Special Issue Article

High-Performance Liquid Chromatographic Enantioseparation of Cyclic β-Amino Acids on Zwitterionic Chiral Stationary Phases Based on *Cinchona* Alkaloids

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ABSTRACT Stereoselective high-performance liquid chromatographic separations of eight sterically constrained cyclic β-amino acid enantiomer pairs were carried out using the newly developed *Cinchona* alkaloid-based zwitterionic chiral stationary phases Chiralpak ZWIX(+) and ZWIX(−). The effects of the mobile phase composition, the nature and concentrations of the acid and base additives, the counterions and temperature on the separations were investigated. The changes in standard enthalpy, $\Delta(\Delta H^\circ)$, entropy, $\Delta(\Delta S^\circ)$, and free energy, $\Delta(\Delta G^\circ)$, were calculated from the linear van't Hoff plots derived from the ln α vs. $1/T$ curves in the studied temperature range (10–50°C). The values of the thermodynamic parameters depended on the nature of the selectors and the structures of the analytes. Unusual temperature behavior was observed on the ZWIX(−) column: decreased retention times were accompanied by increased separation factors with increasing temperature. On the ZWIX(+) column only enthalpically, whereas on the ZWIX(−) column both enthalpically and entropically driven separations were observed. The elution sequence was determined in all cases and was observed to be the opposite on ZWIX(+) and on ZWIX(−). *Chirality 27:563–570, 2015.* © 2015 Wiley Periodicals, Inc.

KEY WORDS: enantiomer separation; HPLC; cyclic β-amino acids; *Cinchona* alkaloid-based chiral stationary phases

The syntheses of both racemic and enantiomeric ß-amino acids have recently gained in importance as a result of their widespread use in different fields of pharmaceutics and chemistry. For example, biologically active carbocyclic ß-amino acids (e.g., the antibacterial or/and antifungal cispentacin, icofungipen and BAY Y9379)[1,2] can be widely used in heterocyclic,[3,4] peptide,[5,6] or combinatorial chemistry[7,8] and in drug research.[9,10] A search is therefore ongoing for new strategies for the synthesis of enantiomeric ß-amino acids.[7,8,11–13]

To investigate the stereochemistry and the enantiomeric excess of the product β-amino acids, highly efficient analytical methods are needed. One of the most frequently applied techniques is enantioselective (chiral) high-performance liquid chromatography (HPLC). HPLC enantioseparations of β-amino acids have been performed by both indirect and direct methods and in the past decade new types of chiral derivatizing agents and chiral stationary phases (CSPs) have been applied for this purpose and reviewed in numerous articles.[14–26]

Enantioselective retention and separation are influenced by temperature.[27–32] For mechanistic considerations to be drawn, a plot of $R \ln \alpha$ vs. $1/T$ has been applied with a slope of $-\Delta(\Delta H^\circ)$ and an intercept of $\Delta(\Delta S^\circ)$, as discussed by Fornstedt et al.[30]

Newly developed *Cinchona* alkaloid-based zwitterionic ion-exchange-type CSPs characterized by a weak basic and a strong acidic site operate best under slightly acidic polar-ionic mobile phase (PIM) conditions.[33–36] They can operate in the anion-exchange mode for the separation of chiral acids, in the cation-exchange mode for the resolution of chiral amines, and in the zwitterionic exchange mode for the enantioseparation of amphoteric compounds such as amino acids and small peptides.

The present article describes the separation of eight pairs of enantiomers (selectands, SAs) of cyclic β-amino acids (Fig. 1) on the *Cinchona* alkaloid-based zwitterionic CSP selectors (SOs)[33] in PIM mode. The effects of the mobile phase composition, the nature of the acid and base additives, the amount of the counterion, temperature, and the structural features of the SAs on the retention and enantioselectivity are discussed. The sequence of elution of the enantiomers was determined in all cases and is used as the basis of a discussion of stereoselective recognition models.

EXPERIMENTAL

Chemicals and Reagents

The enantiomers of racemic *cis*-aminocyclopentanecarboxylic acids (*cis*-ACPC; **1A**,**1B**) were prepared from cyclopentane via chlorosulfonyl isocyanate addition, followed by aqueous HCl treatment and ion-exchange chromatography.[37] *trans*-ACPC (**1C**,**1D**) was synthesized by

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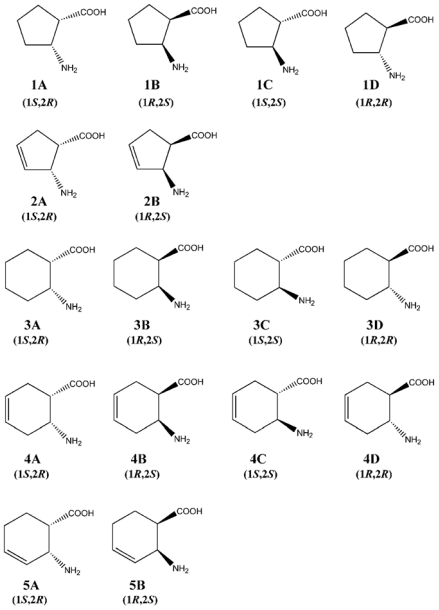

Fig. 1. Chemical structures of Sas.

Michael addition of NH_3 to 1-cyclopentenecarboxylic acid in a steel autoclave at 170°C for 80 h, followed by purification via ion-exchange chromatography.[38] (−)-*cis*-(1*R*,2*S*)-ACPC (**1B**) and (+)-*trans*-(1*S*,2*S*)-ACPC (**1C**) were prepared from the corresponding esters by lipase-catalyzed acylation, followed by hydrolysis.[39] The racemic *cis*- and *trans*-aminocyclohexanecarboxylic acid (*cis*- and *trans*-ACHC, **3A-3D**) and *cis*-(1*S*,2*R*;1*R*,2*S*) and *trans*-ACHC-ene (*1S*,2*S*;1*R*,2*R*) were prepared from the corresponding commercially available *cis*- and *trans*-hexahydro- and tetrahydrophthalic anhydrides by ammonolysis, followed by Hoffmann degradation and ion-exchange purification.[40,41] In the cases of *cis* and *trans*-ACHC-ene (**4A-4D**), Hoffmann degradation was performed with NaOCl instead of the usual NaOBr. For the preparation of the enantiomers of compounds **4A-4D**, lipase PS-catalyzed selective *N*-acylation of the ethyl esters of racemic *cis*- or *trans*-*β*-amino acids was used.[39] 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 10–40 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 HCl 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 that precipitated out were filtered off to give white crystals. The (1*R*,2*S*) and (1*S*,2*S*) enantiomers were obtained after small-scale alkaline hydrolysis of the ethyl esters. Their identities were confirmed by means of melting point determination, mass spectrometry, ^{1}H-NMR spectroscopy, and optical rotation measurement.

Enantiomeric *β*-amino acids **2A**, **2B**, **5A**, and **5B** were prepared through CAL-B-catalyzed enantioselective (E > 200) ring opening of racemic 6-azabicyclo[3.2.0]hept-3-en-7-one and 7-azabicyclo[4.2.0]oct-4-en-8-one (prepared by the above-mentioned cycloaddition of chlorosulfonyl isocyanate to the corresponding cycloalkadienes) with 1 equivalent of H_2O in 2-propyl ether (*iPr_2O*) at 70°C.[42]

Methanol (MeOH) and acetonitrile (MeCN) of HPLC grade, and NH_3, ethylamine (EA), triethylamine (TEA), propylamine (PA) tripropylamine (TPA), butylamine (BA), tributylamine (TBA), formic acid (FA), and glacial acetic acid (AcOH) of analytical reagent grade were purchased from VWR International (Radnor, PA).

Apparatus and Chromatography

Chromatographic measurements were performed on a 1100 Series HPLC system from Agilent Technologies (Waldbronn, Germany), consisting of a solvent degasser, a pump, an autosampler, a column thermostat, a multiwavelength UV–vis detector. and a corona-charged aerosol detector from ESA Biosciences (Chelmsford, MA). Data acquisition and analysis were carried out with ChemStation chromatographic data software from Agilent Technologies.

The Chiralpak ZWIX(+) and ZWIX(−) columns (150 ×3.0 mm I.D., 3-μ m particle size for both columns) were from Chiral Technologies Europe (Illkirch, France).

Chromatography was performed in isocratic mode at a flow rate of 0.6 ml min^{-1} and a column temperature of 25°C (if not otherwise stated). Detection was accomplished by UV at selected wavelengths of 215 and 230 nm or with a corona detector. The void volume of the columns (t_0) was determined by injecting acetone dissolved in MeOH, with detection at 280 nm. All SAs were dissolved in MeOH in the concentration range 0.5–1.0 mg ml^{-1} and further diluted with mobile phase.

RESULTS AND DISCUSSION

Effects of Mobile Phase Composition and Additives on Chromatographic Parameters

All the investigated amino acids (Fig. 1) possess a cycloalkane skeleton. The carboxy and primary amino groups provide the charged sites for the ionic interactions. SAs **A** and **B** are the enantiomers of the *cis*, while **C** and **D** are the enantiomers of the *trans* isomers; all other combinations are diastereomers. Besides carboxy and primary amino groups, analogs **2**, **4**, and **5** possess a double bond. These differences result in different physical properties, such as hydrophobicity, polarity, and rigidity of the molecules and may exert a significant influence on the interactions with the quinine- and quinidine-based chiral zwitterionic SOs.

In the PIM mode, a mixture of MeOH as protic solvent and MeCN as aprotic solvent in combination with acid and base additives ensured the best enantioseparation on the *Cinchona* alkaloid-based zwitterionic CSP ion-exchangers.[34,36] To investigate the effects of the organic bulk solvent, chromatographic separation was carried out in MeOH/MeCN (75/25, 50/50, and 25/75 *v*/*v*) mobile phases containing 25 mM TEA and 50 mM AcOH for representative SAs, i.e., saturated and unsaturated analogs of *cis* isomers (**1A**,**1B** and **4A**,**4B**) and *trans* analogs (**1C**,**1D** and **4C**,**4D**), respectively. In the presence of different MeOH/MeCN mixtures as mobile phase bulk solvent on ZWIX(+) and ZWIX(−), the application of a higher MeCN content in the mobile phase in all cases significantly increased the retention (Fig. 2). The electrostatically driven interactions involved in the formation of the SO-SA complexes with zwitterionic CSPs are obviously less strong in protic MeOH solvents which may be caused 1) by the weakening of the intermolecular hydrogen bonding effect, and 2) by the change of the solvation shells of the electrostatic interacting ionic sites.[33,34,43–45] The observed effect of a higher content of MeCN on the selectivity was not consistent, but it most often led to a slightly enhanced enantioselectivity. In accordance with the former results for *β*-amino acids,[36,43–46] an increase in separation performance was observed at higher MeCN content. However, on ZWIX (+) for the enantiomer pairs **1A**,**1B** and **4A**,**4B** and on ZWIX(−) for **4A**,**4B** slight decreases in α were observed, similar to the case of α-amino acids.[47] The resolution (R_S) was in most cases highest in MeOH/MeCN (50/50 *v*/*v*) containing 25 mM TEA and 50 mM AcOH.

For optimization of the enantioseparation on *Cinchona* alkaloid-based CSPs, not only variation of the main components of the eluent system, but also variation of the nature of the acid and base additives in the mobile phase may offer

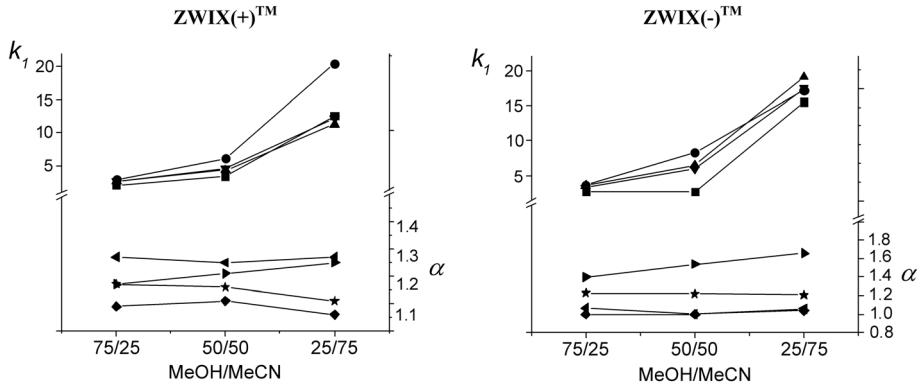

Fig. 2. Effects of the compositions of the bulk solvents on the chromatographic parameters for SAs **1A**,**1B**, **1C**,**1D**, **4A**,**4B**, and **4C**,**4D** on ZWIX(+) or ZWIX(–). Chromatographic conditions: column, Chiralpak ZWIX(+) and ZWIX(–); mobile phase, MeOH/MeCN (75/25, 50/50 or 25/75 *v/v*) containing 25 mM PA and 50 mM AcOH; flow rate, 0.6 ml min^{-1}; detection, 230 nm; temperature 25°C; symbols, k_1 for SA **1A**,**1B**: —■—, for **1C**,**1D**: –●–, for **4A**,**4B**: –▲– and for **4C**,**4D**: –▼–; α for **1A**,**1B**: –◆–α, for **1C**,**1D**: –◀–, for **4A**,**4B**: –★– and for **4C**,**4D**: –▶– .

a relatively easy choice. An acid-to-base ratio of 2:1 was previously found to be most effective for successful enantioseparation,[36,43–46] with the concentration of the acid being kept at 50 mM, and that of the base at 25 mM. The acid excess applied in the mobile phase ensured that the bases (amines) were present in their "ammonium ion" forms.

For comparison of the effects of the nature of the base and the acids as mobile phase additives, separations of saturated and unsaturated analogs of *cis* isomers (**1A**,**1B**, **2A**,**2B**, and **5A**,**5B**) and *trans* analogs (**3C**,**3D** and **4C**,**4D**) were carried out with the same mobile phase composition on both ZWIX (+) and ZWIX(–) columns, the MeOH/MeCN content being kept constant (75/25 *v/v*), while the nature of the base (25 mM base) and the acid (50 mM) was varied. Seven different bases (NH_3, EA, TEA, PA, TPA, BA, and TBA) and two acids (FA and AcOH) were selected for these experiments.

The experimental results for the pairs **1A**,**1B** and **5A**,**5B** on ZWIX(+) and ZWIX(–) columns (Figs. 3 and 4) revealed that the retention factors in several cases slightly increased in the sequences EA<PA<BA and TEA<TPA<TBA (in several cases BA resulted in smaller *k* values than with PA; data not shown). Increasingly apolar and bulkier amine additives, probably having a reduced effect on the competitive ion-ion interactions lead to an increase in *k*. With a few exceptions on both columns, the retention factors increased as the degree of ethyl, propyl or butyl substitution of the nitrogen increased (Fig. 3). The increased retention observed is probably due to the reduced capacity of TBA, TPA, or TEA in their roles as counterions as compared with BA, PA, or EA, respectively. As concerns the influence of the nature of the bases on the separation factor, a slight change in α was observed, but without any general trend. When the effects of the base additives on the retention and selectivity are taken into account, the application of TEA appears favorable.

The nature of the acids exerted a slight effect on the retention. For SAs **1A**,**1B**, **2A**,**2B**, **3C**,**3D**, **4C**,**4D**, and **5A**,**5B**, the presence of FA with a few exceptions resulted in slightly smaller k_1 values with the same base additive, as seen previously.[47–50]

As regards the effect of the acid modifier (FA or AcOH) on the separation factor and resolution, again no general trend could be observed. When separation occurred, α differed slightly in the presence of FA from that obtained with AcOH, while slightly higher R_S values were obtained on both CSPs when AcOH was applied.

For both anion-exchange and cation-exchange SOs under PIM conditions, a predominant ion-exchange-driven retention mechanism has been confirmed[51] and the observed effect could be explained by a simple displacement model.[52] In order to gain deeper insight into the retention mechanism in the cases of the given zwitterionic CSPs, the effects of variation of the concentrations of the base additives on the

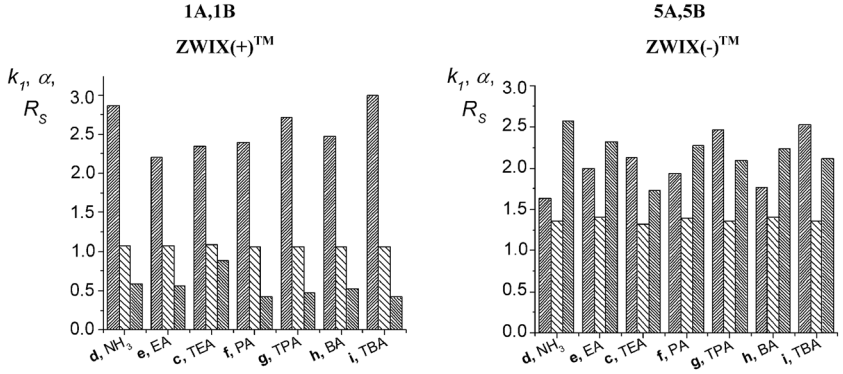

Fig. 3. Effect of the nature of acid and base additives on chromatographic parameters on Chiralpak ZWIX(+) and ZWIX(–) for SAs **1A**,**1B** and **5A**,**5B**. Chromatographic conditions: column, Chiralpak ZWIX(+) and ZWIX(–); mobile phase, MeOH/MeCN (75/25 *v/v*) containing **c**, 50.0 mM AcOH and 25.0 mM TEA, **d**, 50.0 mM AcOH and 25.0 mM NH_3, **e**, 50.0 mM AcOH and 25.0 mM EA, **f**, 50.0 mM AcOH and 25.0 mM PA, **g**, 50.0 mM AcOH and 25.0 mM TPA, **h**, 50.0 mM AcOH and 25.0 mM BA, **i**, 50.0 mM AcOH and 25.0 mM TBA, **j**, 50.0 mM FA and 25.0 mM NH_3, **k**, 50.0 mM FA and 25.0 mM EA, **l**, 50.0 mM FA and 25.0 mM TEA, **m**, 50.0 mM FA and 25.0 mM PA, **n**, 50.0 mM FA and 25.0 mM TPA, **o**, 50.0 mM FA and 25.0 mM BA, **p**, 50.0 mM FA and 25.0 mM TBA; flow rate, 0.6 ml min^{-1}; detection, corona detector; symbols, k_1, ▨, α ▧, R_S ▩.

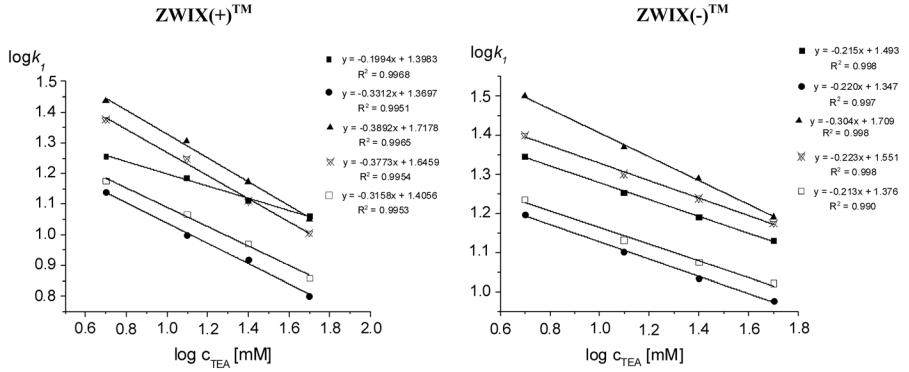

Fig. 4. Influence of the counter-ion concentration on retention of the first eluting enantiomer (k_1) of **1A**,**1B**, **2A**,**2B**, **3C**,**3D**, **4C**,**4D** and **5A**,**5B**. Chromatographic conditions: column, ZWIX(+) and ZWIX(–); mobile phase, MeOH/MeCN (25/75 *v*/*v*) containing 5, 12.5, 25 and 50 mM TEA and 10, 25, 50, and 100 mM AcOH (acid-to-base ratio being kept at 2:1); flow rate, 0.6 ml min^{-1}; detection, corona detector; symbols,■**1A**,**1B**, ●**2A**,**2B**, ▲**3C**,**3D**, ⨳**4C**,**4D**, □**5A**,**5B**.

retention were studied. Data displaying the effects of the TEA on the retention for **1A**,**1B**, **2A**,**2B**, **3C**,**3D**, **4C**,**4D**, and **5A**,**5B** are depicted in Figure 4. Under the studied conditions, linear relationships were found between log k_1 and log c_{TEA}, with slopes varying of around 0.2–0.4. With the earlier-mentioned simple displacement model, the slopes of these plots are determined by the ratio of the effective charges of the solute and the counterions; values of around 0.9 have been found for a cation-exchange CSP and different chiral amines.[49] In our case, the significantly lower slopes observed reveal a marked difference between a zwitterionic and a "single ionic" CSP, as was found by Hoffmann et al.[34] In the case of zwitterionic CSPs with enhancement of the counterion concentration, the retention can be reduced, but in only a rather limited range. (An order of magnitude enhancement of the concentration of the counterions resulted in an ~50% reduction in the retention factor.) Under the studied conditions on both CSPs, practically identical slopes were obtained for each enantiomer, i.e., the enantioselectivity remained almost constant when the counterion concentration was increased. However, these findings indicate that the sterically determined accessibility of the ionizable interaction sites, mainly of the constraint zwitterionic SO but also of the SAs (Fig. 1), may cause a deviation from the simplified stoichiometric displacement model.

Structure–Selectivity and Retention Relationships

The steric arrangement of the carboxylic and amino groups on the constrained SAs (Fig. 1) participating in the SO-SA associate formation influenced retention and chiral recognition. Table 1 presents the k, α and R_S values observed with the most frequently applied mobile phase (eluent **c**) on the ZWIX(+) and ZWIX(−) columns.

The experimental results obtained with the same mobile phase under isocratic conditions allow the following considerations:

1. Stereoselectivity

   (i) For all pairs of *cis* SAs (**1A**,**1B**; **2A**,**2B**, **3A**,**3B**, **4A**,**4B**, and **5A**,**5B**) on the ZWIX(+) column, the (1*R*,2*S*) SAs were consistently eluted before the (1*S*,2*R*) SAs. The SA pair **2A**,**2B** could not be resolved under the conditions of eluent **c**, whereas with mobile phase **d** it could be resolved (Tables 1 and 2) and exhibited the same elution sequence.

TABLE 1. Chromatographic data, retention factor (k), selectivity factor (α), resolution (R_S), and elution sequence of cyclic ß-amino acids on ZWIX(+) and ZWIX(−) at constant mobile phase composition

Pair of analytes	Eluent	k_1	k_2	α	R_S	Elution sequence
			ZWIX(+)			
1A,**1B**	**c**	2.35	2.56	1.09	0.88	*1R*,*2S* < *1S*,*2R*
1C,**1D**	**c**	3.00	3.80	1.27	2.18	*1R*,*2R* < *1S*,*2S*
2A,**2B**	**c**	2.41	2.41	1.00	0.00	—
	d*	2.75	3.41	1.24	1.57	*1R*,*2S* < *1S*,*2R*
3A,**3B**	**c**	2.22	2.60	1.17	1.60	*1R*,*2S* < *1S*,*2R*
3C,**3D**	**c**	3.22	3.22	1.00	0.00	—
	d	2.86	3.09	1.08	0.50	*1R*,*2R* < *1S*,*2S*
4A,**4B**	**c**	2.71	3.16	1.17	1.00	*1R*,*2S* < *1S*,*2R*
4C,**4D**	**c**	2.72	3.19	1.17	1.14	*1R*,*2R* < *1S*,*2S*
5A,**5B**	**c**	2.66	3.65	1.38	2.75	*1R*,*2S* < *1S*,*2R*
			ZWIX(−)			
1A,**1B**	**c**	2.81	2.81	1.00	0.00	—
	a**	11.19	11.86	1.06	0.25	*1R*,*2S* < *1S*,*2R*
1C,**1D**	**c**	3.76	4.04	1.07	0.22	*1S*,*2S* < *1R*,*2R*
2A,**2B**	**c**	3.04	3.04	1.00	0.00	—
3A,**3B**	**c**	2.90	3.72	1.28	1.83	*1S*,*2R* < *1R*,*2S*
3C,**3D**	**c**	3.93	5.22	1.33	2.58	*1S*,*2S* < *1R*,*2R*
4A,**4B**	**c**	3.58	4.42	1.23	1.37	*1S*,*2R* < *1R*,*2S*
4C,**4D**	**c**	3.33	4.65	1.40	2.38	*1S*,*2S* < *1R*,*2R*
5A,**5B**	**c**	2.17	3.06	1.41	2.82	*1S*,*2R* < *1R*,*2S*

Chromatographic conditions: columns, Chiralpak ZWIX(+) and Chiralpak ZWIX(−); mobile phase. **a**, MeOH/MeCN (25/75 *v*/*v*) containing 25 mM TEA and 50 mM AcOH, **c**, MeOH/MeCN (75/25 *v*/*v*) containing 25 mM TEA and 50 mM AcOH, **d**, MeOH/MeCN (75/25 *v*/*v*) containing 25 mM NH_3 and 50 mM AcOH; flow rate, 0.6 ml min^{-1}; detection, 230 nm or corona detector; temperature, 25°C
*10°C
**50°C.

On the pseudo-enantiomeric ZWIX(−) column, the expected reversal of the elution sequence was observed (the **2A**,**2B** could not be resolved under any conditions applied, while **1A**,**1B** could be resolved with mobile phase **a** at 50°C [above the T_{iso} temperature, Tables 1 and 2]).

   (ii) For all the *trans*-SAs (**1C**,**1D**, **3C**,**3D**, and **4C**,**4D**) the (1*R*,2*R*) SA eluted before the (1*S*,2*S*) SA on the ZWIX(+) column, with a reversal of the elution sequence on the ZWIX(−) column. The only exception

TABLE 2. Temperature dependence of retention factor of first eluting enantiomer (k_1), separation factor (α) and resolution (R_S) of cyclic β amino acids on ZWIX(+) and ZWIX(−)

Pair of analyte	Mobile phase	k_1, α, R_S	Temperature (°C) 10	20	30	40	50
		ZWIX(+)					
1A,1B	**c**	k_1	2.39	2.21	1.96	1.76	1.59
		α	1.1	1.09	1.09	1.08	1.07
		R_S	0.32	0.41	0.32	0.22	0.24
2A,2B	**d**	k_1	2.93	2.75	2.58	2.41	2.26
		α	1.25	1.24	1.22	1.21	1.19
		R_S	1.63	1.57	1.45	1.36	1.23
3C,3D	**d**	k_1	3.18	2.86	2.58	2.34	2.12
		α	1.09	1.08	1.07	1.07	1.06
		R_S	0.52	0.48	0.4	0.37	0.44
4C,4D	**c**	k_1	3.12	2.82	2.52	2.26	2.05
		α	1.18	1.17	1.15	1.14	1.13
		R_S	0.88	0.86	0.94	0.75	0.74
5A,5B	**c**	k_1	2.23	1.99	1.8	1.62	1.47
		α	1.46	1.42	1.38	1.34	1.3
		R_S	2.22	2.1	2.00	1.78	1.6
		ZWIX(−)					
1A,1B	**a**	k	16.04	15.66	14.81	13.82	11.99
		α	1.00	1.02	1.04	1.05	1.06
		R_S	0.00	0.08	0.13	0.17	0.21
1C,1D	**a**	k	4.97	4.84	4.70	4.57	4.41
		α	1.10	1.08	1.06	1.04	1.03
		R_S	0.36	0.3	0.27	0.22	0.13
3C,3D	**c**	k	3.83	3.67	3.53	3.39	3.25
		α	1.30	1.27	1.24	1.22	1.19
		R_S	1.5	1.5	1.47	1.81	1.45
4C,4D	**c**	k	3.29	3.15	3.00	2.88	2.76
		α	1.40	1.37	1.34	1.31	1.28
		R_S	1.93	1.85	1.69	1.67	1.62
5A,5B	**c**	k	2.35	2.26	2.17	2.08	1.98
		α	1.47	1.42	1.38	1.34	1.31
		R_S	2.17	2.09	1.89	1.8	1.58

Chromatographic conditions: column, Chiralpak ZWIX(+) and ZWIX(−); mobile phase. **a**, MeOH/MeCN (25/75 *v/v*) containing 25 mM TEA and 50 mM AcOH, **c**, MeOH/MeCN (75/25 *v/v*) containing 25 mM TEA and 50 mM AcOH, **d**, MeOH/MeCN (75/25 *v/v*) containing 25 mM NH_3 and 50 mM AcOH; flow rate, 0.6 ml min^{-1}; detection, corona detector.

was the pair **3C**,**3D**, which could not be resolved on ZWIX(+) with mobile phase **c**, whereas with mobile phase **d** it could (Tables 1 and 2).

(iii) The above elution sequence holds in principle for the five- and six-membered cycloalkanes, although it should be mentioned that the pair **2A**,**2B** could not be separated under the given conditions on the ZWIX(+) and the ZWIX(−) column, although at composition **d** they could (Tables 1 and 2).

There is no clear trend as to whether the *cis* or the *trans* enantiomer pairs can be better separated. However, it is obvious that the similar cyclopentene- and cyclohexene-ß-amino acids (**2A**,**2B** and **5A**,**5B**) have different steric constraints, significantly influencing the intermolecular SO-SA interactions.

At this point it should be mentioned that, when the differences in the retention factors of the SAs are small, the peaks may not be resolved, as this depends on the efficiency of the columns.

2. Retention

In general, the *trans* SAs are better retained than the related *cis* SAs on both the ZWIX(+) and the ZWIX(−) columns.

In summary, the clear selectivity and retention trends did not vary with a change of the MeOH to MeCN ratio or the nature of the acid and the base modifiers. Selected chromatograms are depicted in Figure 5.

Temperature Dependence and Thermodynamic Parameters

In order to investigate the effects of temperature on the chromatographic parameters, a variable-temperature study was carried out over the temperature range 10–50°C. Experimental data for all SAs on both columns in the mobile phases (**a**) MeOH/MeCN (25/75 *v/v*) containing 25 mM TEA and 50 mM AcOH, (**c**) MeOH/MeCN (75/25 *v/v*) containing 25 mM TEA and 50 mM AcOH, and (**d**) MeOH/MeCN (75/25 *v/v*) containing 25 mM NH_3 and 50 mM AcOH, are listed in Table 2. The slightly different mobile phase conditions with regard to the MeOH/MeCN ratio were selected in order to achieve separations for all pairs of enantiomers.

The tabulated data indicate that the retention factor k in most cases decreased with increasing temperature. Transfer of the SA from the mobile phase to the stationary phase is generally an exothermic process and consequently k (and α) decreases with increasing temperature. However, on the ZWIX(−) column with mobile phase **a**, the behavior of the pair **1A**,**1B** was unusual: with increasing temperature, k decreased, but α and R_S increased. Such behavior was recently observed for other unusual amino acids too.[43–45]

This unexpected behavior occurred only on the ZWIX(−) CSP for the pair **1A**,**1B**. In the studied temperature range, the solvation of the SO of the ZWIX(−) column and the SAs probably does not change significantly. However, for the pair **1A**,**1B** an unexpected reversal of the elution sequence was observed above the T_{iso} temperature.

As a general trend, van't Hoff analysis of the separation factors (ln α vs. $1/T$) gave linear plots, as indicated by the correlation coefficients in Table 3. The differences in $\Delta(\Delta H^\circ)$ and $\Delta(\Delta S^\circ)$, are presented in Table 3. $\Delta(\Delta H^\circ)$ ranged from −2.2 to −0.6 kJ mol^{-1} on ZWIX(+), and from −2.2 to +1.1 kJ mol^{-1} on ZWIX(−). The trend in the change in $\Delta(\Delta S^\circ)$ was similar to that in $\Delta(\Delta H^\circ)$. Under the conditions where $\Delta(\Delta H^\circ)$ was negative, $\Delta(\Delta S^\circ)$ was also negative, and positive $\Delta(\Delta H^\circ)$ was accompanied by positive $\Delta(\Delta S^\circ)$. The interactions of **5A**,**5B** with ZWIX(+) and ZWIX(−) were characterized by the highest negative $\Delta(\Delta H^\circ)$ and $\Delta(\Delta S^\circ)$ values at eluent composition **c**.

When the selectivity increased with increasing temperature, $\Delta(\Delta H^\circ)$ and $\Delta(\Delta S^\circ)$ were positive. In these cases, the change in $\Delta(\Delta H^\circ)$ with increasing temperature had a positive effect on the enantioselectivity. On the other hand, the positive $\Delta(\Delta S^\circ)$ compensated the positive $\Delta(\Delta H^\circ)$ and resulted in a negative $\Delta(\Delta G^\circ)$, the selectivity increasing with increasing temperature. Thermodynamically, this unusual behavior may be attributed to the positive $\Delta(\Delta S^\circ)$ values, indicating the importance of the entropy contribution to the chiral separation.

The thermodynamic parameter $-\Delta(\Delta G^\circ)_{298}$ suggests that, both on ZWIX(+) and on ZWIX(−), mobile phases **c** and **d** induced binding to the SO more efficiently, as reflected by the larger $-\Delta(\Delta G^\circ)$ (**1A**,**1B** on ZWIX(−) could be separated only under mobile phase condition **a**).

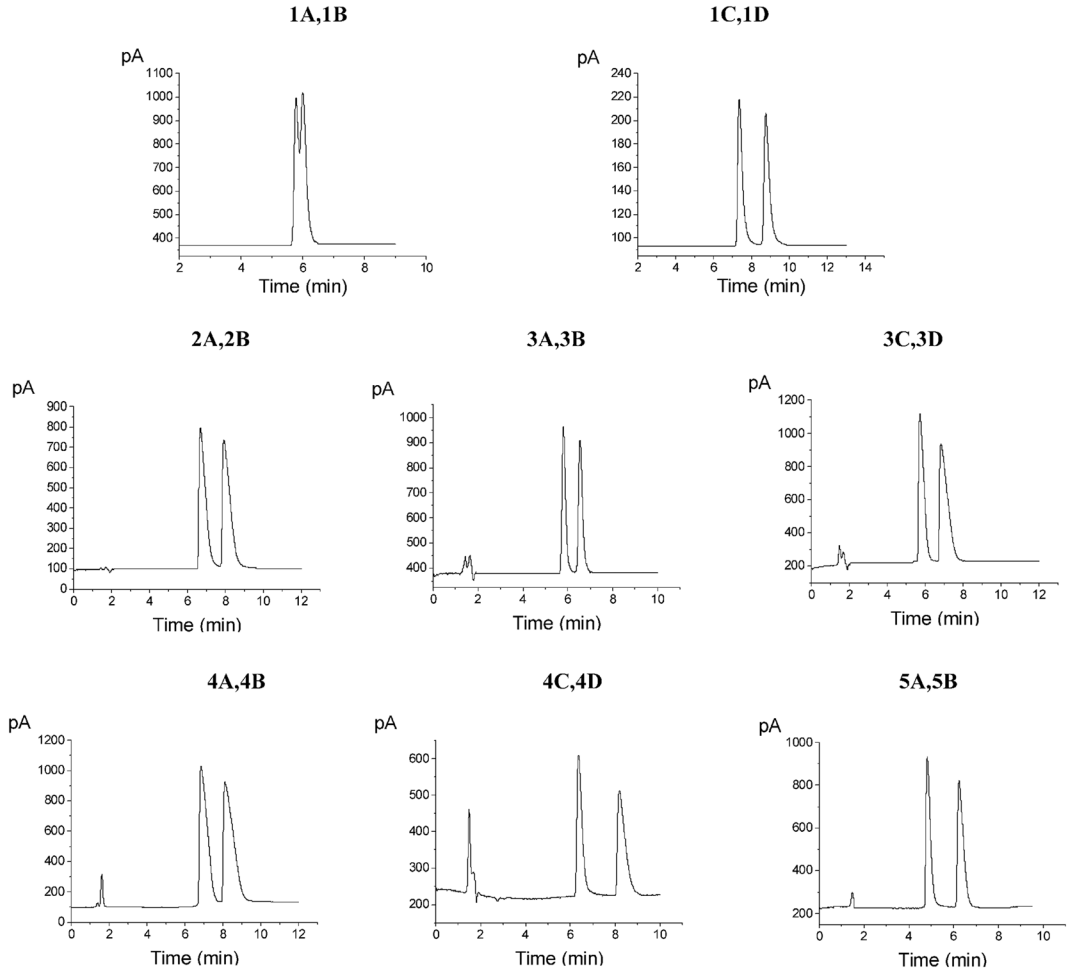

Fig. 5. Selected chromatograms for SAs **1-5**. Chromatographic conditions: columns, ZWIX(+) for SAs **1A**,**1B**, **1C**,**1D**, **2A**,**2B**, and **3A**,**3B**, ZWIX(–) for SAs **3C**,**3D**, **4A**,**4B**, **4C**,**4D**, and **5A**,**5B**; mobile phase: MeOH/MeCN (75/25 *v/v*) containing 25mM TEA and 50 mM AcOH for SAs **1A**,**1B**, **1C**,**1D**, **3A**,**3B**, and **4A**,**4B**, MeOH/MeCN (75/25 *v/v*) containing 25 mM EA and 50 mM FA for SA **3C**,**3D**, MeOH/MeCN (75/25 *v/v*) containing 25 mM NH_3 and 50 mM AcOH for SA **2A**,**2B** and **4C**,**4D** and MeOH/MeCN (75/25 *v/v*) containing 25 mM PA and 50 mM AcOH for SA **5A**,**5B**; flow rate, 0.6 ml min^{-1}; detection, 230 nm or corona detector; temperature, 25ºC for all SAs (with exception for SA **2A**,**2B**, 10ºC).

TABLE 3. Thermodynamic parameters, $\Delta(\Delta H°)$, $\Delta(\Delta S°)$, $Tx\Delta(\Delta S°)$, $\Delta(\Delta G°)$, correlation coefficients, (R^2) and T_{iso} temperature of cyclic β–amino acids on ZWIX(+) and ZWIX(–)

Pair of analyte	Mobile phase	$-\Delta(\Delta H°)$ (kJ mol^{-1})	$-\Delta(\Delta S°)$ (J mol^{-1} K^{-1})	Correlation coefficients (R^2)	$-Tx\Delta(\Delta S°)_{298K}$ (kJ mol^{-1})	$-\Delta(\Delta G°)_{298K}$ (kJ mol^{-1})	T_{iso} (°C)
				ZWIX(+)			
1A,**1B**	**c**	0.6	1.3	0.9936	0.4	0.2	189
2A,**2B**	**d**	0.9	1.3	0.9823	0.4	0.5	431
3C,**3D**	**d**	0.6	1.2	0.9832	0.4	0.2	179
4C,**4D**	**c**	0.7	1.2	0.9955	0.4	0.3	322
5A,**5B**	**c**	2.2	4.7	0.9993	1.4	0.8	201
				ZWIX(–)			
1A,**1B**	**a**	–1.1	–4.0	0.9949	–1.2	0.1	9
1C,**1D**	**a**	1.3	3.8	0.9978	1.1	0.2	69
3C,**3D**	**c**	1.7	3.7	0.9982	1.1	0.6	179
4C,**4D**	**c**	1.7	3.8	0.9978	1.1	0.6	254
5A,**5B**	**c**	2.2	4.5	0.9985	1.3	0.9	211

Chromatographic conditions: column, Chiralpak ZWIX(+) and ZWIX(–); mobile phase. **a**, MeOH/MeCN (25/75 *v/v*) containing 25 mM TEA and 50 mM AcOH,**c**, MeOH/MeCN (75/25 *v/v*) containing 25 mM TEA and 50 mM AcOH, **d**, MeOH/MeCN (75/25 *v/v*) containing 25 mM NH_3 and 50 mM AcOH; flow rate, 0.6 ml min^{-1}; detection, corona detector; R^2, correlation coefficient of van't Hoff plot, ln $\alpha - 1/T$ curves; T_{iso},, temperature of ln $k - 1/T$ curves where enantioselectivity cancels.

On ZWIX(−), the positive $\Delta(\Delta S°)$ for **1A**,**1B** compensated for the positive $\Delta(\Delta H°)$ and resulted in a negative $\Delta(\Delta G°)$ value (Table 3). For **1A**,**1B** in this temperature range, enantioresolution is entropically driven, and the selectivity increases with increasing temperature.

The data were used to calculate the temperature T_{iso} at which the enantioselectivity cancels out (Table 3). In most cases, T_{iso} was considerably higher than room temperature; while for **1A**,**1B** T_{iso} was 9°C and the reversed elution sequence at higher column temperature was observed (Table 1).

CONCLUSION

HPLC methods were developed for separation of the enantiomers of cyclic β-amino acids by using *Cinchona*-based zwitterionic CSPs i.e., Chiralpak ZWIX(+) and ZWIX(−) in PIM mode. The effects of the composition of the mobile phase, the nature of the acid and base additives, the structures of the Sas, and temperature were discussed. The changes in $\Delta(\Delta H°)$, $\Delta(\Delta S°)$, and $\Delta(\Delta G°)$ were calculated from the linear van't Hoff plots derived from the ln α vs. $1/T$ curves in the temperature range 10–50°C. The values of the thermodynamic parameters depended on the nature of the SOs and the structures of the SAs. On the ZWIX(+) column only enthalpically, while on the ZWIX(−) column both enthalpically and entropically driven separations were observed. The latter was registered on the ZWIX(−) column for analyte **1A**,**1B**. By variation of the chromatographic parameters, the separations of the stereoisomers were optimized and baseline resolution was achieved for most of the investigated SAs in at least one chromatographic system. The elution sequence was determined in all cases and was found to be opposite on the ZWIX(+) and ZWIX(−) columns.

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