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High-performance liquid chromatographic separation of the enantiomers of unusual α-amino acid analogues

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Abstract

The direct and indirect stereochemical resolution of the enantiomers of ring- and α-methyl-substituted phenylalanines and phenylalanine amides was attempted by high-performance liquid chromatographic methods. The direct separation was carried out on two chiral stationary phases, the crown-ether-based Crownpak CR(+), and the teicoplanin-based Chirobiotic T, while the indirect resolution was performed by applying pre-column derivatization with 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) and *N*α-(2,4-dinitro-5-fluorophenyl)-L-alanine amide (Marfey's reagent, FDAA). The Chirobiotic T column was efficient in the separation of ring- and α-methyl-substituted phenylalanine analogues, but was ineffective for the amides of these analogues. The Crownpak CR(+) column separated the ring-substituted phenylalanines and amides, whereas the α-methylated analogues were coeluted. Of the two indirect methods, GITC derivatization seemed more effective than FDAA derivatization.

Keywords: Enantiomer separation; Amino acids

1. Introduction

In the past decade, great efforts have been made to obtain receptor-selective ligands with conformationally constrained structures. The different ring- and α-substituted aromatic amino acids in chirally pure form are of interest in bioorganic and medicinal chemistry. α-Methyl amino acids have been incorporated into peptides for conformational studies because of the rigidity they provide to the peptide backbone, and their tendency to promote α-helix or β-turn formation [1–10]. In biochemistry they are believed to be competitive inhibitors of those enzymes that metabolize the corresponding α-unsubstituted proteinogenic amino acids [11].

The synthesis of unusual amino acids generally leads to racemic compounds; even though asymmetric synthetic methods have been elaborated for the synthesis of unnatural amino acids, their enantioselectivity is not 100%, and therefore enantiomerically enriched mixtures are obtained. This holds true for α,α-disubstituted amino acids [12–15]. It is necessary therefore, to know the chiral purity of the mixtures obtained.

Different ring- and α-methyl-substituted phenylalanine analogues were synthesized in racemic form and were converted to the amidated analogues (Fig. 1). Highly selective amidase biocatalysts obtained

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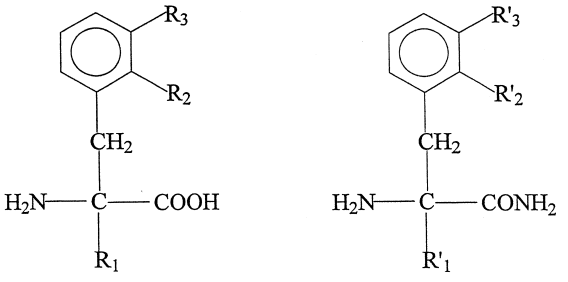

1. R_1: H R_2: H R_3: OCH_3
2. R_1: H R_2: CH_3 R_3: H
3. R_1: H R_2: CN R_3: H
4. R_1: CH_3 R_2: H R_3: OCH_3
5. R_1: CH_3 R_2: CH_3 R_3: H
6. R_1: CH_3 R_2: CN R_3: H
7. R'_1: H R'_2: H R'_3: OCH_3
8. R'_1: H R'_2: CH_3 R'_3: H
9. R'_1: H R'_2: CN R'_3: H
10. R'_1: CH_3 R'_2: H R'_3: OCH_3
11. R'_1: CH_3 R'_2: CH_3 R'_3: H
12. R'_1: CH_3 R'_2: CN R'_3: H

Fig. 1. Structures of phenylalanine analogues. **1**, 2-amino-3-(3-methoxyphenyl)propionic acid; **2**, 2-amino-3-*o*-tolylpropionic acid; **3**, 2-amino-3-(2-cyanophenyl)propionic acid; **4**, 2-amino-3-(3-methoxyphenyl)-2-methylpropionic acid; **5**, 2-amino-2-methyl-3-*o*-tolylpropionic acid; **6**, 2-amino-3-(2-cyanophenyl)-2-methylpropionic acid; **7**, 2-amino-3-(3-methoxyphenyl)propionamide; **8**, 2-amino-3-*o*-tolylpropionamide; **9**, 2-amino-3-(2-cyanophenyl)-propionamide; **10**, 2-amino-3-(3-methoxyphenyl)-2-methylpropionamide; **11**, 2-amino-2-methyl-3-*o*-tolylpropionamide; **12**, 2-amino-3-(2-cyanophenyl)-2-methylpropionamide.

from *Pseudomonas putida* or from *Ochrabactum anthopi* have previously been used to resolve racemic ring-substituted phenylalanine amides and their α-methyl-substituted analogues [16–18]. The application of these enzymes to the phenylalanine analogues reported in this paper necessitated the elaboration of analytical techniques to monitor the resolution.

This paper describes the separation and identification of the enantiomers of phenylalanine and phenylalanine amide analogues **1–12** by using two different, direct and indirect, reversed-phase high-performance liquid chromatographic (RP-HPLC) methods. Direct separation was carried out on two chiral stationary phases, the crown-ether-based Crownpak CR(+) and the teicoplanin-based Chirobiotic T. The indirect separation was carried out by applying pre-column derivatization with 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) and *N*α-(2,4-dinitro-5-fluorophenyl)-L-alanine amide (Marfey's reagent, FDAA). The conditions of chiral resolution were optimized.

2. Experimental

2.1. Chemicals and reagents

Racemic α-hydrogen phenylalanine analogues were prepared by phase-transfer catalysed alkylation of the benzophenone Schiff base of glycine ethyl ester [19], followed by mild acid hydrolysis and transformation into the amide by treatment with aqueous ammonia. Racemic α-methyl phenylalanine amide analogues were prepared similarly from *N*-benzylidene (*R,S*) alanine amide [20]. Characterization of all compounds will be described in a separate paper dealing with the synthesis and resolution.

FDAA and GITC were purchased from Aldrich (Steinheim, Germany) and Pierce (Rockford, IL, USA), respectively; trifluoroacetic acid (TFA), potassium dihydrogenphosphate and perchloric acid of analytical reagent grade, and acetonitrile and methanol of HPLC grade were from Merck (Darmstadt, Germany).

Phosphate buffer was prepared by dissolving 0.01 mol potassium dihydrogenphosphate in ≈950 ml Milli-Q water, adjusting the pH with 5.0 *M* phosphoric acid to pH 3 and diluting to a final volume of 1 l in a volumetric flask. A 0.1% aqueous solution of trifluoroacetic acid was prepared by adding 1 ml trifluoroacetic acid to 1 l Milli-Q water. Perchloric acid solutions of different pH were prepared by dissolving perchloric acid in Milli-Q water. The hydrogen ion concentration of the perchloric acid solution was checked by pH measurement and by potentiometric titration with standardized sodium hydroxide.

The mobile phase was prepared by mixing buffer and organic phase in a given volume ratio and filtering on a 0.45-μm Millipore filter, type HV (Molsheim, France). Mobile phase compositions were: 0.01 *M* perchloric acid (pH 2) (Crownpak CR(+) column); methanol, water–methanol (10:90 v/v) and water–methanol (50:50 v/v) (Chirobiotic T column); 0.1% TFA–acetonitrile (75:25, 70:30, 65:35, 60:40 v/v) (Vydac 218TP54 column). Gradient elutions were run with 0.01 *M* potassium dihydrogenphosphate as mobile phase A and methanol as mobile phase B ranging from 20 to 45% B within 40 min, from 45 to 90% B within 60 min, and from 50 to 95% B within 60 min (Vydac column).

2.2. Apparatus

The HPLC system consisted of an M-600 low-pressure gradient pump, equipped with an M-996 photodiode array detector and a Millenium 2010 Chromatography Manager data system (Waters Chromatography, Milford, MA, USA), and a Rheodyne Model 7125 injector (Cotati, CA, USA) with a 20-μl sample loop.

The chiral stationary phases (CSP) used for direct separation were the teicoplanin-containing Chirobiotic T, the cyclodextrin-bonded Cyclobond III (α-CD-bonded), both 250×4.6 mm I.D., 5-μm particle size (Astec, Advanced Separation Technologies, Whippany, NJ, USA), the chiral crown-ether-based Crownpak CR(+), 150×4 mm I.D., 5-μm particle size (Daicel, Tokyo, Japan) and the α_1-acid glycoprotein-based Chiral AGP, 100×4.0 mm I.D., 5-μm particle size (Chrom Tech AB, Høgersten, Sweden). For achiral separation, Vydac 218TP54 C_{18}, 250×4.6 mm I.D., 5-μm particle size (The Separations Group, Hesperia, CA, USA) was applied. The columns were thermostated with an MK 70 thermostat (Mechanik Prüfgeräte, Medlingen, Germany). The temperature of the columns was 20°C, in the case of Crownpak CR(+) 10°C, and accuracy of temperature adjustment was ±0.1°C.

The chromatographic system was conditioned by passing the eluent through the column until a stable baseline signal and reproducible retention factors were obtained for the subsequent injections. This procedure was always followed when a new mobile phase or temperature was chosen, mainly in the direct separations.

A Radelkis OP/20811 pH-meter (Budapest, Hungary) equipped with a combined glass–calomel electrode was employed for pH measurements.

2.3. Derivatization procedure and sample preparation

Methyl substitution in the α position increase the rigidity of the molecule. The attack of the derivatizing reagent on the α-amino group becomes sterically hindered and the molecule exhibits low reactivity [21–23]. Some modifications to the original protocols were therefore made in order to compensate for the low reactivity of the α-amino group [24,25].

In the procedure of derivatization with GITC, 1 mg of the amino acid or amino acid amide was dissolved in 1 ml of 50% (v/v) aqueous acetonitrile containing 0.4% (v/v) triethylamine. A 100-μl aliquot of this stock solution was mixed with 100 μl of a solution of 0.2% (w/v) GITC in acetonitrile. The reaction mixture was thermostatted at 40°C for 1.5 h and prior to injection was diluted to 1 ml with mobile phase. A further increase of temperature, to 45 or 50°C, accelerated the derivatization procedure: the reaction was completed in 1 or 0.5 h, respectively, but side-products appeared in the chromatograms, therefore the lower temperature was chosen.

Derivatization of the analytes tested, especially α-methyl compounds with FDAA, showed some difficulty. Application of the original protocol of Marfey [25] was unsuccessful. In the modified procedure of derivatization with FDAA, 1 mg of the analyte was dissolved in 1 ml of water. A 100-μl aliquot of this stock solution was mixed with 100 μl of a solution of 1.6% (w/v) FDAA in acetone (molar ratio of FDAA to analyte was about 15:1). Besides the application of a higher reactant concentration, longer reaction times were also used. In some experiments, the higher derivatization temperature of 50°C was also applied. In spite of these modifications, the reaction remained slow: even after overnight standing at 40°C or reaction for 6 h at 50°C, the reaction was not complete. The reaction was stopped by the addition of 2 *M* HCl and the reaction mixture was diluted twofold directly with the mobile phase. The application of a higher temperature and a longer reaction time may promote racemization of the enantiomers which have hydro-

Table 1
Retention factors (k), separation factors (α) and resolutions (R_S) of compounds **1–3** and **7–9** on Crownpak CR(+) column[a]

Compound	k_D	k_L	α	R_S
1	10.34	14.47	1.40	1.60
2	9.68	12.34	1.27	1.05
3	5.81	7.94	1.36	2.10
7	9.20	13.92	1.51	2.95
8	8.00	10.87	1.32	1.95
9	5.24	8.42	1.61	3.65

[a] Column: Crownpak CR(+); flow-rate: 0.6 ml/min; detection: 200 nm; column temperature: 10°C; mobile phase: 0.01 *M* aqueous perchloric acid; t_0=2.28 min.

gen in α position. In the independent experiments, the degree of racemization was followed and was found to be negligible relative to the direct determination of the enantiomers on chiral columns.

For direct separation, 1 mg/ml solutions of the amino acids and amides were made in the eluent. Before injection onto the column, such solutions were further two or five-fold diluted.

3. Results and discussion

3.1. Direct separation of the enantiomers

Table 1 contains results relating to the chiral separation of the enantiomers of the free and the amidated amino acids on the Crownpak CR(+) column. This column contains a chiral crown ether as chiral selector and can resolve compounds bearing a primary amino group near the chiral center. Chiral recognition is achieved when a complex is formed between the crown ether and the ammonium ion of the sample [26]. As shown in Table 1 the Crownpak CR(+) column separated amino acids and amino acid amides containing hydrogen in the α position, but was ineffective in the separation of compounds with methyl substitution in the α position. This behavior can be explained in terms of the mechanism of chiral discrimination on the crown-ether-containing column [26]. The first step in this mechanism is host–guest complex formation involving the protonated α-amino group of the analyte with the cavity of the chiral crown ether. Since the presence of a methyl group in the α position hinders complex formation, no chiral discrimination was observed for these compounds. The separation was carried out with perchloric acid as mobile phase. The pH of the eluent is one of the factors governing the separation. A pH decrease improves the conditions for complex formation, but at the same time reduces the lifetime of the column [27]. Generally pH 2 is optimal. With perchloric acid at pH 2 as mobile phase, ambient temperature and a flow-rate of 1 ml/min, most of the enantiomers were separated only partially; optimization of the analyses was therefore necessary. In a series of experiments, the flow-rate was decreased to 0.8 or 0.6 ml/min. This change improved the resolution, but the optimal $R_S \geq 1.5$ was not reached. Keeping the flow-rate at 0.6 ml/min and decreasing the temperature from ambient to 10°C led to optimal resolution for most of the compounds, but not for **2,** for which $R_S = 1.05$ was achieved. Slight differences were observed in the retention factors obtained for amino acids and amino acid amides, indicating the lower hydrophobicity of the amides which resulted in shorter retention times. The enantiomer with the longer retention time was identified as the L compound by co-chromatography with authentic samples of the respective enantiomers. This is in agreement with literature data [27].

The results of direct separation on the Chirobiotic T column are summarized in Table 2. This column was useful in the analyses of amino acids containing hydrogen or a methyl group in the α position but not for amino acid amides. This behavior lends support to the separation mechanism proposed by Berthod et al. [28], according to which the primary and dominating step in chiral recognition on a teicoplanin-containing CSP is the strong charge–charge interaction between the carboxylate group of the amino acid and the ammonium group of teicoplanin. The lack of the carboxylate group in the amino acid amides could explain the coelution of enantiomers of these types of analytes. Compounds **1–3** underwent good separation in the eluent system of methanol–water 50:50 (v/v). The enantiomers of α-methylated analytes, **4–6**, being more hydrophobic, were co-eluted in this mobile phase system. The Chirobiotic T column exhibited special behavior in the reversed-phase mode. An increase of the organic modifier content generally lowered the mobility of the solute on the column [29]. An increase of the methanol

Table 2
Retention factors (k), separation factors (α) and resolutions (R_S) of compounds **1–3** and **4–6** on Chirobiotic T column[a]

Compound	k_L	k_D	α	R_S
1 (w/m 50:50)	0.75	1.11	1.48	3.60
2 (w/m 50:50)	0.92	1.15	1.25	1.70
3 (w/m 50:50)	0.68	0.88	1.29	1.70
4 (m)	1.45	1.86	1.28	1.60
5 (m)	1.22	1.49	1.22	1.30
6 (w/m 10:90)	1.00	1.26	1.26	1.40

[a] Column: Chirobiotic T; flow-rate: 0.6 ml/min; detection: 210 nm; column temperature: 20°C; mobile phase: (m) methanol, (w/m 10/90) water–methanol 10:90 (v/v), (w/m 50:50) water–methanol 50:50 (v/v); $t_0 = 5.80$ min.

content to 90 or 100% increased the retention factor, separation factor and resolution, but the overall retention time did not exceed 20 min. Good resolutions were obtained for the enantiomers of **4** and **5** in methanol and for those of **6** in water–methanol 10:90 (v/v) mobile phases, at ambient temperature. The elution sequence conformed with the literature observation [29]. The L isomer eluted before the D isomer, the ring and α-methyl-substitution did not influence the elution sequence.

3.2. Indirect separation of the enantiomers

Indirect separation was performed via pre-column derivatization with chiral derivatizing reagents. On use of a longer derivatization time, a higher reactant concentration and a higher temperature than in the original protocols [24,25], the ring- and α-methyl-substituted amino acids and amides tested (except for **12**) reacted cleanly with GITC under alkaline conditions, despite the steric hindrance around the α-amino group. The reactions of compounds containing α-hydrogen with FDAA were quantitative, whereas for the α-methyl-substituted analytes the reactions were incomplete under the test conditions.

The chromatographic separations of derivatized compounds were carried out on a Vydac 218TP54 column in different mobile phase systems. The inorganic component of the eluent contained an 0.1% aqueous solution of TFA or 0.01 *M* aqueous potassium dihydrogenphosphate at pH 3. The organic modifier was methanol or acetonitrile. With decreasing organic modifier content (methanol or acetonitrile), the retention factors generally increased and the resolution improved.

Table 3 presents selected results on the separation of the enantiomers of compounds **1–11** as GITC derivatives. Two types of analyses are reported. For amino acids, better peak shapes were obtained with gradient elution and the application of a phosphate buffer–methanol mobile phase system, while amino acid amides could be separated well under isocratic conditions with the application of aqueous TFA–acetonitrile as mobile phase. The introduction of a methyl group in the α position increased the hydrophobicity of the molecules, which resulted in higher retention factors at the same eluent composition. Unfortunately, appropriate conditions for the derivatization and separation of **12** could not be found. The elution sequence for α-hydrogen-containing compounds (amino acids and amides) was in all

Table 3
Retention factors (k), separation factors (α) and resolutions (R_S) of derivatives of **1–11** with GITC[a]

Compound	k_L	k_D	α	R_S
1[b]	4.75	5.64	1.18	4.50
2[b]	4.86	5.80	1.19	4.57
3[b]	7.71	8.02	1.04	1.10
4[b]*	*5.80*	*5.60*	1.03	1.25
5[b]*	*7.51*	*6.98*	1.07	3.25
6[c]	8.04	8.35	1.04	1.70
7[d]	11.38	13.34	1.17	1.70
8[e]	5.62	6.32	1.12	1.65
9[d]	8.04	9.18	1.14	1.30
10[f]	6.05	6.51	1.07	1.30
11[f]	8.17	9.52	1.16	3.10
12	–	–	–	–

[a] Column: Vydac 218TP54; flow-rate: 0.8 ml/min; detection: 250 nm; column temperature: 20°C; mobile phase: [b] linear gradient, 0 min, 0.01 *M* KH_2PO_4–methanol 55:45 (v/v), 60 min, 0.01 *M* KH_2PO_4–methanol 10:90 (v/v); [c] linear gradient, 0 min, 0.01 *M* KH_2PO_4–methanol 80:20 (v/v), 40 min, 0.01 *M* KH_2PO_4–methanol 55:45 (v/v); [d] 0.1% TFA–acetonitrile 75:25 (v/v); [e] 0.1% TFA–acetonitrile 70:30 (v/v); [f] 0.1% TFA–acetonitrile 65:35 (v/v); t_0=3.60 min; * reverse elution.

Table 4
Retention factors (k), separation factors (α) and resolutions (R_S) of derivatives of **1–11** with FDAA[a]

Compound	k_L	k_D	α	R_S
1[b]	1.38	1.92	1.39	4.00
2[b]	1.96	2.93	1.50	6.05
3[c]	3.80	5.86	1.54	2.60
4[e]	3.73	4.54	1.21	6.90
5[e]	5.01	5.28	1.05	2.45
6[f]	12.17	12.62	1.04	2.25
7[b]	0.90	1.21	1.35	3.55
8[b]	0.90	1.45	1.61	3.35
9[d]*	*5.53*	*3.37*	1.64	9.00
10[e]	3.49	4.82	1.38	8.80
11[e]	6.08	5.72	1.06	2.35
12	–	–	–	–

[a] Column: Vydac 218TP54; flow-rate: 0.8 ml/min; detection: 340 nm; column temperature: 20°C; mobile phase: [b] 0.1% TFA–acetonitrile 60:40 (v/v); [c] 0.1% TFA–acetonitrile 70:30 (v/v); [d] 0.1% TFA–acetonitrile 75:25 (v/v); [e] linear gradient, 0 min, 0.01 *M* KH_2PO_4–methanol 50:50 (v/v), 60 min, 0.01 *M* KH_2PO_4–methanol 5:95 (v/v); [f] linear gradient, 0 min, 0.01 *M* KH_2PO_4–methanol 80:20 (v/v), 40 min, 0.01 *M* KH_2PO_4–methanol 55:45 (v/v); t_0=3.60 min; * reverse elution.

cases L isomer before D isomer, which is in accordance with literature data [24]. The same rule held for α-methylated amino acid amides. However, the GITC-α-methyl-L-amino acids (**4** and **5,** but not **6**) were eluted later than the D isomers under the same chromatographic conditions. Similar behavior was observed by Tian et al. [23] for some α-methylated amino acids. The opposite elution sequence for **6,** despite the α-methylation, may be due the more hydrophilic cyano group on the aromatic ring.

Derivatization with FDAA has some disadvantages. The reaction time for the derivatization procedure of α-methylated analogues is too long and even after overnight standing the reaction was not complete. Incomplete reactions may lead to kinetic resolution effect, which makes the method unreliable for enantiomer purity determination. With a long reaction time, side-reactions can also occur.

Table 4 summarizes selected results concerning the separation of FDAA derivatives. Similarly as with the GITC derivatives, chromatography was performed with gradient or isocratic elution, the same buffers and organic modifiers being applied. The FDAA derivatives display higher enantioselectivity than that of the GITC derivatives. With similar retention factors, the R_S values for the FDAA derivatives are in most cases higher. The ring-substituted α-hydrogen amino acids and amides could be analysed well as FDAA derivatives, while the α-methylated ones exhibited moderate reactivity towards FDAA. For the indirect separation of **4–6** and **10** and **11,** we suggest the GITC method. For compound **12,** FDAA derivatization was not successful, similarly as when GITC derivatives were applied. We found that the L enantiomer eluted earlier than the D enantiomer, except for the case of **9,** which contain a cyano group.

Since application of the methods mentioned above was unsuccessful for the separation of **12,** other chiral columns were tested. The α-cyclodextrin-con-

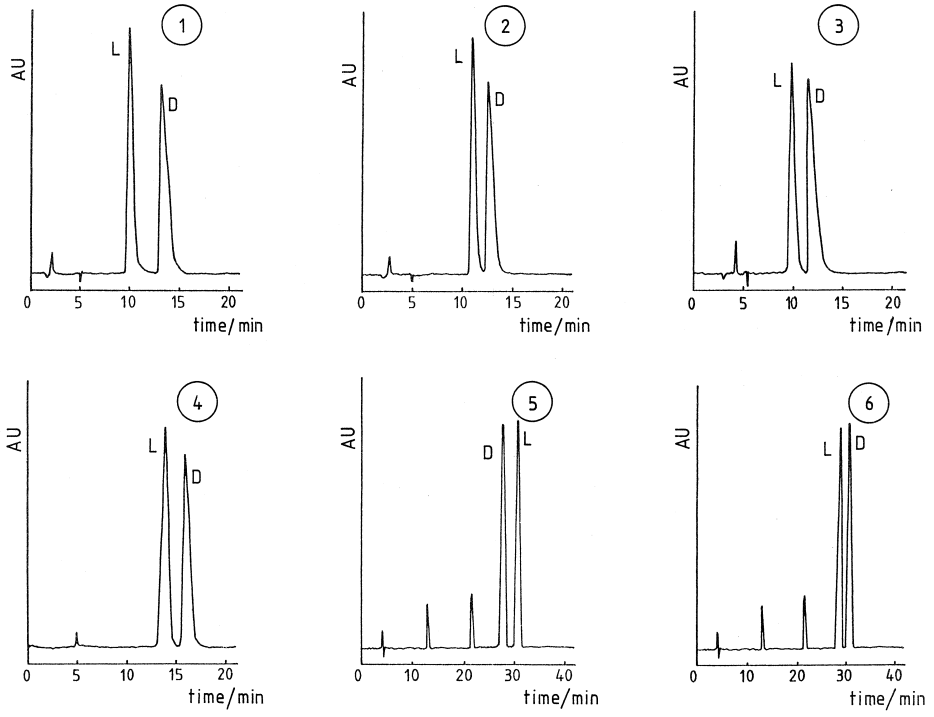

Fig. 2. Selected chromatograms of compounds **1–6**. Columns: **1–4**, Chirobiotic T; **5–6**, Vydac 218TP54; flow-rate, **1–4**, 0.6 ml/min, **5–6**, 0.8 ml/min; detection **1–4**, 210 nm, **5–6**, 250 nm; mobile phase: **1–3**, water–methanol 50:50 (v/v); **4**, methanol; **5**, linear gradient, 0 min, 0.01 *M* KH_2PO_4–methanol 55:45 (v/v), 60 min 0.01 *M* KH_2PO_4–methanol 10:90 (v/v); **6**, linear gradient, 0 min, 0.01 *M* KH_2PO_4–methanol 80:20 (v/v), 60 min, 0.01 *M* KH_2PO_4–methanol 55:45 (v/v); column temperature 20°C; **5–6** were analysed as GITC derivatives.

taining Cyclobond III can form an inclusion complex with the phenyl ring of phenylalanine analogues. Despite the possibility of complex formation, no condition was found for separation of the enantiomers of **12**. The method developed by Armstrong et al. for the separation of α-methylated amino acids on a Cyclobond III column was ineffective as regards the separation of **12** [30]. Similar behavior was observed on a Chiral AGP column. Enantiomers of compound **12** were not separable when a phosphate buffer was utilized in a range of pH 4–7 with methanol as mobile phase.

Selected chromatograms for analytes **1–11** are depicted in Figs. 2 and 3, including direct and indirect methods. Fig. 4 shows two examples of separation of highly enantiomerically enriched mixtures. The L isomer of **2** contains 1.0% D enantiomer while the D isomer of **10** contains less than 0.1% L enantiomer.

The chiral purities of the L-phenylalanine analogues (D contamination) and D-phenylalanine amide analogues (L contamination) after enzymatic resolution are listed in Table 5, where the two direct and the two indirect methods are all represented. The variations between the different methods may be largely ascribed to the errors of integration. The efficiency of the analytical procedure is reported: the limit of determination for the minor isomer is approximately 0.1% when it is present in an excess of the major isomer. The higher degree of chiral contamination is due to an unoptimized enzymatic resolution rather than to the error of chromatography.

4. Conclusions

The described procedures can be applied for the separation, identification and quantification of enantiomers of different phenylalanine analogues. The methods permit a check on the chiral purities of the amino acids and amides after synthesis, and on their incorporation into peptides, and hence allow optimi-

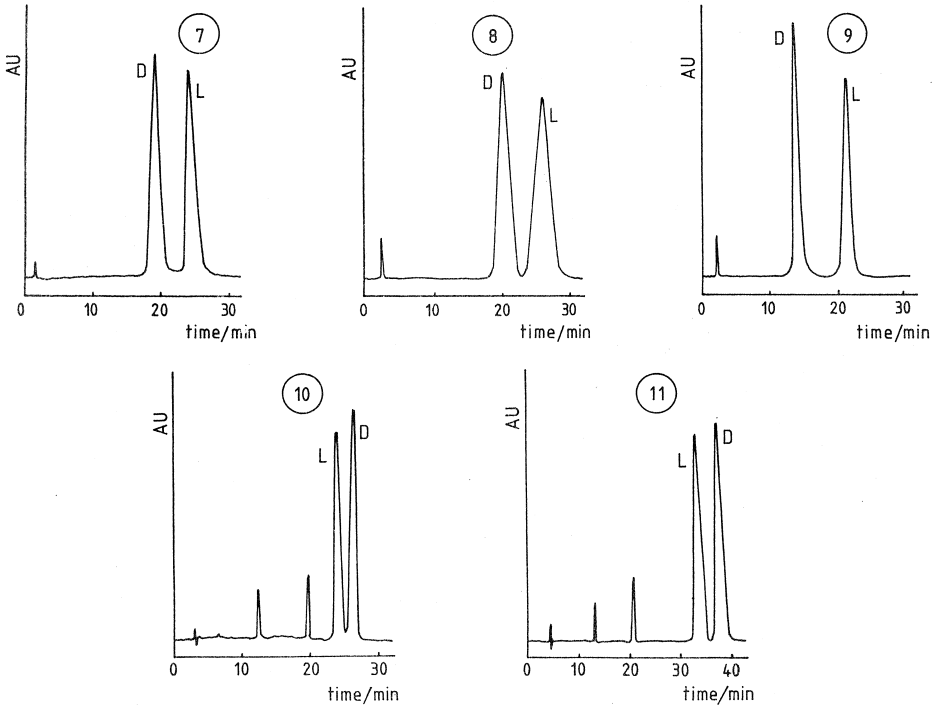

Fig. 3. Selected chromatograms of compounds **7–11**. Columns: **7–9**, Crownpak CR(+); **10–11**, Vydac 218TP54; flow-rate, **7–9**, 0.6 ml/min, **10–11**, 0.8 ml/min; detection **7–9**, 200 nm, **10–11**, 250 nm; mobile phase: **7–9**, 0.01 *M* aqueous perchloric acid; **10–11**, 0.1% TFA–acetonitrile 65:35 (v/v); column temperature: **7–9**, 10°C; **10–11**, 20°C; **10–11** were analysed as GITC derivatives.

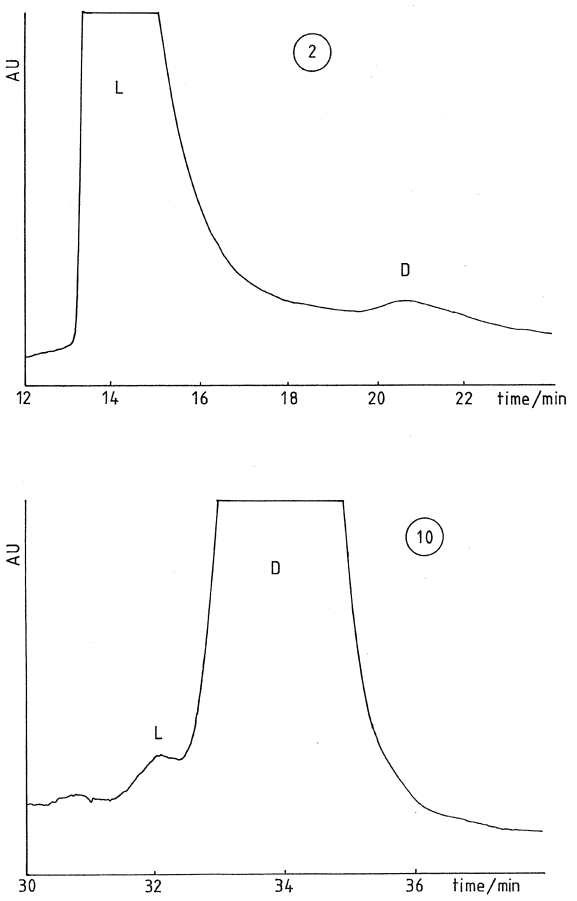

Fig. 4. Separation of enantiomers of **2** and **10** after enzymatic resolution. Columns: **2**, Chirobiotic T; **10**, Vydac 218TP54; flow-rate, **2**, 0.6 ml/min, **10**, 0.8 ml/min; detection **2**, 200 nm, **10**, 250 nm; mobile phase: **2**, methanol; **10**, 0.1% TFA–acetonitrile 70:30 (v/v); column temperature, 20°C; **10** were analysed as GITC derivatives.

zation of the conditions of synthesis of the amino acids and peptides. The Chirobiotic T column was efficient in the separation of ring- and α-methyl-substituted phenylalanine analogues, but was ineffective for the amides of these analogues. The Crownpak CR(+) column separated ring-substituted phenylalanines and amides, whereas α-methylated analogues were coeluted. Of the two indirect methods, GITC derivatization seemed more effective than FDAA derivatization.

Table 5
Chiral in purity (%) of L-phenylalanine analogues (D contamination) and D-phenylalanine amide analogues (L contamination) after enzymatic resolution[a], determined by different methods

Compound	Contamination (%)			
	Method			
	FDAA	GITC	Chirobiotic T	Crownpak
1(D)[b]	2.1	2.8	2.2	2.0
2(D)[b]	1.5	1.6	1.0	1.3
4(D)[b]	4.4	4.6	4.2	–[d]
5(D)[b]	6.4	5.9	6.1	
7(L)[c]	1.4	1.8	–	<0.1
8(L)[c]	1.0	1.0	–	1.0
9(L)[c]	<0.1	<0.1	–	<0.1
10(L)[c]	<0.1	<0.1	–	–
11(L)[c]	8.2	9.7	–	–

[a] Conditions for the enzymatic resolution were not optimized to obtain a maximum enantiomeric purity.
[b] D isomer contamination in L-phenylalanine analogues.
[c] L isomer contamination in D-phenylalanine amide analogues.
[d] –, no data available.

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