner and Hirshbock [16] and Yamazaki et al. [17] reported ligand-exchange chromatographic (LEC) methods. Griffith et al. [18] used π-complex type column, while D'Acquarica et al. [19], Péter et al. [20], Péter [21] and Hyun et al. [22] separated different alicyclic and cyclic β-amino acids on new types of chiral stationary phases (CSPs), containing macrocyclic glycopeptide antibiotic, quinine-derived chiral anion-exchanger and crown ether as chiral selectors.

The aim of the present work was to evaluate direct and indirect HPLC methods for the separation of enantiomers of 18 unusual β-substituted-β-amino acids (β-3-homo amino acids, for structures see Tables 1 and 2). Direct separations were performed on chiral stationary phases containing macrocyclic glycopeptide antibiotics (teicoplanin or teicoplanin aglycone) as chiral selectors. For indirect separations, a new chiral derivatizing agent (*S*)-*N*-(4-nitrophenoxycarbonyl)phenylalanine methoxyethyl ester ((*S*)-NIFE) was applied (Fig. 1), which was earlier successfully applied for the separation of stereoisomers

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Table 1
Chromatographic data, retention factors (k), separation factors (α) and resolutions (R_S) of β-amino acids as (*S*)-NIFE derivatives on Discovery C_{18} and Hyperpep 300 C_{18} columns

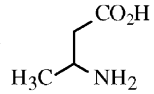
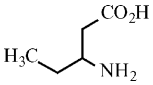
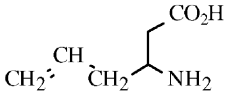
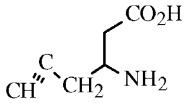
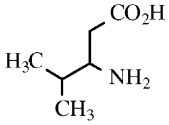
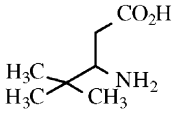
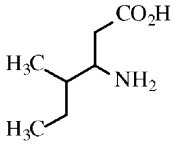
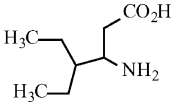
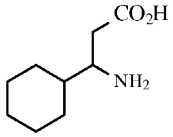
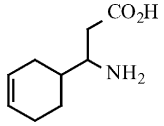
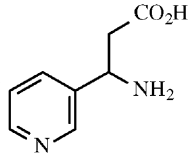
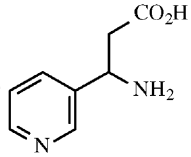
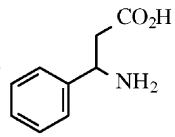
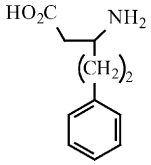
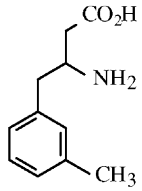
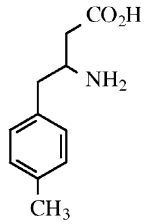
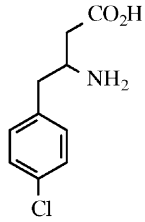
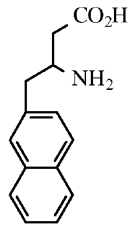
Amino acid	Column and eluent composition	k_1	k_2	α	R_S	Elution sequence
1 	a	8.17	8.44	1.03	0.80	$S < R$
	b	9.90	10.27	1.04	1.21	$S < R$
2[a] 	a	8.56	8.98	1.05	1.20	–
	b	11.29	11.78	1.04	2.00	–
3 	a	10.88	11.39	1.04	1.68	$S < R$
	b	10.55	11.04	1.04	2.59	$S < R$
4 	a	9.10	9.50	1.04	1.25	$S < R$
	b	10.08	10.55	1.05	1.35	$S < R$
5[a] 	a	10.13	10.71	1.06	1.78	–
	b	11.58	12.22	1.06	3.00	–
6[a] 	a	9.78	10.46	1.07	2.25	–
	b	12.31	13.09	1.06	3.50	–
7[a] 	a	10.06	10.63	1.06	2.00	–
	b	12.72	13.36	1.05	2.00	–
8[a] 	a	10.79	11.38	1.05	2.00	–
	b	14.00	14.32	1.05	2.86	–
9 	a	10.98	11.52	1.05	2.00	$R < S$
	b	14.08	14.69	1.04	2.57	$R < S$
10[a] 	a	10.40	10.91	1.05	2.00	–
	b	13.31	13.85	1.04	2.00	–
11 	a	7.18	7.42	1.03	0.76	$R < S$
	b	10.80	11.47	1.06	2.00	$R < S$
12 	a	6.31	7.45	1.18	1.25	$R < S$
	b	7.14	7.63	1.06	2.17	$R < S$
13 	a	10.23	10.63	1.04	1.33	$R < S$
	b	12.96	13.35	1.03	1.50	$R < S$

Table 1 (*Continued*)

Amino acid	Column and eluent composition	k_1	k_2	α	R_S	Elution sequence
14 	a	11.24	11.81	1.05	1.82	$S < R$
	b	14.77	15.48	1.06	2.80	$S < R$
15 	a	11.25	11.73	1.04	1.39	$S < R$
	b	14.55	14.91	1.03	1.50	$S < R$
16 	a	11.35	11.72	1.03	1.20	$S < R$
	b	14.69	15.02	1.02	1.30	$S < R$
17 	a	11.74	11.96	1.02	0.80	$S < R$
	b	15.13	15.44	1.02	1.35	$S < R$
18 	a	12.78	13.44	1.05	1.20	$S < R$
	b	15.78	16.14	1.02	1.66	$S < R$

Column, a, Discovery C_{18}, b, Hyperpep 300 C_{18}; flow rate, 0.8 ml min^{-1}; detection, 205 nm; mobile phase, gradient elution, a and b (see, Section 2).
[a] Elution sequence not determined.

of unusual amino acids [23,24]. By variation of the chromatographic parameters, the separation of the stereoisomers was optimized. The efficiency of the different CSPs and the role of molecular structure of derivatized analytes in the enantioseparation were noted.

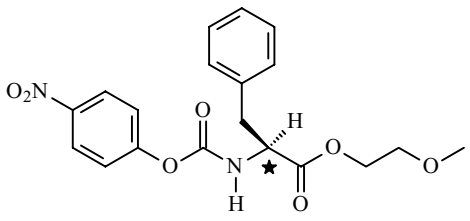


Fig. 1. Structure of chiral derivatizing agent, (*S*)-*N*-(4-nitrophenoxycarbonyl)phenylalanine methoxyethyl ester, (*S*)-NIFE.

2. Experimental

2.1. Chemicals and reagents

Racemic 3-aminobutanoic acid (**1**), (*R*)-3-aminobutanoic acid (**1**), and 3-aminopentanoic acid (**2**) were prepared from the corresponding α,β-unsaturated acids [25–27]. The other racemic β-amino acids, 3-amino-4-methylpentanoic acid (**5**), 3-amino-4,4-dimethylpentanoic acid (**6**), 3-amino-4-methylhexanoic acid (**7**), 3-amino-4-ethylhexanoic acid (**8**), 3-amino-3-cyclohexylpropanoic acid (**9**), 3-amino-3-(3-cyclohexen-1-yl)propanoic acid (**10**) and 3-amino-3-phenylpropanoic acid (**13**), were synthesized by a modified Rodionov procedure [28,29]. 3-Amino-4-methylhexanoic acid (**7**) and 3-amino-3-(3-cyclohexen-1-yl)propanoic acid (**10**) has two chiral centers, two diastereomers (two pairs of enantiomers, *S*,*S* and *R*,*R* or *S*,*R* and *R*,*S*) are possible.

Table 2
Chromatographic data, retention factors (k), separation factors (α) and resolutions (R_S) of β-amino acids on Chirobiotic T and Chirobiotic TAG columns

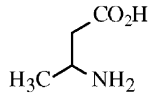
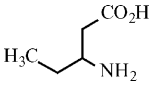
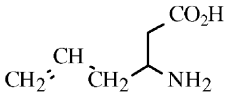
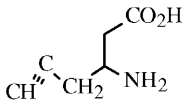
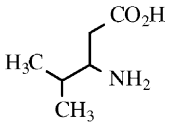
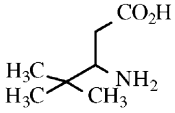
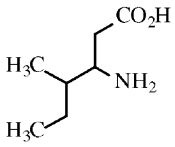
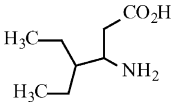
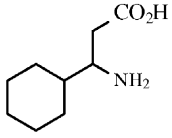
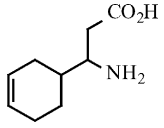
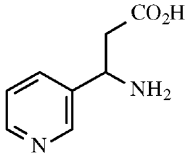
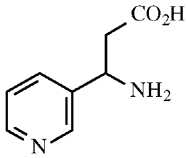
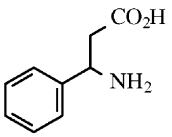
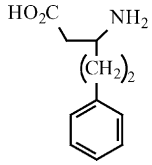
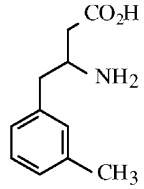
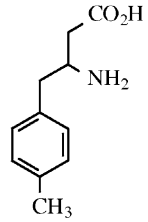
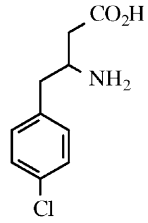
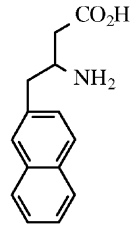
Amino acid	CSP	Mobile phase	k_1	k_2	α	R_S	Elution sequence
1 	T	10/90 b,e	2.26	2.75	1.03	0.39	*R* < *S*
	T	20/80 c,e	3.38	5.71	1.69	2.62	*R* < *S*
	TAG	d,e	4.01	4.45	1.11	0.48	*R* < *S*
2[a] 	T	10/90 b,e	2.45	2.64	1.08	0.47	–
	T	10/90 a,e	2.10	2.36	1.12	1.03	–
	TAG	d,e	2.06	3.38	1.64	1.26	–
3 	T	10/90 b,e	2.49	2.73	1.10	0.75	*R* < *S*
	T	5/95 a,e	2.29	5.46	2.46	3.95	*R* < *S*
	TAG	d,e	2.99	4.43	1.48	0.40	*R* < *S*
4 	T	10/90 b,e	2.75	3.22	1.17	0.90	*R* < *S*
	T	30/70 a,e	2.86	4.21	1.47	2.14	*R* < *S*
	TAG	0/100 a,e	8.28	9.77	1.18	1.28	*R* < *S*
5[a] 	T	10/90 b,e	1.94	2.10	1.08	0.53	–
	T	10/90 a,e	1.91	2.35	1.23	1.55	–
	TAG	d,e	1.97	2.39	1.21	0.75	–
6[a] 	T	10/90 b,e	1.66	1.86	1.12	0.57	–
	T	10/90 a,e	1.44	1.72	1.19	1.47	–
	TAG	0/100 a,e	5.77	6.17	1.06	0.40	–
7[a] 	T	10/90 b,e	1.97	1.97	1.00	0.00	–
	T	d,e	2.27	2.75	1.21	0.93	–
	TAG	d,e	2.06	2.27	1.10	0.47	–
8[a] 	T	10/90 b,e	1.87	1.92	1.03	0.22	–
	T	0/100 a,e	3.26	3.68	1.13	1.35	–
	TAG	d,e	1.90	2.11	1.11	0.71	–
9 	T	10/90 b,e	1.97	2.21	1.12	0.77	*S* < *R*
	T	10/90 a,e	4.12	4.77	1.16	1.45	*S* < *R*
	TAG	d,e	2.11	2.27	1.08	0.50	*S* < *R*
10[a] 	T	10/90 b,e	2.24	2.58	1.15	0.70	–
	T	d,e	2.66	3.29	1.24	0.97	–
	TAG	d,e	3.39	3.90	1.15	0.40	–
11 	T	10/90 b,e	6.68	6.88	1.03	0.50	*S* < *R*
	T	0/100 a,e	18.08	22.28	1.23	0.40	*S* < *R*
	TAG	0/100 a,e	26.74	31.71	1.19	1.00	*S* < *R*
12 	T	10/90 b,e	6.57	7.10	1.08	0.96	*S* < *R*
	T	0/100 a,e	16.10	22.09	1.37	1.68	*S* < *R*
	TAG	d,e	8.27	13.66	1.65	1.95	*S* < *R*
13 	T	10/90 b,e	2.48	2.87	1.16	0.82	*S* < *R*
	T	10/90 a,e	3.90	4.14	1.06	0.83	*S* < *R*
	TAG	d,e	2.99	2.99	1.00	0.00	–

Table 2 (*Continued*)

Amino acid	CSP	Mobile phase	k_1	k_2	α	R_S	Elution sequence
14 	T	10/90 b,e	2.94	2.94	1.00	0.00	–
	T	80/20 c,e	3.84	4.79	1.25	<0.40	$R < S$
	TAG	d,e	4.31	4.31	1.00	0.00	–
15 	T	10/90 b,e	2.35	2.56	1.09	1.00	$R < S$
	T	10/90 a,e	1.72	4.36	2.53	2.88	$R < S$
	TAG	0/100 a,e	7.98	9.56	1.20	1.37	$R < S$
16 	T	10/90 b,e	2.43	2.88	1.19	1.13	$R < S$
	TAG	d,e	2.97	4.19	1.41	1.03	$R < S$
17 	T	10/90 b,e	2.56	2.84	1.11	1.00	$R < S$
	T	5/95 b,e	3.25	3.56	1.09	1.06	$R < S$
	TAG	d,e	3.53	4.49	1.27	0.86	$R < S$
18 	T	10/90 b,e	2.92	2.92	1.00	0.00	–
	T	d,e	3.67	4.00	1.09	<0.40	$R < S$
	TAG	0/100 a,e	10.03	11.20	1.11	0.85	$R < S$

Column, T, Chirobiotic T, TAG, Chirobiotic TAG; mobile phase, a, H_2O/MeOH (v/v), b, 0.1% aqueous TEAA (pH 4.1)/MeOH (v/v), c, 0.1% aqueous TEAA (pH 4.1)/MeCN (v/v), d, MeOH/AcOH/TEA = 100/0.1/0.1 (v/v/v); flow rate, e, 0.4 ml min^{-1}, f, 0.5 ml min^{-1}; detection, 205 nm.

[a] Elution sequence not determined.

Their synthesis led to the mixture of two diastereomers. The hydrochlorides of (*S*)-3-amino-3-cyclohexylpropanoic acid (**9**) and (*S*)-3-amino-3-phenylpropanoic acid (**13**) were prepared by method of Gedey et al. [26].

Enantiomerically pure (*R*)- and (*S*)-3-amino-5-hexenoic acid (**3**), (*R*)- and (*S*)-3-amino-5-hexynoic acid (**4**), (*R*)- and (*S*)-3-amino-4-(3-pyridyl)butyric acid (**11**), (*R*)- and (*S*)-3-amino-4-(4-pyridyl)butyric acid (**12**), (*R*)- and (*S*)-3-amino-5-phenylpentanoic acid (**14**), (*R*)- and (*S*)-3-amino-4-(3-methylphenyl)butyric acid (**15**), (*R*)- and (*S*)-3-amino-4-(4-methylphenyl)butyric acid (**16**), (*R*)- and (*S*)-3-amino-4-(4-chlorophenyl)butyric acid (**17**), (*R*)- and (*S*)-3-amino-4-(2-naphthyl)butyric acid (**18**) were from Solvay-Peptisyntha (Brussels, Belgium).

(*S*)-NIFE was obtained from Solvay-Peptisyntha, but now is available from Fluka (Buchs, Switzerland). Acetonitrile (MeCN) and methanol (MeOH) of HPLC grade were purchased from Merck (Darmstadt, Germany). Triethylamine (TEA), glacial acetic acid (AcOH), trifluoroacetic acid (TFA) and other reagents of analytical reagent grade were also from Merck. Milli-Q water and mobile phases were further purified by filtering them on 0.45 μm filter, type HV, Millipore (Molsheim, France).

0.1% Triethylammonium acetate (TEAA) buffer was prepared by titration of 0.1% (by volume) aqueous solutions of TEA with AcOH to a suitable pH. Mobile phases for reversed-phase (RP) and polar organic (PO) chromatography were prepared by mixing the indicated volumes of buffers and/or solvents.

For gradient elution the starting mobile phases were water (A) and MeCN (B) both of which contained 0.1% TFA. The gradient elutions reported in Table 1 used mobile phases A

and B; the gradient slopes were: (a) 0% B at 0 min, increased to 100% B within 60 min (1.67% B min^{-1}); (b) 95% A + 5% B at 0 min, increased to 25% A + 75% B within 60 min (1.17% B min^{-1}).

2.2. Apparatus

The HPLC measurements were carried out on a Waters HPLC system consisting of an M-600 low-pressure gradient pump, an M-996 photodiode-array detector and a Millenium[32] Chromatography Manager data system; the alternative 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 reversed-phase stationary phases used to perform the indirect analyses were octadecyl-modified, spherical and end-capped silica-based phases. The Discovery C_{18} 250 mm × 4.6 mm i.d. column was from Sigma and had a 5 μm particle size, 180 Å pore size, 200 $m^2 g^{-1}$ surface area, and 12.5% carbon content. The corresponding data for Hyperpep 300 C_{18} 250 mm × 4.6 mm i.d. (Shandon, Rucaron, UK) were 5 μm particle size, 300 Å pore size, 80 $m^2 g^{-1}$ surface area, and 6.5% carbon content.

The columns used for direct separations were teicoplanin-containing Chirobiotic T and teicoplanin aglycone-containing Chirobiotic TAG columns, 5 μm particle size, 250 mm × 4.6 mm i.d., both from Astec (Whippany, NJ, USA). The Chirobiotic columns were thermostated in a water bath, with a cooling–heating thermostat (MK 70, Mechanik Prüfgeräte, Medlingen, Germany). The precision of temperature adjustment was ±0.1 °C.

2.3. Derivatization procedure

Derivatization of the investigated analytes with (*S*)-NIFE was performed according to a method reported in the literature [23,24]. The derivatized amino acids were detected at 205 nm.

3. Results and discussion

3.1. Indirect separation of β-amino acids as (S)-NIFE derivatives

The amino acid analytes evaluated in this study can be arranged into two classes (Tables 1 and 2): β-alanine analogs which contained alkyl- or cycloalkyl (cycloalkene) substituents in the β-position (compounds **1**–**10**), the different chain lengths of the substituents resulting in differences in hydrophobicity and bulkiness of these molecules; and amino acids with aromatic substituents in the β-position (compounds **11**–**18**), the substitution on the aromatic ring influencing the hydrophobicity, bulkiness and rigidity of the molecule.

With gradient profiles (see Section 2), on the Hyperpep 300 C_{18} column all the β-amino acid–(*S*)-NIFE stereoisomers were separated with $R_S > 1.3$, with the exception of **1**, for which only partial resolution ($R_S \sim 1.2$) was obtained. The degree of resolution on the Discovery C_{18} column was somewhat lower (Table 1). Mobile phases without TFA resulted in chromatograms with poor reproducibility of the retention times and in asymmetric fronting peaks.

Of the two stationary phases, the Discovery C_{18} column had a higher carbon content than the Hyperpep 300 C_{18} column (12.5% versus 6.5%), while the Hyperpep 300 C_{18} column had a larger average pore size (300 Å versus 180 Å). At a fixed mobile phase composition, the analyses on the Hyperpep 300 C_{18} column exhibited lower *k* values than those on the Discovery C_{18} column (data not shown). This elution behavior could be explained by the higher carbon content of the Discovery C_{18} column, which resulted in higher retention. Change of the phase ratio (i.e. the amount of hydrophilic medium) alters the retention.

The weaker interactions on the Hyperpep 300 C_{18} column were compensated by applying a lower gradient, 1.67% B min^{-1} versus 1.17% B min^{-1} (Table 1). Under these conditions, the resolution (R_S) was in most cases higher on the Hyperpep 300 C_{18} column than on the Discovery C_{18} column. These results revealed the importance of the pore size of the stationary phase. Since the pore size of the Discovery C_{18} column (180 Å) was smaller than that of the Hyperpep 300 C_{18} column (300 Å), the diffusion and mass transfer in the smaller pores were less. The relatively high surface area of the Discovery C_{18} column could also contribute to the higher retention.

Comparison of the chromatographic data for analogous compounds under the same chromatographic conditions (e.g. gradient a or b) permits observations relating to the structure–retention relationship. The isomers of 3-aminobutanoic acid (**1**) were the least retained, while those of 3-amino-3-cyclohexylpropanoic acid (**9**) eluted last, due to the difference in their hydrophobicities. The number of carbon atoms in **3**–**5** was the same, but **3** and **4** are more polar because of their unsaturation. This difference in polarity was seen in the difference in their retention factors. All of these data indicate that the hydrophobicity of the stereoisomers plays an important role in the retention.

Aromatic substituents supported the hydrophobicity retention trends. The lower retention of **10** or **13** as compared with **9** was due to the presence of a cyclohexene or phenyl ring instead of a cyclohexyl ring. For analytes **11** and **12**, the incorporation of the =N– group into the aromatic ring instead of a methylene group decreased the hydrophobicity of the molecules, and resulted in lower retention factors as compared to **13**. The methyl substitution in **15** and **16**, and the chloro substitution in **17** led to an increased hydrophobic character as compared with **13**. It seemed, that the position

of the heteroatom in the ring (**11** and **12**) or the position of the substituent on the ring (**15** and **16**) had no substantial effect on retention (however, analyte **11** exhibited a somewhat higher retention than that of **12** on the Hyperpep 300 C_{18} column). For stereoisomers with two chiral centers (**7** and **10**) only diastereomeric resolution could be achieved.

The sequence of elution of the stereoisomers was in most cases determined by analyzing stereoisomers with known configurations. The stereoisomers of **1**, **3**, **4** and **14**–**18** displayed the elution sequence $S < R$ and those of **9**, and **11**–**13** the sequence $R < S$. Taking into account the Cahn–Ingold–Prelog rule, the steric arrangement of the substituents around the stereogenic center was the same for all these analytes, i.e. a general trend for the elution sequence was observed. The sequence of elution of compounds **2**, **5**–**8** and **10** was not determined because of the lack of enantiomers.

3.2. Direct separation of β-amino acids on macrocyclic antibiotic phases

On the teicoplanin (Chirobiotic T column) and teicoplanin aglycone (Chirobiotic TAG column) stationary phases, the synthetic β-amino acids were analyzed by working in the reversed-phase or polar organic mode. The analytes were chromatographed and detected without pre- or post-column derivatization. All compounds in Table 2 were evaluated by using both stationary phases with a minimum of three different reversed-phase mobile phases, water–MeOH, 0.1% aqueous TEAA (pH 4.1)–MeOH, 0.1% aqueous TEAA (pH 4, 1)–MeCN, plus 100% MeOH as mobile phase. A polar organic mobile phase, MeOH–AcOH–TEA was also evaluated. To simplify the presentation, in Table 2 for purposes of comparison the chromatographic results obtained at 0.1% TEAA–MeOH (10:90, v/v) mobile phase composition and/or conditions giving the best resolution on both column are tabulated.

On the teicoplanin-containing stationary phase, the retention and selectivity could be controlled by altering the nature and concentration of the organic modifier, but variation of the flow rate sometimes had a beneficial effect on the resolution (data not shown). Results reveal that increase of the MeOH content led to an increase in the retention factor. This unusual behavior in the reversed-phase mode was characteristic of teicoplanin stationary phases. A possible explanation may be the decreased solubility of amino acids in MeOH-rich mobile phase [30,31]. Previously, when more hydrophobic α-amino acids were separated on a teicoplanin CSP, more typical reversed-phase retention behavior was observed [30,32]. Here, at a mobile phase composition of 0.1% TEAA–MeOH (10:90, v/v), with increasing hydrophobicity, i.e. with increasing carbon number on the β-carbon of β-amino acid **1**–**9** a small increase in separation factor and resolution was observed (**7** and **8** were exceptions). However, analyte **4**, which was most polar in this series exhibited the highest retention factor and resolution.

Aromatic substitution on the β-carbon (compounds **11**–**18**) did not result in a significant change in the retention factors of the first-eluting enantiomers (except for **11** and **12**) despite the different substituents on the aromatic ring. The resolutions for these compounds were somewhat higher than those found for alkyl-substituted **1**–**9**, except for analytes **14** and **18**. In these latter two cases, the greater distance of the aromatic ring from the stereogenic center (**14**) or the presence of a naphthyl ring (**18**) probably hindered chiral recognition. For analytes **11** and **12**, significantly higher retention factors were observed in the 0.1% TEAA–MeOH (10:90, v/v) mobile phase. This behavior could be attributed to the possibility of hydrogen bond interactions between the chiral selector and the analytes through the =N– group containing non-bonding electron pair.

To obtain higher or baseline resolution on the native teicoplanin stationary phase, the separation was optimized by: (i) changing the water–MeOH ratio of the mobile phase, (ii) application of 0.1% TEAA pH 4.1 buffer instead of water in the eluent, or (iii) application of MeCN instead of MeOH as the organic modifier. Despite the large variation in conditions, the stereoisomers of **7**, **10**, **13**, **14** and **18** exhibited only partial resolution, $R_S < 1.0$.

For α-amino acids, the teicoplanin aglycone afforded a higher separation capability than the native teicoplanin [33]. This was not valid for β-amino acids, and especially for β-3-homo amino acids. Any variations in the separation conditions resulted in poorer resolution than that obtained on the native teicoplanin CSP. The only exceptions were analytes **11** and **15**, where relatively high R_S factors were obtained.

For α-amino acids use of the polar organic mode with a MeOH–AcOH–TEA mobile phase system generally exhibited good enantioresolution for both native teicoplanin and teicoplanin aglycone stationary phases [33]. For β-3-homo amino acids, use of the polar organic mode did not result in any improvement in enantioresolution, except for analytes **7** and **12**, where better resolution was obtained.

In summary, the native teicoplanin proved more suitable for the separation of stereoisomers of β-3-homo amino acids than the teicoplanin aglycone. This behavior is opposite that observed for α-amino acids [33].

The sequence of elution was determined in most cases. Since the Chirobiotic TAG column proved to be less efficient than the Chirobiotic T column, most of the data on the elution sequence in Table 2 related to the latter column. The stereoisomers of **1**, **3**, **4** and **14**–**18** exhibited the elution sequence $R < S$, while the stereoisomers of **9** and **11**–**13** followed the sequence $S < R$. A general rule could be established for the elution of the stereoisomers of β-3-homo amino acids. Taking into account the Cahn–Ingold–Prelog rule, the steric arrangement around the stereogenic center was the same in both cases, i.e. the elution sequence did not change for these analytes.

4. Conclusions

Direct and indirect reversed-phase high-performance liquid chromatographic methods were developed for the separation of enantiomers of 18 unnatural β-amino acids, β-3-homo amino acids. Of two CSPs the native teicoplanin (Chirobiotic T) proved more suitable for the separation of the stereoisomers of β-3-homo amino acids than the teicoplanin aglycone (Chirobiotic TAG) column. The indirect method involved pre-column derivatization with a new chiral derivatizing agent, (*S*)-*N*-(4-nitrophenoxycarbonyl)phenylalanine methoxyethyl ester. The indirect separation exhibited good resolutions. In conclusion, baseline resolution was achieved for β-3-homo amino acids in at least one chromatographic system.

The elution sequence was determined in most cases and, taking into account the Cahn–Ingold–Prelog rule, a general rule was established for the sequence of elution of stereoisomers for direct and indirect methods. If the substituent on the β-carbon was a methyl group or was attached to the β-carbon through a methylene group, the elution sequence for the (*S*)-NIFE derivatives was $S < R$ in the indirect method and $R < S$ on the teicoplanin phases. These elution sequences were reversed ($R < S$ in the indirect method and $S < R$ on the teicoplanin phases) when the substituents were directly attached to the β-carbon.

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