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High-performance liquid chromatographic separation of enantiomers of 1,1′-binaphthyl-substituted α-aminoisobutyric acid

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Abstract

The direct and indirect stereochemical resolution of the enantiomers of free and N-protected (*R,S*)-2′,1′:1,2;1″,2″:3,4-dinaphthcyclohepta-1,3-diene-6-amino-6-carboxylic acid (Bin) was achieved by high-performance liquid chromatographic methods. The direct separation was carried out on a β-cyclodextrin-based chiral stationary phase, ChiraDex, and the indirect resolution by applying pre-column derivatization with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate. © 1997 Elsevier Science B.V.

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1. Introduction

Open-chain and cyclic α,α-disubstituted glycines such as α-aminoisobutyric acid (Aib) and 1-amino-cycloalkane-1-carboxylic acids (Ac_nc) have been at the focus of many investigations as precise molecular markers in the elaboration of specially folded peptides [1]. The newly designed (*R,S*)-2′,1′:1,2; 1″, 2″: 3, 4-dinaphthcyclohepta-1, 3-diene-6-amino-6-carboxylic acid (Bin), a condensed Ac_7c amino acid which can be regarded as 1,1′-binaphthyl-substituted Aib (Fig. 1), constitutes a new example of a chiral atropoisomeric α-amino acid with an achiral tetra-substituted α-carbon atom. It is expected to induce interesting conformational and optical properties when incorporated into peptides.

With the increasing appreciation that the enantiomers of a chiral drug can differ pharmacokinetically and/or pharmacodynamically, the interest in methods developed for the resolution and quantification of enantiomers is rapidly growing.

This paper describes the separation of the enantiomers of Bin by using two different reversed-phase

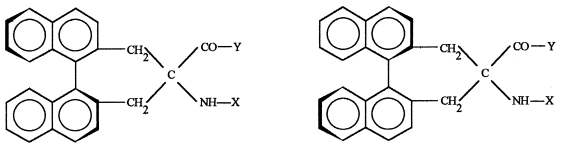

Fig. 1. Structures of enantiomers of (*R,S*)-2′,1′:1,2;1″,2″:3,4-dinaphthcyclohepta-1,3-diene-6-amino-6-carboxylic acid (Bin). Ia: X=H, Y=OtBu, H-Bin-OtBu; Ib: X=H, Y=OH, H-Bin-OH; Ic: X=Boc, Y=OH, Boc-Bin-OH; Id: X=Z, Y=OH, Z-Bin-OH.

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high-performance liquid chromatographic (RP-HPLC) methods (see Fig. 2 for chromatograms of Bin compounds): direct separation on a β-cyclodextrin-containing chiral stationary phase, ChiraDex, and pre-column derivatization with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) as chiral reagent. The conditions of chiral resolution were optimized.

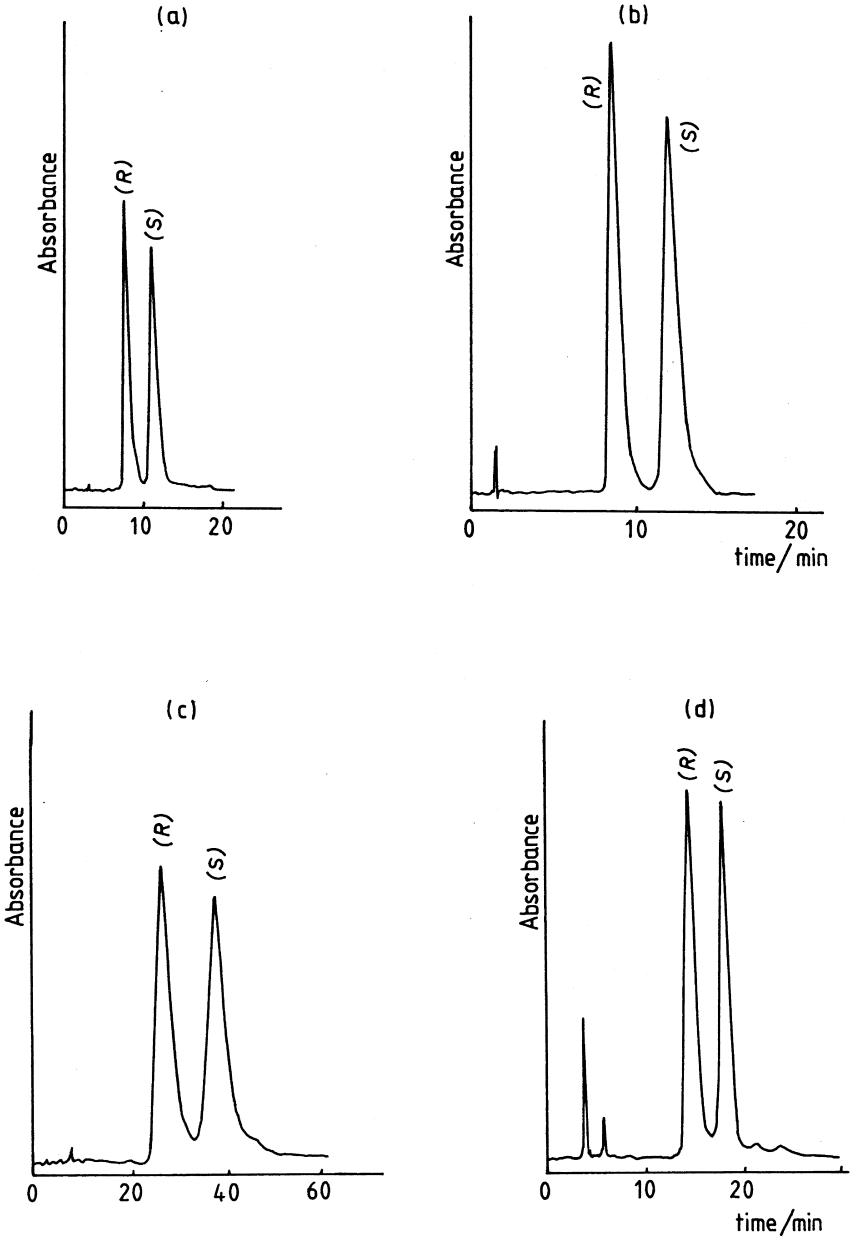

Fig. 2. Chromatograms of Bin compounds. (a) H-Bin-OH, (b) Boc-Bin-OH, (c) Z-Bin-OH, (d) GITC-Bin-OH. Column: (a), (b) and (c) ChiraDex, (d) Vydac 218TP54; mobile phase: (a) and (b) 0.01 *M* potassium dihydrogenphosphate (pH 3)–methanol (75:25, v/v), (c) 0.01 *M* potassium dihydrogenphosphate (pH 3)–methanol (60:40, v/v), (d) 0.01 *M* sodium acetate (pH 3)–methanol (32.5:67.5, v/v); flow-rate: 0.8 ml/min; detection: (a), (b) and (c) at 220 nm, (d) at 250 nm. Peaks: (*R*) = *R*-isomer, (*S*) = *S*-isomer.

2. Experimental

2.1. Chemicals and reagents

The amino esters of racemic or optically pure Ia have been prepared by solid-phase transfer bis-alkylation of a glycine *tert*.-butyl ester Schiff's base [2,3]. The corresponding free amino acids and their N-protected derivatives were synthesized by standard methods.

1-Fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) and GITC were purchased from Pierce (Rockford, IL, USA); trifluoroacetic acid (TFA), sodium acetate (NaOAc), 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 *M* potassium dihydrogenphosphate in ca. 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. Acetate buffer was prepared in the same manner, by dissolving 0.01 *M* sodium acetate in water and adjusting the pH with glacial acetic acid. 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 with different pH were prepared by dissolving perchloric acid in Milli-Q water. The hydrogen ion concentration of the perchloric acid 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).

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).

The columns used for chiral separation were ChiraDex 250×4 mm I.D., 5 μm particle size (Merck), Crownpak CR(+) 150×4 mm I.D., 5 μm particle size (Daicel, Tokyo, Japan) and Chirobiotic T 250×4.6 mm I.D., 5 μm particle size (Astec, Advanced Separation Technologies, Whippany, NJ, USA), and for achiral separation Vydac 218TP54 C_{18} 250×4.6 mm I.D., 5 μm particle size (The Separations Group, Hesperia, CA, USA).

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

2.3. Derivatization procedure and sample preparation

1 mg/ml solutions of H-(*R*,*S*)-Bin-OH and H-(*S*)-Bin-OH were used for derivatization with GITC by the method of Nimura et al. [4] and with FDAA by the modified method of Marfey [5]. In the latter case, the reaction time was increased to 5 h instead of the 1 h originally proposed, and the reaction mixture was diluted directly (without drying) with the mobile phase.

For direct separation, 1–10 m*M* solutions of the free or protected amino acids were made in eluent and filtered through a 0.45 μm Millipore filter before injection onto the column.

3. Results and discussion

3.1. Direct separation of the enantiomers

Table 1 shows the results of the chiral separation of the enantiomers of the free and the N-protected amino acids on a ChiraDex column. With phosphate buffer–methanol as an eluent system, the enantiomers could be separated. Decrease of the organic modifier content increases the retention factors and resolution. Achievement of a similar resolution for the Z-protected Bin-OH demands a longer retention time in spite of the higher methanol content, due to the increased hydrophobicity of the molecule. The enantiomer with longer retention time was generally assigned as the (*S*)-Bin compound by co-chromatography with authentic samples of the respective enantiomers (Fig. 2).

Two other chiral columns were used for the analysis of Bin compounds. In the case of the Chirobiotic T column with 100% methanol as mobile phase at a flow-rate of 1 ml/min, no elution could be

Table 1
Retention factors (k), separation factors (α) and resolutions (R_s) of X-(*R*,*S*)-Bin-OH compounds on ChiraDex column

Eluent composition buffer–CH_3OH (v/v)	k_R	k_S	α	R_s
H-Bin-OH				
70:30	1.08	1.74	1.61	1.30
75:25	1.27	2.11	1.66	2.20
Boc-Bin-OH				
70:30	0.81	1.37	1.69	1.29
75:25	1.38	2.25	1.69	1.95
Z-Bin-OH				
50:50	1.24	1.68	1.35	0.95
55:45	2.13	3.13	1.47	0.98
60:40	6.23	9.35	1.50	1.54

Column: ChiraDex; flow-rate: 0.8 ml/min; detection: 230 nm; buffer, KH_2PO_4: 0.01 *M* aqueous solution of potassium dihydrogenphosphate (pH 3); k_R: retention factor of (*R*)-enantiomer; k_S: retention factor of (*S*)-enantiomer.

observed within 2 h. The Chirobiotic T column exhibits special behaviour in the reversed-phase mode. An increase of the water content generally accelerates the mobility of the solute on the column [6]. In the case of H-(*R*,*S*)-Bin-OH, a decrease of the methanol content decreases the retention factor. At water–methanol mobile phase compositions of 50:50 (v/v) and 80:20 (v/v), values of $k_{R,S}=10.45$ and $k_{R,S}=8.25$ respectively, were observed which means that the two enantiomers were coeluted.

In the cases of Boc-(*R*,*S*)-Bin-OH and Z-(*R*,*S*)-Bin-OH with increasing water content the solubility of the solutes decreased, and therefore the solution should be filtered through a 0.2 μm filter (Merck) prior to injection. On the other hand, the retention factor of Boc-(*R*,*S*)-Bin-OH decreased to $k_{R,S}=3.95$ at a water–methanol mobile phase composition of 90:10 (v/v), but no resolution could be observed for the two enantiomers. Z-(*R*,*S*)-Bin-OH is so strongly adsorbed on the stationary phase, probably because of the bulky Z group, that with the same eluent, as mentioned above, no elution could be observed within 1 h.

The Crownpak CR(+) column contains a chiral crown ether as chiral selector and can resolve compounds bearing a primary amino group near the chiral centre. Chiral recognition is achieved when a complex is formed between the crown ether and the ammonium ion derived from the sample [7].

The analyte and the chiral selector of the Crownpak column have same type of chirality. It was expected that the interaction between the 1,1′-binaphthyl group of H-(*S*)-Bin-OH or H-(*R*)-Bin-OH and the chiral selector would be high enough to allow good separation. Unfortunately, at usual conditions of analysis (mobile phase, perchloric acid pH 2, ambient temperature, flow-rate, 0.5 ml/min) the compounds were not eluted from the column, as very strong adsorption occurred. When conditions of very low inclusion complex stability (pH 6, 50°C) and strong eluent composition (15% methanol content in the mobile phase) were chosen, no elution could be observed within 1 h.

3.2. Indirect separation of the enantiomers

Indirect separation can be carried out via pre-column derivatization with chiral derivatizing reagents. Therefore, the Boc- or Z-N-protected compounds are not suitable for this type of analysis because they are already derivatized. If it is intended to follow the racemization in the course of N-protection by an indirect analytical method, the protecting groups should first be removed.

Table 2 presents the results on the separation of enantiomers of H-(*R*,*S*)-Bin-OH as GITC derivatives. With decreasing organic modifier content (methanol or acetonitrile), the retention factors generally increased and the resolution improved. For both TFA and NaOAc systems, a similar or better resolution was achieved with a shorter retention time by applying methanol as organic modifier instead of acetonitrile. A comparison of the two ‘‘buffer’’ systems (TFA and NaOAc) at the same eluent composition of ‘‘buffer’’–methanol (32.5:67.5, v/v), or ‘‘buffer’’–acetonitrile (52.5:47.5, v/v), revealed similar or better resolutions with smaller retention factors for the NaOAc buffer system than for the aqueous solution of TFA. These results suggest that methanol as organic modifier and NaOAc as buffer were more efficient mobile phase components than acetonitrile and TFA in the separation of GITC-Bin-OH derivatives (Fig. 2).

Table 2
Retention factors (k), separation factors (α) and resolutions (R_s) of H-(*R*,*S*)-Bin-OH derivatives formed with GITC and FDAA

Eluent composition, (v/v)	k_R	k_S	α	R_s
GITC-Bin-OH				
TFA–CH_3OH				
30:70	2.25	2.87	1.27	0.96
32.5:67.5	3.70	4.79	1.29	1.88
35:65	6.21	8.27	1.33	2.43
NaOAc–CH_3OH				
32.5:67.5	3.20	4.12	1.29	1.97
TFA–CH_3CN				
50:50	5.85	6.30	1.07	1.03
52.5:47.5	7.47	7.88	1.06	1.28
NaOAc–CH_3CN				
52.5:47.5	6.59	7.21	1.10	1.58
FDAA-Bin-OH				
TFA–CH_3OH				
45:55	6.33	6.73	1.06	<0.4

Column: Vydac 218TP54 C_{18}; flow-rate: 0.8 ml/min; detection: 250 nm; TFA: 0.1% aqueous solution of trifluoroacetic acid; NaOAc: 0.01 *M* aqueous solution of sodium acetate (pH 3).

The elution sequence determined by standard addition was: (*R*)-Bin compound<(*S*)-Bin compound.

Derivatization with FDAA has some disadvantages. The reaction time for the derivatization procedure is too long and even after 5 h the reaction is not complete. With a long reaction time, racemization or side-reactions can occur. The degree of racemization should be checked, which is difficult if chiral purity control is the task.

The FDAA derivatives of H-(*R*,*S*)-Bin-OH displayed a very pure resolution (Table 2). A decrease of the methanol content lead to a slight improvement in the resolution, but this was far from the optimal value, $R_s>1.5$. For indirect separation of the Bin compounds, therefore, we suggest the application of GITC derivatives.

The limits of determination of Bin compounds for direct and indirect analysis are compared in Table 3. The limit of detection was determined at a signal-to-noise ratio of 3. The higher sensitivity of the indirect analysis is due to the higher molar absorptivity of the derivatives. Further, the determination limit for the minor isomer is less than 0.1% when it is present in an excess of the major isomer.

Table 3
Limit of detection of Bin compounds

Compound	Detection wavelength λ (nm)	Determination limit (p*M*)
H-Bin-OH[1]	219	1
Boc-Bin-OH[2]	220	40
Z-Bin-OH[3]	219	2
GITC-Bin-OH[4]	250	0.5

Columns: [1–3] ChiraDex and [4] Vydac 218TP54 C_{18}; flow-rate, 0.8 ml/min; mobile phase, [1,2] 0.01 *M* KH_2PO_4 (pH 3)–methanol (75:25), [3] 0.01 *M* KH_2PO_4 (pH 3)–methanol (55:45), [4] 0.1% TFA–methanol (35:65); volume injected: 20 μl; signal-to-noise ratio, 3.

4. Conclusions

The described procedures can be applied for the separation and quantification of Bin and its N-protected derivatives. The methods permit a check on the chiral purity of the free and N-protected amino acids after synthesis, and on their incorporation into peptides, and hence allow optimization of the conditions of synthesis of the amino acids and peptides.

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