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Journal of Chromatography A, 761 (1997) 103–113

JOURNAL OF CHROMATOGRAPHY A

High-performance liquid chromatographic methods for separation of enantiomers of alicyclic β-amino acids[1]

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Received 16 July 1996; revised 27 September 1996; accepted 2 October 1996

Abstract

Reversed-phase high-performance liquid chromatographic methods were developed for the separation and quantification of the enantiomers of alicyclic β-amino acids: racemic *cis*- and *trans*-2-aminocyclohexane-1-carboxylic acids (*cis*- and *trans*-ACHC) and racemic *cis*- and *trans*-2-amino-4-cyclohexene-1-carboxylic acids (*cis*- and *trans*-ACHC-ene). The enantioselective separations involved two methods: direct separation on a chiral stationary phase (Crownpak CR(+)) and separation of the diastereomers formed by pre-column derivatization with chiral derivatizing reagents: 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) and 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosylisothiocyanate. The different methods were compared in systematic chromatographic examinations. The effects of pH, mobile phase composition, organic modifier content and temperature on the separation were also investigated.

Keywords: Enantiomer separation; Derivatization, LC; Amino acids

1. Introduction

One of the most exciting of the alicyclic β-amino acids is (1*R*,2*S*)-2-aminocyclopentane-1-carboxylic acid (cispentacin), an antifungal antibiotic. We earlier described a method for separation of the isomers of *cis*- and *trans*-2-aminocyclopentane-1-carboxylic acids (*cis*- and *trans*-ACPC) [1].

In recent years, several investigations have been made with the aim of introducing alicyclic β-amino acids into peptides [2–6]. By insertion of a β-amino acid instead of an α-amino acid into the naturally occurring pharmacologically active peptides, the activity or the effect can be modified and the stability of the natural peptides can be increased.

Besides the pharmacological importance of the β-amino acids, they are used as building blocks for the preparation of modified (unnatural) analogues of biologically active peptides. These investigations are applied for determination of the fine structures of receptors [3,4,7–10]. These amino acids also play a very important role in the synthesis of heterocycles with the aim of preparing potential pharmacons [11,12].

For this purpose, besides *cis*- and *trans*-ACPC,

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[1] Presented at the 10th International Symposium on Advances and Applications of Chromatography in Industry, Bratislava, 30 June–4 July 1996.

PII S0021-9673(96)00832-1

racemic *cis*- and *trans*-2-aminocyclohexane-1-carboxylic acids (*cis*- and *trans*-ACHC) and racemic *cis*- and *trans*-2-amino-4-cyclohexene-1-carboxylic acids (*cis*- and *trans*-ACHC-ene) (Fig. 1) were synthesized.

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. The resolution of enantiomers requires an asymmetric or chiral environment allowing diastereomeric interactions. For this purpose high-performance liquid chromatography (HPLC) is widely used.

This paper describes the separation, identification and quantification of the enantiomers of *cis*- and *trans*-ACHC and *cis*- and *trans*-ACHC-ene by using two different reversed-phase (RP) HPLC methods: direct separation on a chiral stationary phase [Crownpak CR(+) contains a chiral crown ether as a chiral selector] and pre-column derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, Marfey's reagent) and 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl-isothiocyanate (GITC) as chiral reagents. The separations were carried out in different eluent systems. The effects of mobile phase composition, organic modifier content, temperature and pH on the separation were also investigated.

2. Experimental

2.1. Synthesis

The racemic *cis*- and *trans*-ACHC and *cis*- and *trans*-ACHC-ene were prepared from the corresponding commercially available *cis*- and *trans*-hexahydro- and tetrahydrophthalicanhydrides, by ammonolysis, followed by Hoffmann degradation and ion-exchange purification [11,12]. In the case of *cis*- and *trans*-ACHC-ene, Hoffmann degradation was performed with sodium hypochlorite instead of the usual hypobromite. For preparation of the enantiomers of compounds 1–4, a lipase PS catalysed selective N-acylation of the ethyl esters of racemic *cis*- or *trans*-β-amino acids was used [13]. To a solution of the ethyl esters of the racemic compounds and 2,2,2-trifluoroethyl chloroacetate in diethyl ether, a lipase PS preparation was added. The mixture was stirred at room temperature for 10 to 40 min, a few drops of ethanol were then added and the enzyme was filtered off. The ethereal solution was cooled to 0°C and gaseous hydrogen chloride was slowly bubbled through the solution for 30 min. The hydrochlorides of the ethyl esters of the (1*R*,2*S*)- and (1*S*,2*S*)-enantiomers were precipitated. They were filtered off to give white crystals. The (1*R*,2*S*)- and

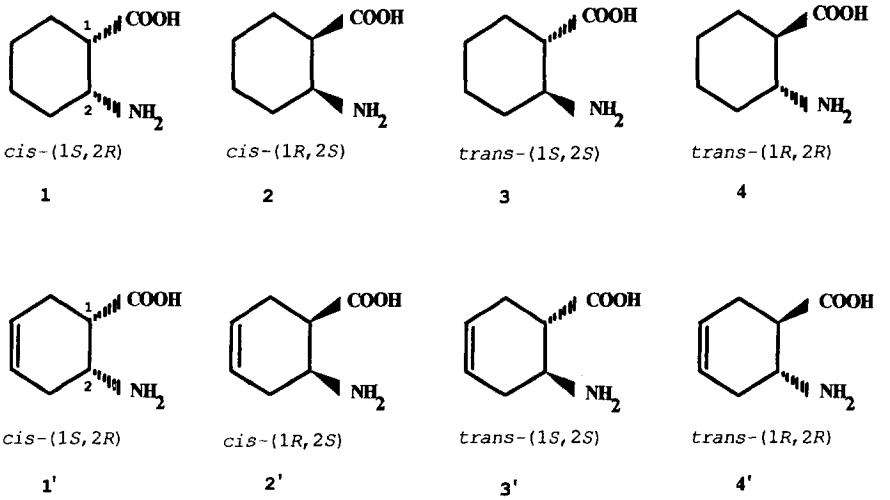

Fig. 1. Structures of four stereoisomers of 2-amino-cyclohexane-1-carboxylic acid and of 2-amino-4-cyclohexene-1-carboxylic acid.

(1*S*,2*S*)-enantiomers were obtained after small-scale alkaline hydrolysis of the ethyl esters.

The identity of the compounds was checked by means of melting point determination, fast atom bombardment (FAB) mass spectrometry, ^{1}H NMR spectroscopy and optical rotation measurement.

2.2. Chemicals and reagents

GITC was purchased from Aldrich (Steinheim, Germany) and FDAA from Sigma (St. Louis, MO, USA). Perchloric acid, potassium dihydrogenphosphate, sodium acetate, trifluoroacetic acid, phosphoric acid, acetic acid of analytical reagent grade and HPLC-grade acetonitrile, methanol and tetrahydrofuran (THF) were obtained from Merck (Darmstadt, Germany).

Lipase PS (*Pseudomonas cepacia*) was obtained from Amano Pharmaceuticals and was immobilized on Celite [14].

Buffers were prepared with triply distilled water or with Milli-Q water purified further by filtering on a 0.45-μm Millipore filter, type HV (Molsheim, France). The phosphate and acetate buffers were prepared by dissolving 0.01 mol potassium dihydrogenphosphate or sodium acetate in water, adjusting the pH with phosphoric or acetic acid to pH 3 and diluting to a final volume of 1 l.

2.3. Apparatus

HPLC measurements were performed on a Waters system consisting of an M-600 low-pressure gradient pump, an M-996 photodiode array detector and a Millenium 2010 Chromatography Manager data system (Waters Chromatography, Division of Millipore, Milford, MA, USA).

The columns used for analytical separations were Vydac 218TP54 C_{18} (250×4.6 mm I.D.), 5 μm particle size (Separations Group, Hesperia, CA, USA), and Crownpak CR(+) (150×4 mm I.D.), 5 μm particle size (Daicel, Tokyo, Japan).

The Crownpak CR(+) column was thermostated with an MK70 thermostat (Mechanik Prüfgerate, Medlingen, Germany). The accuracy of temperature adjustment was ±0.1°C.

2.4. Derivatization procedure and sample preparation

Solutions (1 mg/ml) of *cis*- and *trans*-ACHC and of *cis*- and *trans*-ACHC-ene were used for derivatization with FDAA by the modified method of Marfey [15,16] and with GITC by the method of Nimura et al. [17].

For direct separation, 1–10 m*M* solutions of the investigated β-amino acids were made in 0.01 *M* perchloric acid and injected after filtering on a 0.45-μm Millipore filter, type HV (Molsheim, France).

3. Results and discussion

Each investigated amino acid has two groups which can be protonated. To keep the ionization of the molecules at a constant level along the column, control of the pH is required. For this purpose, three buffer systems were applied: a 0.1% aqueous solution of trifluoroacetic acid which is often used in the separation of peptides and proteins; phosphate buffer has an ion-pairing effect and was applied in 0.01 *M* concentration at pH 3; sodium acetate is a very useful buffer in natural amino acid analysis and was also used in 0.01 *M* concentration at pH 3. Methanol, acetonitrile and THF were used as organic modifiers.

3.1. Separation of GITC derivatives

The results of the separation of ACHC derivatives are summarized in Table 1. With methanol as organic modifier, good resolution could be achieved not only for the enantiomers, but also for the epimers, within a short time in all three buffer systems. There were only slight differences between the buffers; the trifluoroacetic acid-containing system gave good resolution with relatively low *k* values.

The results of the separations in the acetonitrile-containing system show that similar resolutions could be achieved with higher *k* values. On the other hand, sodium acetate was the most efficient of the three buffer systems, giving the best resolution with relatively low *k* values. The elution sequence was independent of the nature of the buffers and organic modifiers: *trans*(1*S*,2*S*)<*trans*(1*R*,2*R*)<*cis*(1*R*,2*S*)< *cis*(1*S*,2*R*). This elution sequence is the same as that

Table 1
Dependence of retention factor (k), separation factor (α) and resolution (R_S) of ACHC–GITC derivatives on eluent composition

Eluent composition (v/v)	k				$\alpha_{t,t}$	$\alpha_{c,c}$	$R_{S;t,t}$	$R_{S;t,c}$	$R_{S;c,c}$
	trans		*cis*						
	(1*S*,2*S*)	(1*R*,2*R*)	(1*R*,2*S*)	(1*S*,2*R*)					
$TFA–CH_3OH$ (50:50)	1.35	1.87	2.47	3.09	1.38	1.25	1.89	2.01	1.86
$KH_2PO_4–CH_3OH$ (52.5:47.5)	1.96	2.89	3.93	5.02	1.47	1.28	2.89	2.76	2.58
$NaOAc–CH_3OH$ (55:45)	2.02	2.97	3.96	5.09	1.47	1.29	3.22	3.05	3.16
$TFA–CH_3CN$ (72.5:27.5)	5.06	6.32	8.12	9.75	1.25	1.20	2.15	2.77	2.06
$KH_2PO_4–CH_3CN$ (75:25)	7.34	9.44	12.31	15.01	1.29	1.22	2.67	2.86	2.30
$NaOAc–CH_3CN$ (70:30)	2.78	3.34	4.23	4.99	1.20	1.18	1.74	1.98	1.49
(72.5:27.5)	3.82	4.80	5.99	7.21	1.26	1.26	4.01	4.26	3.78

Column, Vydac 218TP54 C_{18}; flow-rate, 0.8 ml/min; detection, 250 nm; TFA, 0.1% aqueous solution of trifluoroacetic acid; KH_2PO_4, 0.01 *M* aqueous solution of potassium dihydrogenphosphate (pH 3); NaOAc, 0.01 *M* aqueous solution of sodium acetate (pH 3); $\alpha_{t,t}$ and $R_{S;t,t}$ represent separation of *trans*(1*S*,2*S*) and *trans*(1*R*,2*R*) isomers; $R_{S;t,c}$ represents resolution of *trans*(1*R*,2*R*) and *cis*(1*R*,2*S*) isomers; $\alpha_{c,c}$ and $R_{S;c,c}$ represent separation of *cis*(1*R*,2*S*) and *cis*(1*S*,2*R*) isomers.

observed in the case of 2-aminocyclopentane-1-carboxylic acid [1]. It seems that the configuration of the carbon atom bearing the amino group, which reacts with the derivatizing reagents, is the determining factor in the elution sequence of the *trans–trans* and *cis–cis* isomers. The enantiomers with the *S* configuration at this carbon atom eluted before the enantiomers with the *R* configuration there.

The GITC derivatives of ACHC-ene could be separated in all eluent systems (Table 2). For the

Table 2
Dependence of retention factor (k), separation factor (α) and resolution (R_S) of ACHC-ene-GITC derivatives on eluent composition

Eluent composition (v/v)	k				$\alpha_{t,t}$	$\alpha_{c,c}$	$R_{S;t,t}$	$R_{S;t,c}$	$R_{S;c,c}$
	trans		*cis*						
	(1*S*,2*S*)	(1*R*,2*R*)	(1*R*,2*S*)	(1*S*,2*R*)					
$TFA–CH_3OH$ (50:50)	1.12	1.42	1.82	2.20	1.27	1.21	1.78	2.06	1.72
$KH_2PO_4–CH_3OH$ (52.5:47.5)	1.52	1.98	2.53	3.10	1.30	1.23	1.84	1.92	1.74
$NaOAc–CH_3OH$ (55:45)	1.58	2.08	2.64	3.27	1.31	1.24	2.73	2.57	2.41
$TFA–CH_3CN$ (72.5:27.5)	4.01	4.77	5.63	6.54	1.19	1.62	3.05	2.92	2.77
$KH_2PO_4–CH_3CN$ (75:25)	6.12	7.41	8.87	10.43	1.21	1.18	2.65	2.89	2.86
$NaOAc–CH_3CN$ (70:30)	2.30	2.67	3.10	3.55	1.16	1.15	2.30	2.25	2.27

Column, Vydac 218TP54 C_{18}; flow-rate, 0.8 ml/min; detection, 250 nm; TFA, 0.1% aqueous solution of trifluoroacetic acid; KH_2PO_4, 0.01 *M* aqueous solution of potassium dihydrogenphosphate (pH 3); NaOAc, 0.01 *M* aqueous solution of sodium acetate (pH 3); $\alpha_{t,t}$ and $R_{S;t,t}$ represent separation of *trans*(1*S*,2*S*) and *trans*(1*R*,2*R*) isomers; $R_{S;t,c}$ represents resolution of *trans*(1*R*,2*R*) and *cis*(1*R*,2*S*) isomers; $\alpha_{c,c}$ and $R_{S;c,c}$ represent separation of *cis*(1*R*,2*S*) and *cis*(1*S*,2*R*) isomers.

analysis with methanol, the k values could be kept in the optimal range $1<k<5$ in all buffer systems to get $R_s>1.5$, while for acetonitrile the k values were lowest in sodium acetate. With respect to the two organic modifiers, the methanol-containing systems resulted in a shorter analysis time and hence lower k values. The elution sequence was the same as observed for ACHC derivatives, and the enantiomers with the *S* configuration at carbon 2 eluted first in the separation of the *cis–cis* and *trans–trans* isomers. Comparison of the chromatograms of ACHC and ACHC-ene derivatives at the same eluent composition revealed that the isomers of unsaturated amino acid had shorter retention times (lower k values) than those of the saturated ones, reflecting the decreased hydrophobicity of the double-bond-containing component. Representative chromatograms of the separation of GITC derivatives are shown in Fig. 2.

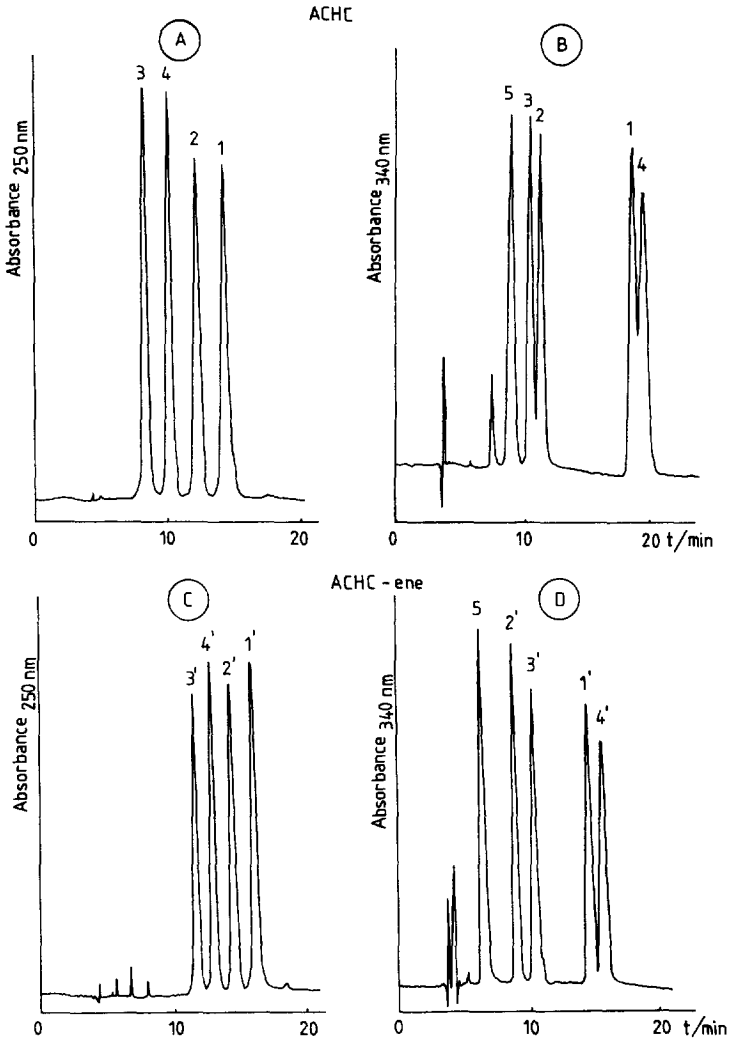

Fig. 2. Representative chromatograms of the separation of four diastereomers of ACHC and ACHC-ene derivatives. (A,C) GITC derivatives, (B,D) FDAA derivatives. Column, Vydac 218TP54 C_{18}; flow-rate, 0.8 ml/min; detection, 250 nm (A,C), 340 nm (B,D); mobile phase, (A) 0.1% trifluoroacetic acid–methanol (50:50, v/v), (B) 0.1% trifluoroacetic acid–acetonitrile–tetrahydrofuran (70:30:3, v/v), (C) 0.01 *M* sodium acetate–acetonitrile (70:30, v/v), (D) 0.1% trifluoroacetic acid–methanol–tetrahydrofuran (50:50:3, v/v). Peaks: 1,1′=*cis*(1*S*,2*R*) derivative; 2,2′=*cis*(1*R*,2*S*) derivative; 3,3′=*trans*(1*S*,2*S*) derivative; 4,4′=*trans*(1*R*,2*R*) derivative; 5=unreacted FDAA.

3.2. Separation of FDAA derivatives

The results of the separation of ACHC derivatives are summarized in Table 3. Change of the derivatizing reagent changed the sequence of isomer elution. The two *cis* enantiomers eluted between the two *trans* enantiomers, but for the enantiomer pairs the same rule is observed as for the GITC derivatives. The enantiomers with the *S* configuration at carbon 2 eluted before those with the *R* configuration there. The elution sequence was: *trans*(1*S*,2*S*)< *cis*(1*R*,2*S*)<*cis*(1*S*,2*R*)<*trans*(1*R*,2*R*). FDAA demonstrated much higher R_s values for the *cis–cis* and *trans–trans* isomers than those observed for the GITC derivatives, e.g. $R_{S;c,c}$ and $R_{S;t,t}$ were higher than 8, while for GITC the corresponding values were lower than 3. On the other hand, the *cis*(1*S*,2*R*) and *trans*(1*R*,2*R*) isomers eluted together in one peak. Decrease of the organic modifier content was not accompanied by any change in the resolution of these isomers ($R_{S;c,t}$=0). This led us to add a third organic component, THF, to the mobile phase. By variation of THF content of the eluent in the interval 1–5% (v/v), a partial separation of these isomers could be achieved in some cases, but $R_{S;c,t}$ never exceeded 0.8, and a baseline separation was not attained. Table 3 also shows that for the FDAA derivatives, there was no pronounced difference between the two organic modifiers, methanol and acetonitrile, as was observed for the GITC derivatives.

The results of the separation of ACHC-ene-FDAA derivatives are collected in Table 4. The sequence of elution of the first two peaks was reverse of that observed for the ACHC-FDAA derivatives: the *cis*(1*R*,2*S*) enantiomer eluted before the *trans*(1*S*,2*S*) enantiomer, but the first peaks for the *cis–cis* and *trans–trans* pairs were those of the isomers which had the *S* configuration at carbon 2. The behaviour of the *cis*(1*S*,2*R*) and *trans*(1*R*,2*R*) isomers was similar to that of the ACHC–FDAA derivatives: they co-eluted or the separation was only partial. Application

Table 3
Dependence of retention factor (k), separation factor (α) and resolution (R_S) of ACHC–FDAA derivatives on eluent composition

Eluent composition (v/v)	k				$\alpha_{c,c}$	$\alpha_{t,t}$	$R_{S;t,c}$	$R_{S;c,c}$	$R_{S;c,t}$	$R_{S;t,t}$
	trans (1*S*,2*S*)	*cis* (1*R*,2*S*)	*cis* (1*S*,2*R*)	*trans* (1*R*,2*R*)						
TFA–CH_3OH										
(50:50)	1.72	2.13	5.85	5.85	2.69	3.51	1.08	7.33	0.00	8.66
KH_2PO_4–CH_3OH										
(52.5:47.5)	2.18	2.95	8.40	8.40	2.84	3.86	1.21	8.44	0.00	10.25
NaOAc–CH_3OH										
(55:45)	1.77	2.43	7.53	7.53	3.10	4.25	1.87	8.61	0.00	10.28
TFA–CH_3CN										
(70:30)	2.38	2.81	6.43	6.43	2.25	2.75	1.29	8.20	0.00	9.24
(75:25)	5.17	6.30	17.06	17.06	2.71	3.30	1.64	8.40	0.00	10.06
TFA–CH_3CN–THF										
(70:30:3)	1.67	1.87	3.77	3.94	2.02	2.36	1.01	8.43	0.57	8.21
TFA–CH_3CN–THF										
(72.5:27.5:5)	2.05	2.30	4.53	4.72	1.96	2.23	1.35	5.88	0.70	10.25
KH_2PO_4–CH_3CN										
(75:25)	4.37	5.20	14.72	14.72	2.82	3.38	2.45	17.25	0.00	19.95
NaOAc–CH_3CN										
(70:30)	1.95	2.25	5.20	5.20	2.25	2.74	1.08	8.14	0.00	9.78
NaOAc:CH_3CN–THF										
(70:30:3)	1.34	1.51	2.92	2.92	1.93	2.18	1.00	6.15	<0.4	7.45

Column, Vydac 218TP54 C_{18}; flow-rate, 0.8 ml/min; detection, 340 nm; TFA, 0.1% aqueous solution of trifluoroacetic acid; KH_2PO_4, 0.01 *M* aqueous solution of potassium dihydrogenphosphate (pH 3); NaOAc, 0.01 *M* aqueous solution of sodium acetate (pH 3); $R_{S;t,c}$ represents resolution of *trans*(1*S*,2*S*) and *cis*(1*R*,2*S*) isomers; $\alpha_{c,c}$ and $R_{S;c,c}$ represent separation of *cis*(1*R*,2*S*) and *cis*(1*S*,2*R*) isomers; $R_{S;c,t}$ represents resolution of *cis*(1*S*,2*R*) and *trans*(1*R*,2*R*) isomers; $\alpha_{t,t}$ and $R_{S;t,t}$ represent separation of *trans*(1*S*,2*S*) and *trans*(1*R*,2*R*) isomers.

Table 4
Dependence of retention factor (k), separation factor (α) and resolution (R_S) of ACHC-ene-FDAA derivatives on eluent composition

Eluent composition (v/v)	k				$\alpha_{c,c}$	$\alpha_{t,t}$	$R_{S;c,t}$	$R_{S;t,c}$	$R^*_{S;c,t}$	$R_{S;c,c}$	$R_{S;t,t}$
	cis (1*R*,2*S*)	*trans* (1*S*,2*S*)	*cis* (1*S*,2*R*)	*trans* (1*R*,2*R*)							
TFA–CH_3OH											
(50:50)	1.90	2.33	4.37	4.67	2.30	2.00	2.01	7.18	0.85	8.59	8.11
(52.5:47.5)	2.26	2.80	5.39	5.72	2.38	2.04	2.44	8.33	0.77	10.07	8.49
TFA–CH_3OH–THF											
(50:50:3)	1.33	1.72	2.86	3.19	2.15	1.85	1.98	4.89	1.29	6.78	6.33
KH_2PO_4–CH_3OH											
(52.5:47.5)	3.51	4.07	7.00	7.00	1.90	1.72	0.71	3.28	0.00	3.81	3.89
NaOAc–CH_3OH											
(55:45)	2.96	3.54	6.74	6.74	2.28	1.90	1.53	4.43	0.00	5.96	5.02
TFA–CH_3CN											
(70:30)	2.39	2.98	4.99	4.99	2.09	1.67	3.39	7.37	0.00	10.45	7.71
TFA–CH_3CN–THF											
(70:30:3)	1.80	2.14	2.30	3.46	1.27	1.61	1.55	5.00	0.80	6.21	5.37
TFA–CH_3CN–THF											
(72.5:27.5:5)	2.16	2.59	3.91	4.14	1.19	1.06	1.63	5.00	0.97	6.75	5.80
KH_2PO_4–CH_3CN											
(70:30)	2.01	2.38	4.18	4.18	2.08	1.76	1.82	6.73	0.00	10.24	7.03
NaOAc–CH_3CN											
(70:30)	2.16	2.47	4.06	4.06	1.88	1.64	1.59	6.87	0.00	8.23	7.04

Column, Vydac 218TP54 C_{18}; flow-rate, 0.8 ml/min; detection, 340 nm; TFA, 0.1% aqueous solution of trifluoroacetic acid; KH_2PO_4, 0.01 *M* aqueous solution of potassium dihydrogenphosphate (pH 3); NaOAc, 0.01 *M* aqueous solution of sodium acetate (pH 3); $R_{S;c,t}$ represents resolution of *cis*(1*R*,2*S*) and *trans*(1*S*,2*S*) isomers; $R_{S;t,c}$ represents resolution of *trans*(1*S*,2*S*) and *cis*(1*S*,2*R*) isomers; $R^*_{S;c,t}$ represents resolution of *cis*(1*S*,2*R*) and *trans*(1*R*,2*R*) isomers; $R_{S;c,c}$ and $R_{S;t,t}$ represent resolution of *cis*(1*R*,2*S*) and *cis*(1*S*,2*R*) isomers and *trans*(1*S*,2*S*) and *trans*(1*R*,2*R*) isomers, respectively.

of THF as a third mobile phase component in the TFA-containing system improved the resolution of the last two peaks, and with the ternary system TFA–CH_3OH–THF almost baseline separation could be achieved for all four isomers. The enantioselectivity of FDAA was much higher than of GITC, especially in acetonitrile. The $R_{S;c,c}$ and $R_{S;t,t}$ values differed by a factor of 2–5. Table 4 also demonstrates that there was no significant difference in separation capability of the two organic modifiers, methanol and acetonitrile. Chromatograms showing the best resolution for the four enantiomers as FDAA derivatives are prersented in Fig. 2.

3.3. Direct separation on chiral column

For the direct separation, a Crownpak CR(+) column was used. This column contains a chiral crown ether as a 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 derived from the sample.

During the analysis, the effects of pH, flow-rate and temperature on the separation were investigated. The results for ACHC are summarized in Table 5. The separation was started at 20°C with perchloric acid of pH 2 as eluent, at a flow-rate of 0.5 ml/min. Under such conditions, the enantiomers of *trans*-ACHC could be separated, whereas the resolution of the enantiomers of *cis*-ACHC was not complete. The elution sequence was: *cis*(1*S*,2*R*)<*cis*(1*R*,2*S*)< *trans*(1*S*,2*S*)<*trans*(1*R*,2*R*). The effect of pH was more significant, due to the stronger complex formation under acidic conditions. On decrease of the pH (pH 1.5 and 1.0), complete separation was achieved for the *cis*-ACHC isomers. On the other hand a decrease of the pH decreased the resolution of the *cis*(1*R*,2*S*) and *trans*(1*S*,2*S*) enantiomers. pH 1 provided adequate enantiomeric resolution of the

Table 5
Dependence of retention factor (k), separation factor (α) and resolution (R_s) of ACHC isomers on temperature, pH and flow-rate of the eluent

Temperature (°C)	pH	Flow-rate (ml/min)	k				$\alpha_{c,c}$	$\alpha_{t,t}$	$R_{S;c,c}$	$R_{S;c,t}$	$R_{S;t,t}$
			cis		*trans*						
			(1*S*,2*R*)	(1*R*,2*S*)	(1*S*,2*S*)	(1*R*,2*R*)					
25	1.0	0.50	3.28	3.63	4.10	5.17	1.10	1.26	0.96	1.40	2.64
	2.0	0.50	2.36	2.58	3.02	3.65	1.09	1.21	0.78	1.54	1.82
		0.25	2.36	2.56	3.11	3.70	1.08	1.19	0.50	1.21	1.30
20	1.5	0.50	3.14	3.46	4.01	4.94	1.10	1.23	1.00	1.42	2.00
		0.25	3.14	3.44	4.00	4.92	1.10	1.23	0.88	1.32	1.87
	1.0	0.50	3.54	4.00	4.48	5.87	1.13	1.31	1.40	1.26	3.25
		0.25	3.50	3.95	4.46	5.80	1.13	1.30	1.32	1.20	3.04
15	1.0	0.50	3.85	4.46	4.95	6.80	1.15	1.37	1.47	1.20	3.40
10	1.0	0.50	4.20	5.03	5.52	7.95	1.19	1.44	1.84	1.18	4.60
	2.0	0.50	3.45	3.97	4.60	6.17	1.15	1.34	1.46	1.65	3.49
		0.25	3.50	3.98	4.63	6.14	1.14	1.32	1.22	1.47	3.18
5	1.5	0.50	4.38	5.12	5.76	8.02	1.17	1.40	1.75	1.58	4.56
		0.25	4.37	5.10	5.83	7.94	1.16	1.36	1.70	1.35	3.62
	1.0	0.50	4.80	5.89	6.44	9.61	1.22	1.48	2.25	1.12	4.83
		0.25	4.88	5.95	6.52	9.66	1.22	1.48	2.11	1.02	4.69

Column, Crownpak CR(+); eluent, aqueous perchloric acid; detection, 205 nm; $\alpha_{c,c}$ and $R_{S;c,c}$ represent separation of *cis*(1*S*,2*R*) and *cis*(1*R*,2*S*) isomers; $R_{S;c,t}$ represents resolution of *cis*(1*R*,2*S*) and *trans*(1*S*,2*S*) isomers; $\alpha_{t,t}$ and $R_{S;t,t}$ represent separation of *trans*(1*S*,2*S*) and *trans*(1*R*,2*R*) isomers.

cis–cis and *trans–trans* isomers, but not the (1*R*,2*S*) and (1*S*,2*S*) enantiomers.

A decrease of the flow-rate in the case of ACHC was not favourable. The decrease from 0.5 to 0.25 ml/min doubled the retention and thus the analysis time. On the other hand, the resolution did not improve, due to the skew shape of the peaks. The increased band broadening led to a poorer resolution of all isomers.

It was earlier reported [18–21] that lowering the temperature of a crown ether column leads to improved enantioselectivity. Table 5 shows that decrease of the temperature increased the enantioselectivity at all investigated pH values. $R_{S;c,c}$ increased from 0.96 to 2.25, and $R_{S;t,t}$ from 2.64 to 4.83 on decrease of the temperature from 25°C to 5°C, but the resolution of the *cis*(1*R*,2*S*) and *trans*(1*S*,2*S*) enantiomers did not improve. The *cis* enantiomers eluted before the *trans* enantiomers, and in the *cis–cis* and *trans–trans* pairs the first peaks related to the variants with the *S* configuration at carbon atom 1. Similar elution behaviour was observed for 2-aminocyclopentane-1-carboxylic acid and for β-amino acids with a bicyclo[2,2,1]heptane skeleton (A. Péter, unpubl. results). The literature data show that (+)–*R* enantiomers elute before (−)–*S* ones, in the case of α-amino acids with one chiral centre, and dipeptides containing two chiral centres [20,22].

The separation of the ACHC-ene isomers is surveyed in Table 6. Unfortunately, on variation of the temperature, pH and flow-rate, no condition was found for separation of the *cis*(1*S*,2*R*) and *cis*(1*R*,2*S*) enantiomers; they always coeluted. In spite of the lack of success of the separation of *cis* enantiomers, some interesting results were obtained, which differed from those for ACHC analysis. Decrease of the flow-rate and temperature increased the resolution values $R_{S;c,t}$ and $R_{S;t,t}$. This was probably due to the more symmetric peak shape: the band broadening was lower than for ACHC. Representative chromatograms for the direct separation of the ACHC and ACHC-ene isomers are shown in Fig. 3.

Measurement of the temperature dependence of

Table 6
Dependence of retention factor (k), separation factor (α) and resolution (R_S) of ACHC-ene isomers on temperature, pH and flow-rate of the eluent

Temperature (°C)	pH	Flow-rate (ml/min)	k			$\alpha_{c,c}$	$\alpha_{t,t}$	$R_{S:c,c}$	$R_{S:c,t}$	$R_{S:t,t}$
			cis	*trans*						
			(1*S*,2*R*) & (1*R*,2*S*)	(1*S*,2*S*)	(1*R*,2*R*)					
25	1.0	0.50	2.05	3.72	4.83	0.00	1.29	0.00	3.65	2.38
	2.0	0.50	1.51	2.81	3.51	0.00	1.25	0.00	3.23	1.87
		0.25	1.53	2.86	3.55	0.00	1.25	0.00	3.49	1.87
20	1.5	0.50	2.03	3.73	4.78	0.00	1.28	0.00	3.75	2.15
		0.25	2.02	3.72	4.75	0.00	1.27	0.00	5.18	3.45
	1.0	0.50	2.33	4.35	5.83	0.00	1.34	0.00	4.65	3.41
		0.25	2.33	4.41	6.00	0.00	1.36	0.00	5.06	4.00
15	1.0	0.50	2.54	5.05	7.09	0.00	1.40	0.00	4.75	4.02
10	1.0	0.50	2.88	5.97	8.72	0.00	1.46	0.00	5.60	4.16
	2.0	0.50	2.31	4.73	6.49	0.00	1.37	0.00	3.82	2.91
		0.25	2.33	4.89	6.72	0.00	1.37	0.00	4.40	3.30
5	1.5	0.50	3.00	6.27	8.96	0.00	1.42	0.00	4.83	3.75
		0.25	2.97	6.28	8.95	0.00	1.43	0.00	7.20	4.90
	1.0	0.50	3.52	7.57	11.31	0.00	1.50	0.00	5.70	4.38
		0.25	3.56	7.60	11.44	0.00	1.49	0.00	8.00	5.84

Column, Crownpak CR(+); eluent, aqueous perchloric acid; detection, 195 nm; $R_{S:c,t}$ represents resolution of *cis*[(1*S*,2*R*)+(1*R*,2*S*)] and *trans*(1*S*,2*S*) isomers; $\alpha_{t,t}$ and $R_{S:t,t}$ represent separation of *trans*(1*S*,2*S*) and *trans*(1*R*,2*R*) isomers.

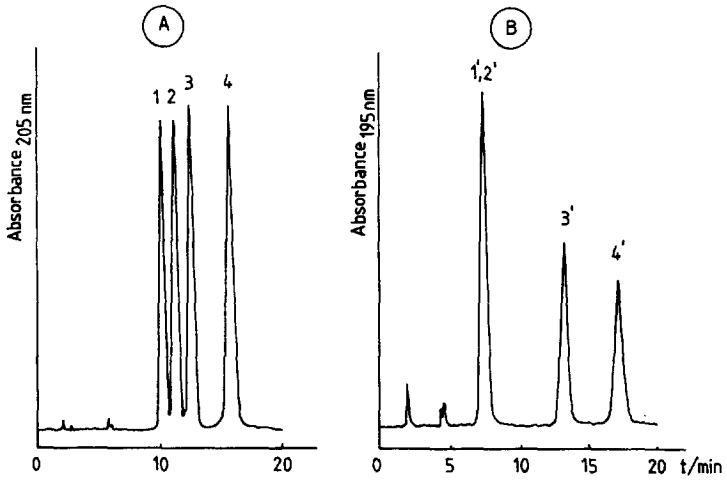

Fig. 3. Representative chromatograms of separation of four isomers of ACHC and ACHC-ene. (A) ACHC, (B) ACHC-ene. Column, Crownpak CR(+); flow-rate, 0.5 ml/min; detection 205 nm (A), 195 nm (B); column temperature, 5°C; mobile phase, perchloric acid, pH 2; peaks, numbered as Fig. 1.

Table 7
Free-energy differences $\Delta(\Delta G°)$ for the complex formation of ACHC and ACHC-ene with the crown ether at various temperature

Temperature (K)	$\Delta(\Delta G°)$(cal/mol)		
	cis-ACHC	*trans*-ACHC	*trans*-ACHC-ene
298	−56.4	−136.8	−150.8
293	−71.2	−157.1	−170.4
288	−80.0	−180.2	−192.6
283	−97.8	−205.1	−212.8
278	−109.8	−216.5	−224.0

Column, Crownpak CR(+); eluent, perchloric acid pH 1; flow-rate, 0.5 ml/min.

the separation gives a possibility for determination of the enthalpy and entropy of complex formation. Assuming a stoichiometry of 1:1 between the crown ether and the amines, the free-energy difference can be calculated from the equation $\Delta(\Delta G°) = -RT \ln\alpha$ [23]. The free-energy differences for the complexation of the investigated β-amino acids with the crown ether are listed in Table 7. The temperature dependence of $\Delta(\Delta G°)$ indicates that the complex formation of the *cis*(1*R*,2*S*)-ACHC enantiomer with the crown ether depends to a large extent on the enthalpy term, whereas the process that results in the formation of the less stable complex [containing the (1*S*,2*R*) enantiomer] is more dependent on the entropy term [24]. The same tendencies hold for the *trans*-ACHC and *trans*-ACHC-ene isomers, where the complexation of the (1*R*,2*R*) enantiomers is enthalpy-determined, while that of the (1*S*,2*S*) enantiomers is entropy-determined. Additionally, when the temperature is elevated from ambient to 5°C, discrimination of the *trans* isomers takes place with larger free-energy difference than that of the *cis* isomers. This indicates that the formation of the crown ether-amine complexes with the *trans* isomers is more dependent on the enthalpy term than the complexation with the *cis* isomers.

Table 8
Detection limits for ACHC and ACHC-ene analysis in different methods

Method	Detection λ (nm)	Detection limit (pmol)	
		ACHC	ACHC-ene
Direct	205 & 195	1700	100
Indirect			
GITC	250	16	13
FDAA	340	27	14

Columns, Crownpak CR(+) and Vydac 218TP54 C_{18}; volume of injection, 20 μl; signal-to-noise ratio 3.

4. Quantification

For separation and identification of the ACHC and ACHC-ene isomers, direct and indirect methods were applied. It is interesting to compare the smallest detectable amount in the different methods. The limit of detection was determined as a signal-to-noise ratio of 3. Table 8 shows the detection limits for ACHC and ACHC-ene determination in the direct and indirect methods. In the direct separation, the detectable concentration was much larger than on pre-column derivatization. The reason is that in the former case the UV absorption of the amino acids was quite poor (especially if they had an alkane skeleton). In the indirect methods, the derivatizing agents displayed good UV absorption, so the derivatives were detected at the absorption maximum of the reagents (in our case λ_{max}^{GITC}=250 nm and λ_{max}^{FDAA}=340 nm). Accordingly, the derivatives could be determined at concentrations one or two orders of magnitude lower. This fact calls attention to achiral derivatization (e.g. using a fluorescent derivatizing reagent) followed by enantiomer resolution on a chiral column. The lower detection limit for ACHC-ene as FDAA derivatives than as ACHC derivatives indicates a hyperchromic effect of the double bond.

5. Conclusions

The enantiomers of ACHC and ACHC-ene can be

separated with good resolution (R_S>1.6) as their GITC derivatives. FDAA as derivatizing reagent exhibits a larger enantioselectivity, but the resolution of the *cis*(1*S*,2*R*) and *trans*(1*R*,2*R*) enantiomers is not satisfactory. In some cases, application of THF as a second organic modifier improves the resolution of these two isomers. In general, with methanol as organic modifier similar resolutions can be achieved with smaller retention factors (k) than with acetonitrile.

The direct determination on a Crownpak CR(+) column proceeds with a good resolution for the ACHC enantiomers, while for ACHC-ene the *cis* enantiomers are not separable. A general elution sequence rule relating to the configuration of the amino acids does not seem to be warranted, since the reverse elution sequence may occur.

Acknowledgments

This work was supported by OTKA grants T14898 and T20454.

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