Journal of Chromatography A, 1217 (2010) 1075–1082

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

High-performance liquid chromatographic enantioseparation of β²-amino acids using a long-tethered (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-based chiral stationary phase

István Ilisz[a], Zoltán Pataj[a], Róbert Berkecz[a], Aleksandra Misicka[b,c], Dagmara Tymecka[b], Ferenc Fülöp[d], Hee Jung Choi[e], Myung Ho Hyun[e], Antal Péter[a,*]

[a] Department of Inorganic and Analytical Chemistry, University of Szeged, Dóm tér 7, H-6720 Szeged, Hungary
[b] Department of Chemistry, University of Warsaw, Pasteura str. 1, 02-093 Warsaw, Poland
[c] Medical Research Center, Polish Academy of Sciences, 02-106 Warsaw, Poland
[d] Institute of Pharmaceutical Chemistry, University of Szeged, Eötvös utca 6, H-6720 Szeged, Hungary
[e] Department of Chemistry and Chemistry Institute for Functional Materials, Pusan National University, Busan 609-735, South Korea

ARTICLE INFO

Article history:
Available online 8 July 2009

Keywords:
β²-Amino acids
Column liquid chromatography
Long-tethered
(+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-based chiral stationary phase
Spacer length effect
Temperature effect

ABSTRACT

Reversed-phase high-performance liquid chromatographic methods were developed for the separation of enantiomers of eleven unnatural β²-amino acids on a new chiral stationary phase, using the 11-methylene-unit spacer of aminoundecylsilica gel for the bonding of (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid as selector. The nature and concentration of the acidic and organic modifiers, the pH, the mobile phase composition, and the structures of the analytes substantially influenced the retention and resolution. Separations were carried out at constant mobile phase compositions in the temperature range 7–40 °C and the changes in enthalpy, $\Delta(\Delta H^\circ)$, entropy, $\Delta(\Delta S^\circ)$, and free energy, $\Delta(\Delta G^\circ)$ were calculated. The elution sequence was determined in some cases: the *S* enantiomers eluted before the *R* enantiomers.

1. Introduction

In view of owing to their presence in biologically active compounds with outstanding pharmacological properties, interest in β-amino acids in the past 20 years has continuously increased [1–8]. With an extra carbon atom between the amino and carboxylic groups, these β-amino acids have even greater potential for structural diversity than their α analogs; the availability of a number of stereo- and regioisomers, together with the possibility for further functionalization on the ring, adds to their structural diversity. Moreover, these β-amino acids are generally more stable to hydrolysis or enzymatic degradation than their α analogs, which leads to the enhanced stability of the peptides in which they are incorporated. β-Amino acids are used as starting substances for the synthesis of heterocyclic compounds, potential pharmacons and analogs of natural products. Their enantiomerically pure forms can serve as chiral auxiliaries in asymmetric transformations.

The enantioselective syntheses of β-amino acids require analytical methods with which to evaluate the enantiopurity of the final products. The separation and identification of β-amino acid enantiomers have mainly been performed by indirect and direct high-performance liquid chromatographic (HPLC) methods. In the past decade, new types of chiral stationary phases (CSPs) have been applied for the enantioseparation of β-amino acids (mainly β³-amino acids) by D'Acquarica et al. [9] and Péter et al. [10–14]. Since the introduction of chiral crown ethers as CSPs by Cram and co-workers [15], a (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-based CSP has been successfully applied for the enantioseparation of β³-amino acids by Hyun et al. [16–21] and Péter et al. [22,23]. Péter and co-workers [24] recently separated new β²-amino acids bearing aliphatic and aromatic side-chains on a CSP of (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid attached to silica with a short, 3-methylene-unit spacer: all aromatic β²-amino acid enantiomers were baseline-resolved, while amino acids with alkyl side-chains exhibited only partial separation.

Enantioselective retention mechanisms are sometimes influenced by temperature to a greater extent than are ordinary separations. This has been noted for some time in chiral gas chromatography [25,26]. It is additionally known that there are both achiral and chiral contributions to retention that can vary with a wide variety of experimental parameters [27–29]. Accordingly, the column temperature has often been optimized in enantioselective HPLC separations [30–36].

* Corresponding author. Tel.: +36 62 544000/3656; fax: +36 62 420505.
E-mail address: apeter@chem.u-szeged.hu (A. Péter).

0021-9673/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2009.07.003

The dependence of the retention of an analyte on the temperature can be expressed by means of the van't Hoff equation, which may be interpreted in terms of mechanistic aspects of chiral recognition:

$$\ln\ k' = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln\,\phi \quad (1)$$

This equation reveals that a plot of $\ln k'$ vs. $1/T$ is linear, with a slope of $-\Delta H^\circ/R$ and an intercept of $\Delta S^\circ/R + \ln\phi$, if ΔH° is invariant with temperature. In chiral chromatography, however, the van't Hoff plot often deviates from linearity, possibly as a result of the inhomogeneity of the CSP surface, leading to a mixed retention mechanism.

Chromatographic chiral separations are determined by the difference in free energy $\Delta(\Delta G^\circ)$ of adsorption of the enantiomers, and in a plot of $R\ln\alpha$ vs. $1/T$, the slope is $-\Delta(\Delta H^\circ)$ and the intercept is $\Delta(\Delta S^\circ)$:

$$\Delta(\Delta G^\circ) = -\mathrm{RT}\ \ln\alpha = \Delta(\Delta H^\circ) - T\Delta(\Delta S^\circ) \quad (2)$$

In the present paper, direct HPLC methods are described for the enantioseparation of new racemic β^2-amino acids (Fig. 1), with the application of a (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid selector connected to silica gel through a relatively long, 11-methylene-unit spacer [19]. The long spacer of the CSP is purely lipophilic and flexible, and consequently it improves the mobility of the residual aminoalkyl groups and the chiral selector moiety of the CSP. The longer spacer of the CSP is expected to exert significant effects on the separation of amino acids with alkyl side-chains. The effects on the separation of parameters such as the natures and concentrations of acidic and organic (alcoholic) modifiers, the mobile phase composition, the structure of the analyte and temperature are examined and discussed. The elution sequence was determined in some cases.

2. Experimental

2.1. Chemicals and reagents

Two-step syntheses were applied for the production of racemic 3-amino-2-methylpropionic acid (**1**), 2-aminomethylbutanoic acid (**2**), 2-aminomethylpentanoic acid (**3**), 2-aminomethylhexanoic acid (**4**), 2-aminomethyl-3-methylbutanoic acid (**5**), 2-aminomethyl-4-methylpentanoic acid (**6**), 3-amino-2-benzylpropionic acid (**7**), 3-amino-2-(4-hydroxybenzyl)propionic acid (**8**), 3-amino-2-(3-hydroxybenzyl)propionic acid (**9**), 3-amino-2-(4-ethoxybenzyl)propionic acid (**10**) and 3-amino-2-benzo[1,3]-dioxol-5-yl-methylpropionic acid (**11**) (Fig. 1). In the first step, the starting material, methyl cyanoacetate, was either alkylated [37] or condensed with aromatic aldehydes [38,39], and in the next step it was reduced (and *N*-protected). Enantiomers (*S*)-**1**, (*S*)-**5** and (*S*)-**6** were generous gifts from Prof. D. Tourwé (Vrije Universiteit Brussels, Brussels, Belgium). The addition of benzylamine to ethyl ethacrylate furnished the corresponding *N*-benzylamino ester, which was transformed to racemic ethyl 3-amino-2-ethylpropanoate, the ethyl ester of **2**, by catalytic hydrogenolysis. Compound (*R*,*S*)-**2** was resolved via *Candida antarctica* lipase-A-catalyzed *N*-acylation in *tert*-amyl alcohol with ethyl butanoate, yielding (*R*)-**2** [40].

Acetonitrile (MeCN), methanol (MeOH) of HPLC grade and glacial acetic acid (AcOH) were purchased from Merck (Darmstadt, Germany). Formic acid (HCOOH), trifluoroacetic acid (TFA), ethanol (EtOH), 1-propanol (PrOH), 2-propanol (IPA) and other reagents of analytical reagent grade were from Sigma–Aldrich Kft (Budapest, Hungary). Ultrapure Milli-Q water was further purified by filtration on a 0.45-μm Millipore filter, type HV (Molsheim, France).

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-2996 photodiode-array detector and a Millenium^{32} Chromatography Manager data system; the alternative Waters Breeze system

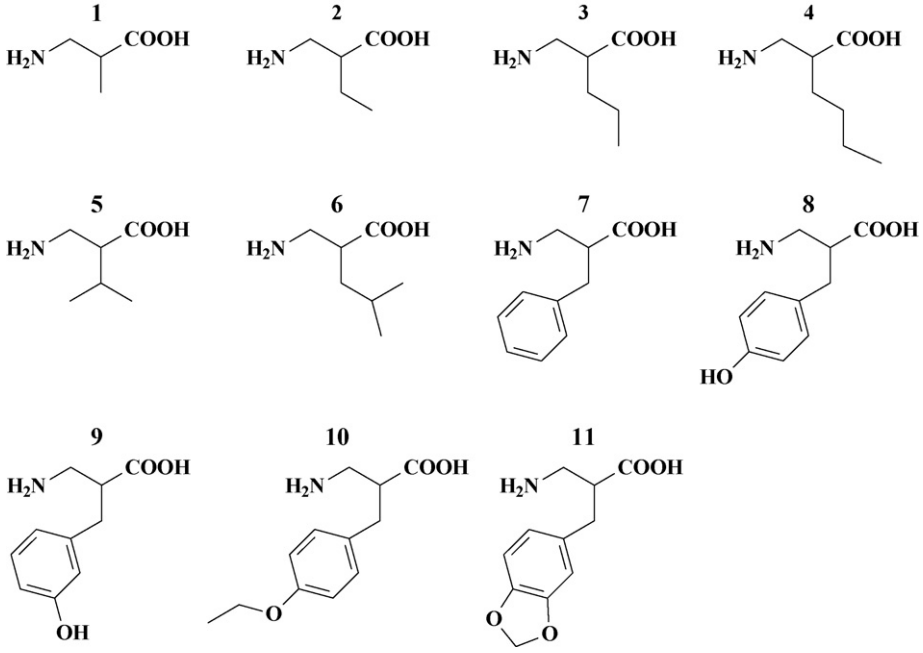

Fig. 1. (**1**) 3-Amino-2-methylpropionic acid, (**2**) 2-aminomethylbutanoic acid, (**3**) 2-aminomethylpentanoic acid, (**4**) 2-aminomethylhexanoic acid, (**5**) 2-aminomethyl-3-methylbutanoic acid, (**6**) 2-aminomethyl-4-methylpentanoic acid, (**7**) 3-amino-2-benzylpropionic acid, (**8**) 3-amino-2-(4-hydroxybenzyl)propionic acid, (**9**) 3-amino-2-(3-hydroxybenzyl)propionic acid, (**10**) 3-amino-2-(4-ethoxybenzyl)propionic acid, and (**11**) 3-amino-2-benzo[1,3]dioxol-5-yl-methylpropionic acid.

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 (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-based CSP containing the 11-methylene-unit spacer, 5-μm particle size, 150 mm × 4.0 mm I.D., was prepared via the method described in a previous paper [19]. The column was thermostated in a Spark Mistral column thermostat (Spark Holland, Emmen, The Netherlands). The precision of the temperature adjustment was ±0.1 °C.

Mobile phases were prepared by mixing the indicated volumes of solvents and were further purified by filtration through a 0.45-μm Millipore filter, type HV. The eluents were degassed in an ultrasonic bath and He gas was purged through them during the analysis. Stock solutions of analytes (1 mg ml^{-1}) were prepared by dissolution in the starting mobile phase.

2.3. HPLC operating conditions applied for validation process

To determine validation characteristics of the analytical procedure separations were carried out repeatedly on the investigated CSP with a mobile phase H_2O/IPA = 62/38 (v/v) + 10 mM AcOH for two representative analytes, compound **6** bearing an alkyl side-chain and **7** bearing an aromatic side-chain, at a flow rate of 0.5 ml min^{-1} maintaining the column at 25 °C. The injection volume was 20 μl, while the detector was set at 205 nm.

3. Results and discussion

The analytes in this study (Fig. 1) can be arranged into two classes. Compounds **1**–**6** bear alkyl groups, while compounds **7**–**11** bear aromatic rings. It has generally been accepted that, for a crown ether-based CSP, the most important interaction is the host–guest complexation of the primary ammonium ion (R–NH_3^+) and the oxygen atoms in the crown ether ring. Lee et al. [41] reported that the high enantioselectivity of this type of CSP for α-amino acids was due to the H-bonding between one carboxylic acid in the CSP and a carbonyl group oxygen in the amino acid. Functional groups on β^2-homoamino acids are potentially able to contribute to hydrophobic, π–π complexation, dipole stacking, H-bonding, electrostatic, short-distance van der Waals interactions, and steric/rigidity effects with the CSP.

3.1. Effects of acidic modifier

In order to investigate the effects of acidic modifiers on the resolution behavior of the CSP, analyte **4** with an alkyl and **7** with an aryl side-chain were chosen as model compounds (Table 1). The concentration of MeOH was fixed at 20% (v/v) and that of the acidic modifier at 10 mM. In example separations, the largest k' values were in most cases obtained on the application of HCOOH or AcOH, while $HClO_4$ and H_2SO_4 resulted in the lowest k' values (Table 1).

The application of HCOOH, AcOH, TFA, $HClO_4$ or H_2SO_4 at the same concentration (10 mM) resulted in different levels of pH_a (the actual pH measured in the hydro-organic mobile phase). In a detailed investigation of the effects of pH, analytes **1**, **4**, **6**, **7**, **8** and **10** were chosen as model compounds. A decrease of the pH from 5.22 to 3.65 in the H_2O/MeOH = 50/50 (v/v) + AcOH aqueous mobile phase system decreased the retention factors by 70–80%. Stronger decreases (80%) were observed for the analytes bearing alkyl side-chains (**1**, **4** and **6**). As the acid content was elevated, the ionic strength of the mobile phase increased, while the pH decreased. The hydrophilic interaction of the ionic analyte with the polar mobile phase was then expected to increase, with a consequent decrease in the retention time. However, the change of the pH exhibited only slight effects on the α and R_S values. On decrease of the pH from 5.22 to 3.65, α exhibited a slight change (decrease), while R_S decreased by about 10–15%. This behavior differed from that observed on short-tethered CSP, where α and R_S increased with decreasing pH [24]. The length of the spacer may have significant influence on the enantiorecognition and it may help or impede the achiral interactions [42]. Steric reasons may also contribute to the observed behavior.

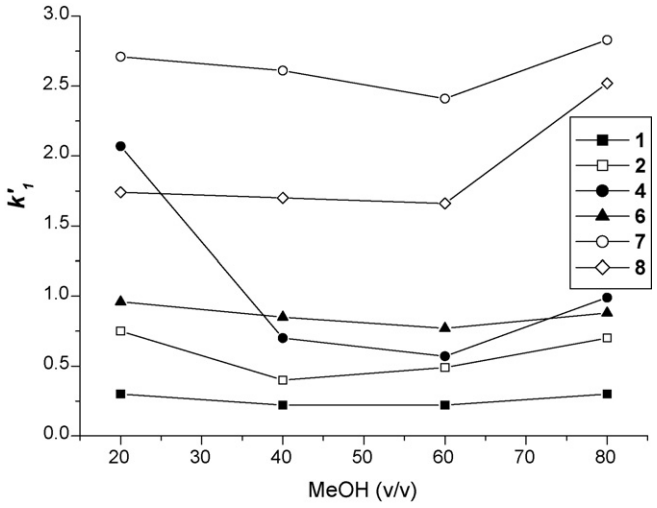

Fig. 2. Effects of MeOH content on retention factor of first-eluting enantiomer (k'_1) for analogs **1**, **2**, **4**, **6**, **7** and **8**. Chromatographic conditions: column, long-tethered (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-based CSP; mobile phase, H_2O/MeOH (v/v) + 10 mM AcOH; flow rate, 0.5 ml min^{-1}; detection, 205 nm; analyte, **1** (■); **2** (□); **4** (●); **6** (▲); **7** (○); **8** (◊).

Table 1 reveals that, on the application of different acidic modifiers at constant pH_a the differences in k'_1 for analytes **4** and **7** were not as high as when the different acidic modifiers were used at the same concentration. For analyte **4**, k'_1 ranged between 1.58 and 2.13, while for analyte **7** it was between 3.65 and 4.51. The somewhat higher k' for $HClO_4$ was due to the low complex-formation ability of ClO_4^- [43], indicating the weakest stereoselective complexation of primary ammonium ions of analytes with ClO_4^-. It was established that the use of different acidic modifiers with the same pH_a may result in similar chromatographic results, but the chromatographic parameters may be influenced by other effects, such as the charge/ionic radius (e/r) value of the anion, the ability of the anions to form complexes and ion-pairs, *etc*.

3.2. Effects of organic modifier

The effects of the MeOH content of the mobile phase on the retention, selectivity and resolution for analytes **1**, **2**, **4** and **6** containing an alkyl side-chain, and for analytes **7** and **8** containing a phenyl or a hydroxyphenyl side-chain, are depicted in Fig. 2. For all these analytes, a U-shaped curve was observed. At higher water content, the retention factor increased with increasing water content, especially for analyte **4**, due to the enhanced hydrophobic interactions in the water-rich mobile phase. Analyte **4** bears a long lipophilic alkyl side-chain capable of hydrophobic interactions with the 11-methylene-unit spacer. As the content of MeOH in the aqueous mobile phase was increased from 50 to 80% (v/v), k' increased again. With increasing MeOH content, the mobile phase became less polar and more hydrophobic. In this instance, the hydrophilic interactions between the mobile phase and the polar analytes decreased, and consequently the retention increased. Here, as earlier for macrocyclic antibiotic-based CSPs, the inflection point and the slope of the U-shaped curve at higher and lower MeOH

Table 1
Chromatographic data, retention factors (k'), separation factors (α) and resolutions (R_S) for analytes **4** and **7** on long-tethered (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid CSP with variation of the type and concentration (pH) of the acidic modifier of the mobile phase.

Mobile phase	Analyte							
	4				**7**			
	k'_1	k'_2	α	R_S	k'_1	k'_2	α	R_S
20% MeOH + 10 mM HCOOH (pH_a 3.03)	2.23	3.63	1.63	0.95	4.03	5.83	1.45	1.40
20% MeOH + 10 mM AcOH (pH_a 3.54)	2.07	3.07	1.48	1.60	2.71	3.96	1.46	1.40
20% MeOH + 10 mM TFA (pH_a 2.11)	1.00	1.61	1.61	1.85	1.91	2.47	1.29	1.25
20% MeOH + 10 mM $HClO_4$ (pH_a 2.10)	0.78	1.27	1.63	1.92	1.41	1.96	1.39	1.29
20% MeOH + 10 mM H_2SO_4 (pH_a 1.91)	0.86	1.38	1.60	2.00	1.60	2.15	1.34	1.60
20% MeOH + HCOOH (pH_a 3.99)	2.06	3.50	1.70	1.60	4.05	5.71	1.41	1.25
20% MeOH + AcOH (pH_a 3.98)	1.78	3.05	1.71	2.30	4.03	5.84	1.45	1.40
20% MeOH + TFA (pH_a 3.96)	1.58	2.63	1.66	2.10	3.80	5.43	1.43	1.65
20% MeOH + $HClO_4$ (pH_a 3.95)	2.13	3.68	1.72	2.35	4.51	6.29	1.39	1.95
20% MeOH + H_2SO_4 (pH_a 3.97)	2.09	3.33	1.60	1.80	3.65	5.73	1.47	2.95

Chromatographic conditions: mobile phase: H_2O/MeOH = 80/20 (v/v) + acidic modifier; flow rate: 0.5 ml min^{-1}; detection: 205 nm; pH_a: actual pH, measured in hydro-organic mobile phase; temperature, ambient.

concentrations differed somewhat for each compound [44]. Comparison of the retention behavior of β^2-homo-amino acids on the short- or the long-tethered CSPs demonstrated that the increase of k' with increasing water content was missing in the case of the short-tethered CSP [24], *i.e.* the hydrophobic and steric interactions were more significant on the long-tethered CSP.

For β^2-amino acids possessing alkyl side-chains, α and R_S slightly increased with increasing MeOH content (the increases in R_S were more progressive), while for amino acids with aromatic side-chains, both α and R_S slightly decreased with increasing MeOH content. Such a difference in the behavior of amino acids possessing aliphatic and aromatic side-chains was not observed on the CSP with the short tethering group, where decreases in α and R_S with increasing alcohol content were observed for both types of analytes [24].

The nature of the alcohol influenced the retention and resolution. Table 2 reveals that for analytes **1**, **4**, **6**, **7**, **8** and **10** at constant alcohol concentration (4.93 mol l^{-1}), the change in k' did not appear to correlate with the carbon number of the alcohols. From these six examples (Table 2), it can be concluded that MeOH and EtOH in most cases gave larger k' values than those for PrOH or IPA. The apolar character of the mobile phase increased in the sequence MeOH < EtOH < PrOH < IPA and, due to the decreased polar interactions between the mobile phase and the analytes, an increase in retention in this sequence would therefore be expected, as reported in the case of the short-tethered CSP [24] (only analyte **6** exhibited a slight increase in k' with increasing number of carbon atoms in the alcohol). The decrease in retention was more pronounced for IPA. The enantioselectivity changed when the different alcohols were applied at the same molar concentration, and a slight increase in α was generally observed with increasing number of carbon atoms in the alcohol. The nature of the alcohol exhibited a significant effect on the resolution but no general rule could be established. In most cases, the smallest resolution was observed on the application of PrOH (an exception was **6**), whereas the application of MeOH or IPA ensured the largest R_S values. The reason for this chromatographic behavior is not yet clear; the long-tethered selector probably swells more in these solvents, resulting in better mass-transfer kinetics and higher R_S values.

3.3. Structure–retention relationship

The k' values may vary with the structures of the analytes. With the mobile phase H_2O/MeOH = 80/20 (v/v) + 10.0 mM AcOH, k' var-

Table 2
Retention factor of first-eluting enantiomer (k'_1), separation factor (α) and resolution (R_S) of enantiomers of β^2-homo-amino acids as a function of nature of alcohol.

Analyte	k'_1, α, R_S	Alcohol				Analyte	k'_1, α, R_S	Alcohol			
		MeOH	EtOH	PrOH	IPA			MeOH	EtOH	PrOH	IPA
1	k'_1	0.30	0.33	0.32	0.21	**7**	k'_1	2.71	2.83	1.54	1.52
	α	1.29	1.54	1.40	1.55		α	1.46	1.41	1.34	1.57
	R_S	1.55	0.80	0.90	1.00		R_S	1.40	1.70	1.35	2.15
2	k'_1	0.75	–	–	–	**8**	k'_1	1.74	1.72	1.02	0.85
	α	1.60	–	–	–		α	1.39	1.31	1.21	1.32
	R_S	1.40	–	–	–		R_S	1.90	1.40	0.95	1.25
3	k'_1	1.30	–	–	–	**9**	k'_1	2.00	–	–	–
	α	1.70	–	–	–		α	1.41	–	–	–
	R_S	1.65	–	–	–		R_S	1.60	–	–	–
4	k'_1	2.07	2.02	1.22	0.97	**10**	k'_1	7.10	5.89	1.89	1.66
	α	1.48	1.67	1.57	2.10		α	1.53	1.49	1.38	1.60
	R_S	1.55	1.75	0.90	1.45		R_S	1.74	1.38	1.40	1.82
5	k'_1	0.84	–	–	–	**11**	k'_1	6.20	–	–	–
	α	1.45	–	–	–		α	1.55	–	–	–
	R_S	1.55	–	–	–		R_S	1.60	–	–	–
6	k'_1	0.94	1.22	1.17	0.99						
	α	1.28	1.83	1.70	2.11						
	R_S	1.55	1.50	2.45	2.80						

Column, long-tethered (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-based CSP; mobile phase, H_2O/MeOH = 80/20 (v/v) + 10 mM AcOH, H_2O/EtOH = 71/29 (v/v) + 10 mM AcOH, H_2O/PrOH = 62/38 (v/v) + 10 mM AcOH, H_2O/IPA = 62/38 (v/v) + 10 mM AcOH; flow rate, 0.5 ml min^{-1}; detection, 205 nm; temperature, ambient.

Table 3
Retention factor of first-eluting enantiomer (k'_1), separation factor (α) and resolution (R_S) of enantiomers of β²-homo-amino acids as a function of temperature.

Analyte	Mobile phase	Temperature (°C)														
		7			15			20			30			40		
		k'_1	α	R_S	k'_1	α	R_S	k'_1	α	R_S	k'_1	α	R_S	k'_1	α	R_S
1	**a**	0.35	1.33	0.65	0.32	1.29	0.70	0.31	1.26	0.75	0.28	1.22	0.35	0.26	1.18	0.20
	b	0.26	1.63	1.25	0.23	1.59	1.30	0.21	1.55	1.00	0.19	1.48	0.95	0.16	1.43	0.40
2	**b**	0.78	1.69	1.90	0.67	1.64	1.95	0.62	1.62	1.90	0.52	1.58	1.20	0.45	1.53	0.80
3	**b**	0.89	2.11	1.95	0.79	1.96	2.45	0.73	1.92	2.80	0.60	1.79	2.60	0.50	1.71	1.45
4	**a**	2.62	1.36	1.10	2.21	1.32	1.20	2.07	1.27	1.80	1.79	1.23	1.20	1.50	1.18	0.90
	b	1.33	2.31	1.85	1.09	2.17	1.90	0.97	2.10	3.45	0.81	1.92	2.80	0.66	1.83	2.00
5	**b**	0.99	1.76	1.75	0.84	1.72	1.80	0.75	1.69	2.10	0.59	1.64	2.30	0.48	1.60	1.60
6	**a**	1.21	1.31	1.20	1.03	1.29	1.75	0.94	1.28	2.80	0.79	1.26	3.00	0.65	1.23	1.05
	b	1.52	2.22	2.40	1.12	2.16	2.45	0.99	2.11	2.75	0.71	2.03	2.35	0.54	1.95	2.10
7	**a**	4.14	1.54	1.85	3.11	1.50	1.90	2.71	1.46	1.45	2.08	1.42	1.30	1.50	1.39	1.20
	b	2.36	1.65	1.95	1.70	1.60	2.15	1.52	1.57	2.15	1.07	1.48	1.95	0.78	1.42	1.80
8	**a**	2.83	1.57	2.10	2.09	1.46	2.20	1.74	1.39	1.85	1.27	1.31	1.20	0.97	1.21	1.00
	b	1.25	1.36	1.15	0.95	1.34	1.25	0.85	1.32	1.20	0.61	1.30	1.10	0.44	1.28	0.85
10	**a**	9.04	1.57	1.30	7.43	1.54	1.60	6.21	1.51	1.75	4.88	1.47	1.90	3.46	1.42	1.80
	b	2.54	1.83	1.70	1.96	1.64	1.70	1.66	1.60	1.80	1.26	1.44	1.70	0.95	1.35	1.65

Column, long-tethered CSP; mobile phase, **a**, H_2O/MeOH = 80/20 (v/v) + 10 mM AcOH, **b**, H_2O/IPA = 62/38 (v/v) + 10 mM AcOH; flow rate, 0.5 ml min^{-1}; detection, 205 nm.

ied with the length of the alkyl chain in the molecules (Table 2). Molecules **1**–**4**, with increasing *n*-alkyl chains, are more hydrophobic and flexible, and hydrophobic/steric interactions between the analyte and the long-tethered CSP are therefore favored: k' increased. The relatively long spacer of CSP is lipophilic and flexible, and consequently it improves the mobility of the chiral selector moiety of the CSP. For analytes **5** and **6**, in spite of the longer alkyl chain, the smaller k' values indicate that a branched alkyl group diminishes the stabilization of the molecule–CSP complex. The presence of an extra CH_2 group in analyte **6** as compared with **5** increases the flexibility and lipophilicity of the molecule: k' again increased.

For analytes **7**–**11**, bearing an aromatic ring, k' proved higher as compared with **1**–**6**. The π character of the analytes contributes to the retention through the interaction with the polar moieties of the selector. The presence of –OH or –O– groups in analytes **8**–**11** may improve the interaction with the selector through the H-bonding or may decrease the apolar interaction ability with CSP due to the change in polarity. The decrease in the retention factor when **8** and **9** are compared with **7** and **11** is compared with **10** underlines the importance of lipophilic interactions with the long-tethered CSP.

The elution sequences for analytes **1**, **2**, **5** and **6** were determined by injecting configurationally known samples. The elution sequences were consistent, the *R* enantiomers being retained longer than the *S* enantiomers. Selected chromatograms for the enantioseparation of analytes **1**–**11** are depicted in Fig. 3.

3.4. Temperature effects and thermodynamic parameters

In order to investigate the effects of temperature on the chromatographic parameters, a variable-temperature study was carried out between 7 and 40 °C at mobile phase compositions of H_2O/MeOH = 80/20 (v/v) + 10 mM AcOH and H_2O/IPA = 62/38 (v/v) + 10 mM AcOH (the concentration of MeOH or IPA was 4.93 M in both cases) (Table 3). A comparison of the retention factors in Table 3 shows that k'_1 and α decreased with increasing temperature. It is evident that an increase in separation temperature lowers the separation factor, α, but it may also improve the peak symmetry. Since the effect of temperature on the separation was more complex, an extensive study dealing with the thermodynamics of enantiomer separation was carried out.

Several papers have been published that discuss the effects of temperature on enantiomers HPLC separation [27,45,46,34,47]. In order to calculate the thermodynamic parameters, van't Hoff plots were constructed [Eq. (1)]. The ΔH° and $\Delta S^{\circ *} = \Delta S^\circ + \ln \phi$, values calculated from the slopes and intercepts of the plots of Eq. (1) for the enantiomers in both eluent systems were negative. Further, the ΔH° and $\Delta S^{\circ *}$ values for the first-eluting enantiomer were always less negative than those for the second-eluting enantiomer. The

Table 4
Thermodynamic parameters, $\Delta(\Delta H^\circ)$, $\Delta(\Delta S^\circ)$, $\Delta(\Delta G^\circ)$ and correlation coefficient (R^2) of analytes measured with different mobile phase additives.

Analyte	Mobile phase H_2O/MeOH = 80/20 (v/v) + 10 mM AcOH				Mobile phase H_2O/IPA = 62/38 (v/v) + 10 mM AcOH			
	$-\Delta(\Delta H^\circ)$ (kJ mol^{-1})	$-\Delta(\Delta S^\circ)$ (J mol^{-1} K^{-1})	Correlation coefficient, R^2	$-\Delta(\Delta G^\circ)_{293\,K}$ (kJ mol^{-1})	$-\Delta(\Delta H^\circ)$ (kJ mol^{-1})	$-\Delta(\Delta S^\circ)$ (J mol^{-1} K^{-1})	Correlation coefficient, R^2	$-\Delta(\Delta G^\circ)_{293\,K}$ (kJ mol^{-1})
1	2.4	6.2	0.9986	0.56	3.0	6.5	0.9921	1.07
2	–	–	–	–	2.1	3.2	0.9958	1.17
3	–	–	–	–	4.6	10.2	0.9906	1.60
4	3.2	8.8	0.9944	0.59	5.3	12.0	0.9936	1.80
5	–	–	–	–	2.2	3.1	0.9977	1.27
6	1.3	2.5	0.9959	0.59	2.9	3.7	0.9992	1.82
7	2.3	4.6	0.9910	0.92	3.3	7.5	0.9923	1.09
8	5.6	16.2	0.9975	0.81	1.3	2.1	0.9908	0.68
10	2.2	4.0	0.9928	1.31	6.7	19.0	0.9901	1.14

Column, long-tethered CSP; R^2 correlation coefficient of plot $R \ln \alpha$ vs. $1/T$ curves.

differences in ΔH° and ΔS° values of the second- and first-eluting enantiomers are depicted in Table 4.

For the amino acids bearing an aliphatic side-chain (**1**–**6**), the $-\Delta H^\circ$ and $-\Delta S^{\circ *}$ values in both eluent systems increased with increasing lipophilicity. For analytes **1**–**6** in the H_2O/MeOH system, $-\Delta H^\circ$ ranged between 6.3 and 14.8 kJ mol^{-1}, and in the H_2O/IPA system between 10.3 and 25.8 kJ mol^{-1}. In the H_2O/MeOH system, $-\Delta S^{\circ *}$ ranged between 31.4 and 49.8 J mol^{-1} K^{-1}, and in the H_2O/IPA system between 48.1 and 82.0 J mol^{-1} K^{-1}. It was also observed that for the same analyte the ΔH° and $\Delta S^{\circ *}$ values in the H_2O/IPA system were in most cases more negative than in the H_2O/MeOH eluent system.

For amino acids bearing an aromatic side-chain, somewhat larger $-\Delta H^\circ$ and $-\Delta S^{\circ *}$ values were observed and these values did not differ appreciably in the two eluent systems (for analytes **7**, **8** and **10** in both eluent systems, the $-\Delta H^\circ$ values ranged between 22.0 and 28.0 kJ mol^{-1} and the $-\Delta S^{\circ *}$ values between 60.0 and 88.0 J mol^{-1} K^{-1}).

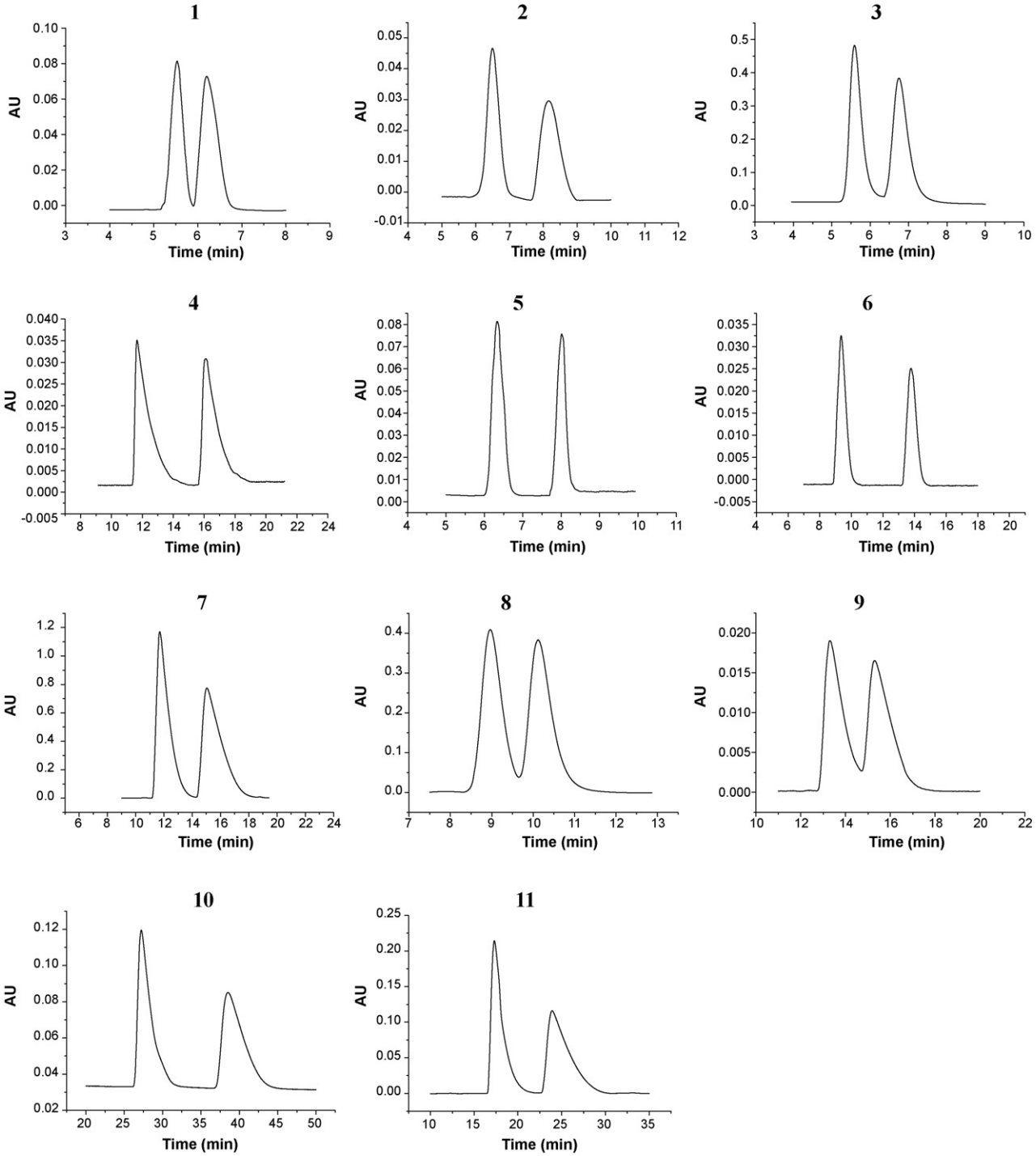

Fig. 3. Selected chromatograms for analytes **1**–**11**. Chromatographic conditions: column, long-tethered (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-based CSP; mobile phase, H_2O/IPA = 62/38 (v/v) + 10 mM AcOH for analytes **1**–**3**, **5**–**8**, H_2O/MeOH = 80/20 (v/v) + 10 mM AcOH for analyte **4**, H_2O/MeOH = 50/50 (v/v) + 10 mM AcOH for analytes **9** and **11** and H_2O/EtOH = 71/29 (v/v) + 10 mM AcOH for analyte **10**; flow rate, 0.5 ml min^{-1}; detection, 205 nm; temperature, 15 °C for analyte **2**, 40 °C for analyte **3**, 30 °C for analyte **5**, and ambient temperature for the others.

The data on the changes in $\Delta(\Delta H^\circ)$, $\Delta(\Delta S^\circ)$ and $\Delta(\Delta G^\circ)$ are depicted in Table 4. For the β^2-amino acid analogs bearing an alkyl side-chain, the $-\Delta(\Delta H^\circ)$ and $-\Delta(\Delta S^\circ)$ were most negative for analyte **4** in both eluent systems, indicating that the interaction of the long alkyl side-chain of the analyte with the long-tethered CSP contributes not only to the retention, but also to the selectivity. The data on the changes in $-\Delta(\Delta G^\circ)$ indicate that enantioseparation on the long-tethered CSP in the H_2O/IPA = 62/38 (v/v) + 10 mM AcOH mobile phase system is more favorable for β^2-amino acids bearing an alkyl side-chain (Table 4). The largest negative $\Delta(\Delta G^\circ)$ values for **4** and **6** suggest that the lipophilic or steric/rigid interactions induce highly efficient binding to the selector. The difference in $-\Delta(\Delta G^\circ)$ values for **5** and **6** may be explained by the differences due to the steric and lipophilic effects of the one methylene group difference in the alkyl side-chains. The $-\Delta(\Delta G^\circ)$ values of analytes **7**, **8** and **10** indicate that the more polar H_2O/MeOH = 80/20 (v/v) + 10 mM AcOH mobile phase favors the interactions between the aromatic side-chain and the CSP, resulting in higher $-\Delta(\Delta G^\circ)$ values.

In summary, the complex formation that involved multiple intermolecular interactions was generally exothermic, and the corresponding entropic contribution was also negative. The negative $\Delta(\Delta G^\circ)$ values originated from the negative $\Delta(\Delta H^\circ)$ values and consequently the separations for all the investigated analytes on this CSP were enthalpically favored.

However, the exact chiral recognition mechanism on the long-tethered CSP is not yet clear; further investigations are needed to clarify the effects of acidic/alcoholic modifiers and interactions governing the chiral recognition.

4. Method validation

4.1. Linearity

Solutions of the racemates were prepared at six different concentration levels, from 0 to 875 $\mu g\,ml^{-1}$ and 0 to 10 $ng\,ml^{-1}$ for analytes **6** and **7**, respectively. Three parallel injections of each solution were made under the chromatographic conditions described above. The peak area response of the first and the second-eluting enantiomers was plotted against the corresponding concentration and the linear regression was computed by the least square method using Microsoft Excel program. Very good linearity was observed in the investigated concentration range with the following regression equations; $y = 1538x + 4703$ ($R^2 = 0.9997$) and $y = 132{,}993x + 1313$ ($R^2 = 0.9999$) for compounds **6** and **7**, respectively. (The difference of the regression parameters of the enantiomers was within the standard error.)

4.2. Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ were determined based on the calibration curve according to the ICH guidelines [48]. LOD was 10 $\mu g\,ml^{-1}$ and 0.02 $ng\,ml^{-1}$, while LOQ 30 $\mu g\,ml^{-1}$ and 0.06 $ng\,ml^{-1}$ for **6** and **7**, respectively.

4.3. Precision

Replicate HPLC analysis showed that the relative standard deviation was no more than 6% for the peak area response and less than 4% for the retention time.

5. Conclusions

This study has demonstrated that the long-tethered CSP is quite successful for the direct enantioseparation of the investigated β^2-amino acids. The chromatographic retention and resolution behavior was found to be dependent on the natures and concentrations of the acidic and alcoholic modifiers and the nature of the substituents in the β position. On the basis of the thermodynamic parameters, the separation proved to be enthalpically favored. In contrast with the short-tethered CSP, the long-tethered CSP exhibited excellent resolution for β-amino acids, either bearing aliphatic or aromatic side-chains in the β position. The elution sequence followed the general rule ($S < R$) established earlier for α- and β-amino acids.

Acknowledgements

This work was supported by OTKA grant K 67563, by UE grant LSHC-CT-2006-037733 and by the Korea Science and Engineering Foundation (NCRC program: R15-2006-022-03001-0). I.I. wishes to express his thanks for a Bolyai János Postdoctoral Research Scholarship to support his work.

References

[1] E. Juaristi (Ed.), Enantioselective Synthesis of β-Amino Acids, Wiley–VCH, New York, 1997.
[2] F. Juaristi, H. López-Ruiz, Curr. Med. Chem. 6 (1999) 983.
[3] F. Fülöp, Chem. Rev. 101 (2001) 2181.
[4] J.-A. Ma, Angew. Chem., Int. Ed. 42 (2003) 4290.
[5] E. Juaristi, V.A. Soloshonok (Eds.), Enantioselective Synthesis of β-Amino Acids, second edition, Wiley–VCH, Hoboken, NJ, 2005.
[6] E. Forró, F. Fülöp, Mini Rev. Org. Chem. 1 (2004) 93.
[7] A. Liljeblad, L.T. Kanerva, Tetrahedron 62 (2006) 5831.
[8] F. Fülöp, T.A. Martinek, G.K. Tóth, Chem. Soc. Rev. 35 (2006) 323.
[9] I. D'Acquarica, F. Gasparrini, D. Misiti, G. Zappia, C. Cimarelli, G. Palmieri, A. Carotti, S. Cellamare, C. Villani, Tetrahedron Asymm. 11 (2000) 2375.
[10] A. Péter, L. Lázár, F. Fülöp, D.W. Armstrong, J. Chromatogr. A 926 (2001) 229.
[11] A. Péter, J. Chromatogr. A 955 (2002) 141.
[12] A. Péter, A. Árki, E. Vékes, D. Tourwé, L. Lázár, F. Fülöp, J. Chromatogr. A 1031 (2004) 171.
[13] A. Péter, R. Török, K. Wright, M. Wakselman, J.P. Mazaleyrat, J. Chromatogr. A 1021 (2003) 1.
[14] I. Ilisz, R. Berkecz, A. Péter, J. Sep. Sci. 29 (2006) 1305.
[15] E.P. Kyba, M.G. Siegel, L.R. Sousa, G.D.Y. Sogah, D.J. Cram, J. Am. Chem. Soc. 95 (1973) 2691.
[16] M.H. Hyun, Y.J. Cho, J.S. Jin, J. Sep. Sci. 25 (2002) 648.
[17] M.H. Hyun, S.C. Han, S.H. Whangbo, J. Chromatogr. A 992 (2003) 47.
[18] M.H. Hyun, S.C. Han, S.H. Whangbo, Biomed. Chromatogr. 17 (2003) 292.
[19] M.H. Hyun, D.H. Kim, Chirality 16 (2004) 294.
[20] M.H. Hyun, H.J. Choi, B.S. Kang, G. Tan, Y.J. Cho, Bull. Korean Chem. Soc. 27 (2006) 1775.
[21] M.H. Hyun, Y. Song, Y.J. Cho, H.J. Choi, J. Sep. Sci. 30 (2007) 2539.
[22] R. Berkecz, A. Sztojkov-Ivanov, I. Ilisz, E. Forró, F. Fülöp, M.H. Hyun, A. Péter, J. Chromatogr. A 1125 (2006) 138.
[23] R. Berkecz, I. Ilisz, F. Fülöp, Z. Pataj, M.H. Hyun, A. Péter, J. Chromatogr. A 1189 (2008) 285.
[24] R. Berkecz, I. Ilisz, A. Misicka, D. Tymecka, F. Fülöp, H.J. Choi, M.H. Hyun, A. Péter, J. Sep. Sci. 32 (2009) 981.
[25] R. Charles, U. Beitler, B. Feibush, E. Gilav, J. Chromatogr. 112 (1975) 121.
[26] B. Koppenhoefer, E. Bayer, Chromatographia 19 (1984) 123.
[27] V. Schurig, J. Ossig, R. Link, Angew. Chem. 101 (1989) 197.
[28] T. Fornstedt, P. Sajonz, G. Guiochon, Chirality 10 (1998) 375.
[29] G. Gotmar, T. Fornstedt, G. Guiochon, Anal. Chem. 72 (2000) 3908.
[30] T. Fornstedt, P. Sajonz, G. Guichon, J. Am. Chem. Soc. 119 (1997) 1254.
[31] T. Fornstedt, G. Götmar, M. Andersson, G. Guichon, J. Am. Chem. Soc. 121 (1999) 1164.
[32] A. Péter, G. Török, D.W. Armstrong, G. Tóth, D. Tourwé, J. Chromatogr. A 828 (1998) 177.
[33] A. Péter, E. Vékes, D.W. Armstrong, J. Chromatogr. A 958 (2002) 89.
[34] E. Peyrin, Y.C. Guillaume, C. Guinchard, Anal. Chem. 69 (1997) 4979.
[35] N. Morin, Y.C. Guillaume, E. Peyrin, J.C. Rouland, Anal. Chem. 70 (1998) 2819.
[36] A. Cavazzini, G. Nadalini, F. Dondi, F. Gasparrini, A. Ciogli, C. Villani, J. Chromatogr. A 1031 (2004) 143.
[37] A. Gaucher, Y. Zuliani, D. Cabaret, M. Wakselman, J.P. Mazaleyrat, Tetrahedron Asymm. 12 (2001) 2571.
[38] F. Texier-Boullet, A. Foucaud, Tetrahedron Lett. 23 (1982) 4927.
[39] J. Lee, D. Gauthier, R.A. Rivero, J. Org. Chem. 64 (1999) 3060.
[40] M. Fitz, E. Forró, E. Vigóczki, L. Lázár, F. Fülöp, Tetrahedron Asymm. 19 (2008) 1114.
[41] W. Lee, J.Y. Jin, C.S. Back, Microchem. J. 80 (2005) 213.

[42] L. Thurnberg, S. Allenmark, A. Friberg, F. Ek, T. Frejd, Chirality 16 (2004) 614.
[43] E. Högfeldt, Stability Constants of Metal-ion Complexes, Pergamon Press, Oxford, 1982.
[44] D.W. Armstrong, Y. Liu, K.H. Ekborg-Ott, Chirality 7 (1995) 474.
[45] T. Fornstedt, P. Sajonz, G. Guichon, J. Am. Chem. Soc. 119 (1997).
[46] T. Fornstedt, G. Götmar, M. Anderson, G. Guichon, J. Am. Chem. Soc. 121 (1999) 1664.
[47] J. Oxelbark, S. Allenmark, J. Chem. Soc., Perkin Trans. 2 (1999) 1587.
[48] Q2(R1) Document:Validation of Analytical Procedures: Text and Methodology, International Conference on Harmonization (ICH), Geneva, 2005.