Applied zwitterionic phases

ZWIX(+)TM

ZWIX(+A)

ZWIX(-)TM

ZWIX(-A)

Mechanistic considerations of enantiorecognition on novel *Cinchona* alkaloid-based zwitterionic chiral stationary phases from the aspect of the separation of *trans*-paroxetine enantiomers as model compounds

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Highlights

Enantiomers of *trans*-paroxetine were separated on zwitterionic chiral stationary phases; Molecular recognition events were studied by computer modelling;

26 The elution sequence was readily changed through variation of the chiral stationary phase

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ABSTRACT

The enantiomers of *trans*-paroxetine were separated on four chiral stationary phases (CSPs) based on chiral zwitterionic *Cinchona* alkaloids fused with (*R,R*)- or (*S,S*)-*trans*-2-aminocyclohexanesulfonic acid. The enantioseparations were carried out in polar-ionic or in hydro-organic mobile phases with MeOH/THF, MeCN/THF, MeCN/THF/H₂O and MeOH/MeCN/THF containing organic acid and base additives, in the temperature range 0–50 °C. The effects of the mobile phase composition, the natures and concentrations of the additives and temperature on the separations were investigated. Thermodynamic parameters were calculated from plots of $\ln \alpha$ vs $1/T$. $\Delta(\Delta H^\circ)$ ranged between -3.0 and +1.5 kJ mol⁻¹, and $\Delta(\Delta S^\circ)$ between -8.8 and +5.9 J mol⁻¹ K⁻¹. The enantioseparation was generally enthalpically controlled, the retention factor and separation factor decreasing with increasing temperature, but entropically controlled separation was also observed. The elution sequences of the paroxetine enantiomers on the two pairs of pseudo-enantiomeric CSPs were investigated, and an attempt was made to explain the observed anomalies *in silico* in order to gain an insight into the underlying molecular recognition events between the four chiral selectors and the analyte enantiomers.

1. Introduction

The stereoselective analysis of pharmaceutical compounds is extremely important for the pharmaceutical industry since many drug compounds are chiral molecules whose stereoisomers usually possess quite different pharmacological and toxicological properties. Often only one isomer exhibits the required activity (that is the eutomer), while the other(s) (defined as distomer(s)) may possibly be inactive or even toxic. For this reason, the chiral purity of enantiomers and their analysis is essential.

Paroxetine contains two chiral carbon centres, and therefore has four stereoisomers, two in a *cis* and two in a *trans* configuration (Fig. 1). The active ingredient is *trans*-(*-*)-paroxetine [(3*S*,4*R*)-3-[(1,3-benzodioxol-5-yloxy)methyl]-4-(4-fluorophenyl)piperidine], which is a well-known antidepressant drug of the most specific serotonin reuptake inhibitor type. It is used to treat depression, panic disorder, social anxiety/social phobia disorder, post-traumatic stress disorder, generalized anxiety disorder and obsessive-compulsive disorder [1–3].

Several methods of analysis have been reported for the non-stereoselective determination of paroxetine and its key intermediate (precursor) or metabolites in human serum, human plasma or pharmaceutical formulations. High-performance liquid chromatography (HPLC) has frequently been coupled to a diode array [4], ultraviolet (UV) [5], electrochemical [6], fluorescence (without [7] or with derivatization [8]) or mass spectrometric (MS) detection [9]; gas chromatography has been coupled to MS detection [10] or to ultra-HPLC [11]. Two of the important precursors in the synthesis of *trans*-(*-*)-paroxetine, *trans*-3-ethoxycarbonyl-4-(4'-fluorophenyl)-1-methylpiperidine-2,6-dione and paroxol, have earlier been analysed stereoselectively on polysaccharide-derived chiral stationary phases (CSPs) by means of supercritical fluid chromatography as a powerful alternative technique to HPLC [12, 13].

For the chromatographic resolution of the enantiomers of paroxetine, a number of diverse CSPs and “chiral” columns thereof have been investigated successfully. They include β - and γ -cyclodextrin [14], macrocyclic glycopeptide [14], Pirkle or “brush”-type optically active metal complexes (Ru adsorbed to porous spherical ceramics by cation exchange) [14], polysaccharides (amylose and cellulose) [14, 15], glycoprotein (ovomucoid) [16] or tartardiamide-based (*O,O'*-bis(4-*tert*-butylbenzoyl)-*N,N'*-diallyl-*L*-tartardiamide) [17] columns. The present study involves the use of chiral ion exchangers as a promising alternative with a view to solving this analytical challenge.

In liquid chromatography, the retention and selectivity are strongly influenced not only by the mobile phase conditions, but also by temperature. This is especially true for chiral separations. The relationship between the selectivity and the column temperature is as follows:

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

where α is the selectivity factor ($\alpha = k_2/k_1$) and $\Delta(\Delta H)^\circ$ and $\Delta(\Delta S)^\circ$ are the differences in standard enthalpy and standard entropy, respectively, for a given pair of enantiomers. In general, $\Delta(\Delta H^\circ)$ is invariant with temperature, a plot of $R \ln \alpha$ vs $1/T$ having slope $-\Delta(\Delta H^\circ)$ and intercept $\Delta(\Delta S^\circ)$. It is important to note that there are both “achiral” and “chiral” contributions to the retention that can vary with a wide variety of experimental parameters [18-23]. In this study we did not process and quantify the thermodynamic coefficients for the chiral and nonchiral sites, *i.e.* the van't Hoff plots yield apparent enthalpy and entropy values, in which the respective contributions of the chiral and achiral interactions are combined. (Linear plots might be expected when the retention mechanism would not change with

temperature, or the relative weights of the achiral and chiral interactions would not change dramatically.) As described by Fornstedt, for a more realistic approach, the contributions of the enantioselective and nonselective sites should be distinguished [24].

The present work involves mechanistic considerations of enantiorecognition relating to the separation of *trans*-paroxetine enantiomers as model compounds (Fig. 1) on four newly developed *Cinchona* alkaloid quinine (QN) or quinidine (QD)-based zwitterionic ion-exchange-type selectors (SOs) chemically fused with (*R,R*)- and (*S,S*)-aminocyclohexanesulfonic acid (ACHSA) (Fig. 2). A polar-ionic mode (PIM) and a hydro-organic mobile phase were applied. The effects of the composition of the mobile phase, the natures of the additives, the amount of the counter-ion, the temperature and the combination of the chiral subunits of the ampholytic ion-exchangers (Fig. 2) on the retention and enantioselectivity are discussed. Computer modelling was carried out in an effort to achieve a better understanding of the underlying molecular recognition mechanism and the observed elution sequences of the enantiomers.

2. Materials and methods

2.1. Chemicals

Methanol (MeOH), acetonitrile (MeCN) and tetrahydrofuran (THF) of HPLC grade were purchased from VWR International (Arlington Heights, IL, USA). NH₃, ethylamine (EA), diethylamine (DEA), triethylamine (TEA), *n*-propylamine (PA), tri-*n*-propylamine (TPA), *n*-butylamine (BA), tri-*n*-butylamine (TBA) and formic acid (FA) of analytical reagent grade were from Sigma-Aldrich (St. Louis, MO, USA). Synthesis chemicals were purchased

from ABCR G.m.b.H. (Karlsruhe, Germany) and LachNer (Neratovice, Czech Republic). The Milli-Q water was further purified by filtration on a 0.45 μm HV Millipore filter (Molsheim, France). All eluents were degassed in an ultrasonic bath, and helium gas was purged through them during the HPLC analyses.

Standard selectands (SAs) of *trans*-(-)-paroxetine and a *trans*-(+)-paroxetine (Fig. 1) were obtained from Gedeon Richter Ltd. (Budapest, Hungary). Analytes were dissolved in MeOH at a concentration of 1.0 mg ml⁻¹ or 5 mg ml⁻¹ and further diluted with mobile phases when appropriate.

2.2. Apparatus and chromatography

The analytical chromatographic 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 an Empower 2 Chromatography Manager data system (Waters Chromatography, Milford, MA, USA). This system was equipped with a Rheodyne Model 7125 injector (Cotati, CA, USA) with a 20 μl loop. For thermostating of the columns, a Lauda Alpha RA 8 cooling thermostat with a temperature adjustment precision of ± 0.1 °C (Lauda Dr. R. Wobser GmbH & Co. Kg, Königshofen, Germany) was used. Nuclear magnetic resonance spectroscopy (NMR) measurements were carried out on an Agilent 400-MR DDR2 spectrometer. CD₃OD was used as solvent and the signals of the solvent served as an internal standard, *J* values being given in Hz.

Of the four zwitterionic CSPs (Fig. 2 and Table 1), ZWIX(+)TM [QN-(*S,S*)-ACHSA] and ZWIX(-)TM [QD-(*R,R*)-ACHSA] are commercially available from Chiral Technologies Europe (CTE, Illkirch, France), while ZWIX(-A) [QD-(*S,S*)-ACHSA] was prepared as described in the Supplementary Material. Each of these three CSPs comprised 3 μm particles

packed into 150×3.0 mm I.D. columns. ZWIX(+A) [QN-(*R,R*)-ACHSA] comprised $5 \mu\text{m}$ or $3 \mu\text{m}$ particles packed into 150×4.0 mm I.D. or 150×3 mm I.D., respectively. The synthesis of this CSP material was described previously [25]. All columns were provided by CTE (Illkirch, France).

All chromatographic experiments were carried out in isocratic mode at a flow rate of 0.6 ml min^{-1} , with UV detection at 295 nm. The void volume of the columns (t_0) was determined by injecting a solution of acetone in MeOH.

2.3. Molecular modelling methods

With the same protocol as used previously [26–28], a cubic box was built with a $30 \text{ \AA}$ side length. For a realistic reproduction of the stationary phase environment, 4 mercaptopropyl-functionalized silanols ($\sim 1.97 \mu\text{mol m}^{-2}$), 8 free silanols ($\sim 8.0 \mu\text{mol m}^{-2}$) and 45 silicon atoms were considered for each grafted SO unit ($\sim 0.5 \mu\text{mol m}^{-2}$), at the base of the box. All the silicon atoms and their bonded hydrogen atoms in the base layer were set frozen during the molecular dynamics. A custom solvent of MeOH/THF (80:20 v/v) was created in house with the aid of the gromacs tools [29,30] and added to the simulation systems. All the (eight) simulations with the four CSP systems were performed in the canonical ensemble at 298 K. The temperature in the simulation cell was maintained constant through use of a Nosé-Hoover thermostat [31, 32]. All the other parameters in the simulation study were left to default values in the *Desmond Molecular Dynamics System*, version 4.0 [33–35]. A production run produced 3000 frames during the 300 ns dynamics (100 ps recording interval), with an integration time of 2 fs. Two descriptors were calculated: the interaction energy between the SA unit and the whole CSP system (INTER, in kcal mol^{-1}) and

the conformational energy of the SA (SELF, in kcal mol⁻¹) relative to its minimum energy registered by the collected snapshots.

2.4. Method validation

For the determination of linearity, solutions of the standards were prepared at six different concentration levels, from 0.5 to 500 µg ml⁻¹. Three parallel injections of each solution were made onto the ZWIX(+A) (3 µm particle size) column, MeOH/THF (50/50 v/v) containing 50 mM FA and 25 mM NH₃ serving as mobile phase. The peak area responses of the first and second-eluting enantiomers were plotted against the corresponding concentration and the linear regression was computed by the least square method with the Microsoft Excel program. The limit of detection (LOD) and the limit of quantitation (LOQ) were determined from the standard deviation for a calibration curve and its slope. For determination of the precision of peak areas and retention times, replicate HPLC analyses were carried out.

3. Results and discussion

3.1. Effects of mobile phase compositions

The separations of anionic, cationic and amphoteric analytes on zwitterionic *Cinchona* alkaloid- and (*R,R*)- or (*S,S*)-ACHSA-based CSPs are mostly carried out in non-aqueous polar organic solvents containing acid and base additives (often referred to as PIM [36]). In this context, MeOH is applied as protic bulk solvent component (which can suppress H-bonding interactions) and MeCN as aprotic bulk solvent component (which is advantageous for ionic interactions, but disadvantageous for π - π interactions [37]). However, in the case of the

enantioseparation of *trans*-paroxetine, any combination of volume ratios of MeOH/MeCN as bulk solvent containing 50 mM FA and 25 mM DEA proved disadvantageous, no enantiomer separation being observed. However, on the change of MeOH or MeCN to THF, a significant enantioselectivity improvement was observed. The MeOH/THF or MeCN/THF ratio exerted a strong effect on the chromatographic parameters. On all four investigated zwitterionic CSPs with MeOH/THF mobile phase systems containing 50 mM FA and 25 mM DEA, we observed a significant decrease in k_I with increasing MeOH content, whereas the opposite trend was registered in the MeCN/THF system, *i.e.* the k_I values increased with increasing MeCN content (Fig. 3). The higher the MeOH content in the protic solvent, the progressively weaker the ionic interaction between the SA and the SO. This also held for water as solvent component. As a higher amount of aprotic solvent promoted formation of the SO–SA complex, k_I increased. As concerns the selectivity, α changed from 1.00 to 1.17 when the MeOH or MeCN content was increased from 10 to 80 v%. The resolution both increased and decreased with increasing MeOH or MeCN content.

Addition of a small amount of water to a PI mobile phase generally reduces the retention, but in several cases better peak shapes and resolution can be achieved [38]. On ZWIX(-)TM in the MeCN/THF (20/80 v/v) mobile phase containing 50 mM FA and 25 mM DEA, increase of the H₂O content from 0.0 to 5.0 v% was accompanied by a decrease of about 30% in k_I , while α remained practically constant ($\alpha = 1.07$ – 1.10), and R_S exhibited a maximum curve; its values were highest in the mobile phases containing 1.0–2.0 v% H₂O.

3.2. Effects of the natures of base additives

Under the applied acidic mobile phase conditions, *trans*-paroxetine contains a protonated free secondary amino group which will interact electrostatically with the

226 sulfohexyl moiety of the SOs, primarily through an ion-pairing/ion-exchange retention
227 mechanism. Different alkylamines in the presence of an excess of FA in the mobile phase
228 may compete with the SA for the sulfohexyl moiety of the SO. The experimental results
229 depicted in Fig. 4 reveal that, in the MeOH/THF (20/80 v/v) mobile phase system containing
230 50 mM FA and 25 mM alkylamine, k_I increased as the degree of alkyl substitution on the N
231 atom increased (EA<DEA<TEA, PA<TPA and BA<TBA). Moreover, in most cases k_I also
232 increased as the alkyl chain length on the amino group increased (EA<PA<BA and
233 TEA<TPA<TBA). However, NH_4^+ behaved somewhat similarly to Et_2NH_2^+ .

234 Besides the protonated amino group, the present ampholytic SOs (Fig. 2) also
235 intrinsically contain a sulfonic acid group, which interacts both with the salt components and
236 with the counter-ions. The basic components of the mobile phase may therefore compete for
237 CSP binding sites with basic SAs and this may influence the band width and peak symmetry.
238 The larger the cationic component of the mobile phase additive, the less effective it is in
239 displacing a protonated SA from the SO–SA ion-pair complex. As a result, the k values
240 increase. In order to facilitate the enantioselectivity, additional SO–SA binding events are
241 necessary, which can be of a π - π or H-bonding character, for instance.

242 In this context, the nature of the base exerted merely a slight effect on the overall
243 selectivity, α varying in only the narrow range 1.11–1.15 on ZWIX(-)TM, and slight increases
244 were registered in the sequences EA<DEA<TEA, PA<TPA and BA<TBA. The best R_S values
245 were obtained for DEA and BA.

247 3.3. Effects of the counter-ion concentration

248

249 For both anion-exchange and cation-exchange SOs under PIM conditions, a predominant
250 ion-exchange-driven retention mechanism has been confirmed [39] and the observed effect

has been explained by a simple displacement model [40]. In the case of cationic-type SAs, long-range ionic interactions between the cationic SA sites and the anionic SO sites have been detected as primary interactions, with additional intermolecular SO–SA interactions responsible for chiral discrimination. In the ion-pairing process, the counter-ions present in the mobile phase act as competitors, and the retention can be adjusted through variation of the concentrations of the counter-ions. The effects of variation of the concentrations of basic counter-ion additives on the retention were studied. Data demonstrating the effects of DEA on the retention are depicted in Fig. 5. Under the studied conditions, linear relationships were found between $\log k_I$ and $\log c_{DEA}$, with slopes varying in the interval from -0.7 to -0.9. A clear exception to the otherwise similar trends was found for the ZWIX(+A) column with the mobile phase MeOH/MeCN/THF (49/49/2 v/v/v), where the slope was -0.37. The reason for this abnormality is not yet clear.

According to the displacement model of Kopaciewicz *et al.* [40], the slopes of these plots are determined by the ratio of the effective charges of the solute and the counter-ions; slopes in the interval from -0.7 to -0.9 correspond with the values of around 0.9 found for a cation-exchange CSP and different chiral amines [41]. Under the studied conditions on the four zwitterionic CSPs, practically identical slopes were obtained for each enantiomer, *i.e.* the enantioselectivity remained almost constant when the counter-ion concentration was changed. This is in line with the general observation concerning the behaviour of enantioselective ion-exchangers.

3.4. Enantiomer elution sequence and *in silico* simulations

The investigated *Cinchona* QN and QD-based and (*R,R*)- and (*S,S*)-*trans*-2-ACHSA-based chiral SOs, *i.e.* the ZWIX(+)TM and ZWIX(-)TM columns and the ZWIX(+A) and

ZWIX(-A) columns, are frequently termed pseudo-enantiomeric, though they are actually diastereomers, like QN and QD (see Fig. 2). On switching from QN to QD-based SOs, therefore, the sequence of elution of the enantiomers of SAs should in principle be reversed, as observed in several investigations [42-44]. However, the resolution of the enantiomers *trans*-(+)- and (-)-paroxetine does not fully fit this pattern. The sequence of elution of (+)- and (-)-paroxetine on the pseudo-enantiomeric ZWIX(+)TM and ZWIX(-)TM columns was the same, whereas on the other pseudo-enantiomeric pair of ZWIX(+A) and ZWIX(-A) it was reversed. This inconsistency was unexpected. For a better understanding of the interactions of the paroxetine enantiomers with the four SOs, *in silico* simulations of the eight SO-SA systems were computed with a MeOH/THF (80:20 v/v) virtual eluent system. The data listed in Table 1 indicate that the four zwitterionic CSPs permit *trans*-paroxetine enantioseparation at this MeOH/THF volume ratio. However, the α values are typical of low $\Delta(\Delta G)$. The limited difference in thermodynamic stability between the diastereomeric ion-pairing couples limits the amount of mechanistic information that can be derived from the *in silico* simulations.

Nevertheless, a pool of previous evidences [26-28] clearly suggests that the developed molecular dynamic (MD) protocol can be useful to gain more insight into the identification of the key interactions governing the overall enantiorecognition process. Indeed, in spite of the intrinsically limited predictive character of this type of molecular modelling approaches, in cases of low α values, it allowed the qualitative rationalization of the chromatographic behavior of dafachronic acids (DAs) ($\alpha = 1.14$ for Δ^4 -DA and 1.19 for Δ^7 -DA) [26], ketoprofen ($\alpha = 1.09$) [27] and the dipeptidic *N* ^{α} -Boc-*N*⁴-(hydroorotyl)-4-aminophenylalanine ($\alpha_{D,D-/D,L-1} = 1.08$; $\alpha_{D,L-/L,D-1} = 1.08$; $\alpha_{L,D-/L,L-1} = 1.40$) [28], when analysed with similar ion-exchange-type CSPs.

Accordingly, the application of the MD methodology in this study proved useful for a basic understanding of the main features governing the sequence of elution of the *trans*-paroxetine enantiomers. In most of the cases, the chromatographic outcome can be explained through analysis of the SELF and INTER values of the most strongly interacting frames.

Analysis of the most stable frames (best INTER value recorded) with ZWIX(+)TM led to a situation opposite to that observed experimentally, (–)-paroxetine being characterized by a SELF energy ~ 2.7 kcal mol^{–1} lower than that computed for the (+) enantiomer and a more favourable INTER value (~ 1.9 kcal mol^{–1}) (Table 1). This intriguing result is compatible with what may be expected from the pseudo-enantiomeric character of ZWIX(+)TM and ZWIX(–)TM. Figure 6 illustrates the modelled association between the paroxetine enantiomers and ZWIX(+)TM. As a common feature of the virtual models in Fig. 6, an ionic interaction always occurs between the SA piperidine nitrogen and the sulfonic acid residue on the SO. In some cases, additional SA–SO interactions are involved though they influence the estimated INTER value only slightly.

Discrepancies between the experimental and the computational data may be due to the intrinsic limitation of the *in silico* calculation in considering the entropic contributions of mobile phase elements to the overall chromatographic process. Furthermore, possible kinetic processes such as molecular aggregations and competitive mechanism of interactions occurring with the CSP are not taken into account in the simplified virtual model. In some cases, however, these effects become highly significant and should not be ignored. In fact, the mobile phase components are anything but passive transporters of the SA enantiomers along the column, but rather define the degree of complementarity between the functional groups on the SO and SA units and the main forces controlling the sequence of enantiomer elution.

A firm conclusion can be drawn from the analysis of the data obtained with the system simulating the ZWIX(–)TM phase, with which (+)-paroxetine displayed a SELF energy of the

most interacting frame $\sim 6.2 \text{ kcal mol}^{-1}$ lower than that computed for the corresponding (–) enantiomer, while there was only a negligible difference in the case of the INTER energies (Table 1). Figure 6 demonstrates the modelled association between the paroxetine enantiomers and ZWIX(–)TM.

For the ZWIX(+A) and ZWIX(–A) phases, full agreement was observed between the computational and experimental results. With the simulated ZWIX(+A), the SELF and INTER energies of the most interacting frames (Figs. 6C, D) agreed well with the observed sequence of elution (+) < (–) (Table 1). In this setting, the quite large difference in the SELF energies ($\sim 22.5 \text{ kcal mol}^{-1}$) in favour of (–)-paroxetine deserves to be highlighted (Table 1).

With the *in silico* reproduced ZWIX(–A), (+)-paroxetine exhibited a SELF energy of the most interacting frames $\sim 10.5 \text{ kcal mol}^{-1}$ lower than that of the corresponding (–) enantiomer, but similar INTER energies (Table 1, Figs. 6E, F). This result is in line with the experimental sequence of elution (–) < (+). Interestingly, the simulation of ZWIX(–A) represents a paradigmatic case where the less retained enantiomer, (–)-paroxetine, triggers two π –cation-type interactions between the SA aromatic rings and the charged nitrogen of the QD moiety of the SO, without impacting the INTER energies to a relevant extent (Table 1, Fig. 6F).

With the aim of gaining more information from the pool of computational outcomes, a deeper analysis of the conformations assumed by the four SOs during the MD simulation was carried out. The percentage occurrences of the different intramolecular interactions (π – π -stacking, π –cation and H-bonding) were calculated for each of the four virtual systems. Only an intramolecular ion-pairing materializes between the sulfonic acid moiety and the QD nitrogen of each SO unit. Interestingly, this intramolecular coulombic interaction was found to occur more often in QN-(*R,R*)-ACHSA than in its pseudo-enantiomeric variant QD-(*S,S*)-ACHSA: in 12.1% and 3.6%, respectively, of the frames recorded during the MD simulation. The

resulting conformational difference between QN-(*R,R*)-ACHSA and QD-(*S,S*)-ACHSA can explain the different stereorecognition mechanisms activated by ZWIX(+A) and ZWIX(-A). Only a slight difference in intramolecular counter-ion effect was found between QN-(*S,S*)-ACHSA and QD-(*R,R*)-ACHSA 1.3% and 2.3%, respectively, which could account for the observed elution behaviour.

Interesting results also emerged from the analysis of the π - π -stacking interactions taking place between the paroxetine enantiomers and the SO during the MD run. The case of ZWIX(+A) may be highlighted, where (+)-paroxetine engaged in π - π -stacking interactions with aromatic portions of QN-(*R,R*)-ACHSA in 12.0% of the 3000 frames, whereas the frequency was much lower (1.5%) for (-)-paroxetine. A much less pronounced difference (1.9 percentage points) emerged for QD-(*S,S*)-ACHSA in ZWIX(-A). This finding supports the previous assumption of dissimilar SO-SA associations in the pseudo-enantiomeric QD-(*S,S*)-ACHSA and QN-(*R,R*)-ACHSA. Negligible differences were observed for the QN-(*S,S*)-ACHSA and QD-(*R,R*)-ACHSA pseudoenantiomeric pair.

3.5. Temperature dependence and thermodynamic parameters

The effects of temperature on the chromatographic parameters on the four *Cinchona* alkaloid-based CSPs were studied over the temperature range 0–50 °C. Experimental data with the mobile phases (ensuring the best separation) containing 50 mM FA and 25 mM DEA are presented in Supplementary Table S1.

In most cases, the k values on all four CSPs decreased with increasing temperature, as might be expected when α and R_s decrease in parallel. However, in the mobile phase MeOH/THF (20/80 v/v) containing 50 mM FA and 25 mM DEA on ZWIX(+A) in the temperature range 0–40 °C, k_I increased from 2.47 to 2.53 with increasing temperature, which

is rather unusual. Such behaviour, *i.e.* an increase in k , but a decrease in α with increasing temperature, was earlier reported by Adlof and List [45], Wu *et al.* [46] and Yogo *et al.* [47] for non-chiral separations, and by Matarashvili *et al.* [48] and Ilisz *et al.* [42, 46, 49] for chiral separations. The changes observed in selectivity with temperature in the present study were inconsistent. As usual, α and R_S (Table S1) decreased with increasing temperature, but on ZWIX(+)TM α increased from 1.04 to 1.16 and R_S from 0.38 to 0.99 when the temperature was increased from 0 °C to 50 °C.

Since the effects of temperature on enantiomer separation are complex, the thermodynamic parameters were established on the basis of the chromatographic data through the application of van't Hoff plots [Eq. (1)]. The differences in the changes in standard enthalpy and entropy, $-\Delta(\Delta H^\circ)$ and $-\Delta(\Delta S^\circ)$, calculated from the $\ln \alpha$ vs $1/T$ curves, are presented in Table 2.

The $\Delta(\Delta H^\circ)$ values, which ranged from -3.0 to +1.5 kJ mol⁻¹, were more negative on ZWIX(-)TM and ZWIX(-A) than on ZWIX(+)TM or ZWIX(+A). When $\Delta(\Delta H)^\circ$ and $\Delta(\Delta S)^\circ$ are both negative, the second-eluted enantiomer forms a more stable complex with SO than does the first-eluted enantiomer, and the negative entropy is more unfavourable for enantioseparation. In this case, the selectivity decreases with increasing temperature. When $\Delta(\Delta H)^\circ$ and $\Delta(\Delta S)^\circ$ are both positive, which was the case for ZWIX(+)TM (Table S1 and Table 2), the separation factor increases with increasing temperature, in parallel with decreasing retention. In this case, the change in the adsorption enthalpy 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 enantioseparation is entropically driven (Table 2 3). 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 on

ZWIX(-)TM and ZWIX(-A) were characterized by the highest negative $\Delta(\Delta H^\circ)$ and $\Delta(\Delta S^\circ)$ values.

The thermodynamic parameter $\Delta(\Delta G^\circ)_{298}$ suggests that the binding to the SO was induced more efficiently on ZWIX(-)TM and ZWIX(-A), as reflected by the lower $\Delta(\Delta G^\circ)$ values.

The relative contributions of the enthalpic and entropic terms to the free energy of adsorption can be visualized through the enthalpy/entropy ratio Q [$Q = \Delta(\Delta H^\circ) / [298 \times \Delta(\Delta S^\circ)]$] calculated at 298 K (Table 2). Comparison of the Q values for the individual analytes revealed that the enantioselective discrimination was enthalpically driven ($Q > 1.0$) in most cases, indicating the higher contribution to the free energy, while $Q < 1.0$ was registered on ZWIX(+)TM, reflecting the higher contribution of entropy to the free energy.

3.6. Enantiomeric excess analysis

Satisfactory linearity was observed for the investigated concentration range (0.5–500 $\mu\text{g ml}^{-1}$), with $R^2 = 0.999$. However, for samples with lower concentrations, limiting the concentration range to 0.5–50 $\mu\text{g ml}^{-1}$ offers more reliable results, with the regression equation $y = 3.4 \times 10^4 x - 8550$ ($R^2 = 0.999$) for *trans*-(-)-paroxetine, and $y = 3.5 \times 10^4 x - 11090$ ($R^2 = 0.999$) for *trans*-(+)-paroxetine. (The differences in the regression parameters of the enantiomers were within the standard error.)

LOD and LOQ were determined via the overall performance of the calibration curve, *i.e.* the standard error of the y-intercept and the slope. LOD was 3 ng and 3.2 ng, while LOQ was 9 ng and 10 ng for *trans*-(-)-paroxetine and *trans*-(+)-paroxetine, respectively. Figure 7 presents a chromatogram when the ratio of the minor and major components was 1:1000. The minor isomer can be determined when it is present at a level of at least 0.1%, though an LOQ

of 0.02% should be possible on the use of adjusted calibration standards. (European Pharmacopeia 8.0 specifies that the amount of *trans*-(+)-paroxetine in *trans*-(-)-paroxetine should be <0.2%.)

For the determination of precision, replicate HPLC analysis demonstrated that the relative standard deviation did not exceed 5.5% for the peak area response, and was <2.8% for the retention time.

4. Conclusions

The enantiomers of *trans*-paroxetine (representing a group of chiral basic pharmaceuticals) were separated on zwitterionic Chiralpak ZWIX(+)TM, ZWIX(-)TM, ZWIX(+A) and ZWIX(-A) columns based on chiral SOs composed of QN- or QD- or (*R,R*)- or (*S,S*)-*trans*-aminocyclohexane sulfonic acid subunits. The chromatographic parameters were found to depend on the mobile phase composition, the natures and concentrations of the mobile phase additives and temperature.

The four zwitterionic CSPs behaved as chiral cation exchangers; under optimized conditions, baseline resolution was achieved. The elution sequence was interpreted means of by computational modelling studies. The elution sequence (+) < (-) on ZWIX(+A) was advantageous from the aspect of the chiral separation, as the minor component elutes before the major one, where a mobile phase of MeOH/THF (50/50 (v/v) containing 25 mM NH₃ and 50 mM FA at 25 °C offered the best resolution. The results obtained in this study add new insight concerning the enantiodiscrimination power of such chiral systems.

Conflict of interest

The authors declare no conflict of interest

448

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612

613 **Figure legend**

614 **Figure 1**

615 Structures of *trans*-(-)- and *trans*-(+)-paroxetine

616

617 **Figure 2**

618 Structures of the selectors ZWIX(+)TM, ZWIX(-)TM, ZWIX(+A) and ZWIX(-A), comprising
619 the chiral subunits of quinine (QN), quinidine (QD) and (*R,R*)- or (*S,S*)-ACHSA

620

621 **Figure 3**

622 Effects of the bulk solvent composition on the retention factor of the first-eluting enantiomer
623 (k_I), the separation factor (α) and the resolution (R_S)

624 Chromatographic conditions: columns, ZWIX(-)TM, ZWIX(+)TM, ZWIX(-A) or ZWIX(+A);
625 mobile phases, MeOH/THF and MeCN/THF (80/20, 50/50, 20/80 and 10/90 v/v) containing
626 25 mM DEA and 50 mM FA; flow rate, 0.6 ml min⁻¹; detection, 295 nm; symbols: for k_I : ■;
627 for α : ●; and for R_S : ▲

628

629 **Figure 4**

630 Effects of the nature of the alkylamines on the retention of the first-eluting enantiomer (k_I),
631 the separation factor (α) and the resolution (R_S)

632 Chromatographic conditions: column, ZWIX(+A); mobile phase, MeOH/THF (20/80 v/v)
633 containing 25.0 mM alkylamine and 50.0 mM FA; flow rate, 0.6 ml min⁻¹; detection, 295 nm;

634 symbols, k_I : ▨; α : ▩; and R_S : ▤

635

636 **Figure 5**

Influence of the counter-ion concentration on the retention of the first-eluting enantiomer (k_I)
Chromatographic conditions: column, **A** and **B**, ZWIX(-)TM; **C** and **D**, ZWIX(+)TM; **E**, ZWIX(+A); and **F**, ZWIX(-A); mobile phase, **A** and **C**, MeOH/THF/H₂O (20/78/2 v/v/v); **B** and **D**, MeCN/THF/H₂O (20/79/1 v/v/v); **E**, MeOH/THF (20/80 v/v); **F**, MeOH/MeCN/THF (49/49/2 v/v/v), all mobile phase containing DEA/FA (mM/mM), 6.125/12.5, 12.5/25, 25/50, 50/100 and 100/200 (in all cases the base-to-acid ratio being kept at 1:2); flow rate, 0.6 ml min⁻¹; detection, 295 nm

Figure 6

Best interacting frames recorded with the four CSPs

(A) (+)-Paroxetine with ZWIX(-)TM; (B) (-)-paroxetine with ZWIX(-)TM; (C) (+)-paroxetine with ZWIX(+A); (D) (-)-paroxetine with ZWIX(+A); (E) (+)-paroxetine with ZWIX(-A); (F) (-)-paroxetine with ZWIX(-A). H-bonds, π - π -stacking, and π -cation-type interactions are also displayed. An ionic interaction always occurs between the SA piperidine nitrogen and the sulfonic acid residue on the SO

Figure 7

Chromatogram for the determination of 0.1% *trans*-(+)-paroxetine in the presence of *trans*-(-)-paroxetine

Chromatographic conditions: column, ZWIX(+A) (3 μ m particle size); mobile phase MeOH/THF (50/50 (v/v) containing 25 mM NH₃ and 50 mM FA; temperature, 25 °C; flow rate, 0.3 ml min⁻¹; detection, 295 nm

Table 1

SELF and INTER values (in kcal mol⁻¹) with CSPs 1-4.

Analyte	ZWIX(+)TM QN-(<i>S,S</i>)-ACHSA		ZWIX(-)TM QD-(<i>R,R</i>)-ACHSA		ZWIX(+A) QN-(<i>R,R</i>)-ACHSA		ZWIX(-A) QD-(<i>S,S</i>)-ACHSA	
	<i>SELF</i>	<i>INTER</i>	<i>SELF</i>	<i>INTER</i>	<i>SELF</i>	<i>INTER</i>	<i>SELF</i>	<i>INTER</i>
(+)-paroxetine	23.5	-83.3	35.3	-85.4	42.7	-86.6	7.5	-86.6
(-)-paroxetine	20.8	-85.2	41.5	-86.0	20.2	-89.4	18.0	-85.8
<i>α</i>	1.07		1.16		1.11		1.10	
EES*	(-) < (+)		(-) < (+)		(+) < (-)		(-) < (+)	

*enantiomeric elution sequence

Table 2

Thermodynamic parameters, $\Delta(\Delta H^\circ)$, $\Delta(\Delta S^\circ)$, $T_x\Delta(\Delta S^\circ)$, $\Delta(\Delta G^\circ)$, correlation coefficients (R^2) and Q values of paroxetine enantiomers on ZWIX(+)TM, ZWIX(-)TM, ZWIX(+A) and ZWIX(-A) columns measured in the temperature range 0–50 °C

Column	Eluent (+25 mM DEA and 50 mM FA)	$-\Delta(\Delta H^\circ)$ kJ mol ⁻¹	$-\Delta(\Delta S^\circ)$ J mol ⁻¹ K ⁻¹	Corr. coeff. (R^2)	$-T_x\Delta(\Delta S^\circ)_{298K}$ kJ mol ⁻¹	$-\Delta(\Delta G^\circ)_{298K}$ kJ mol ⁻¹	Q
ZWIX(-) TM	20/80 MeOH/THF	3.0*	8.8*	0.9969	2.6*	0.4*	1.2
	20/80 MeCN/THF	2.0**	5.9**	0.9917	1.7**	0.2**	1.2
	20/79/1 MeCN/THF/H ₂ O	1.2*	3.1*	0.9907	0.9*	0.2*	1.3
ZWIX(+) TM	80/20 MeOH/THF	-1.5	-5.9	0.9977	-1.8	0.2	0.8
ZWIX(+A)	20/80 MeOH/THF	0.7	1.4	0.9912	0.4	0.3	1.8
	20/80 MeCN/THF	0.3	0.5	0.9985	0.1	0.1	3.0
ZWIX(-A)	49/49/2 MeOH/MeCN/THF	2.3	6.3	0.9915	1.9	0.4	1.2

Chromatographic conditions: flow rate, 0.6 ml min⁻¹; detection, 295 nm; R^2 , correlation coefficient of van't Hoff plot, $\ln \alpha$ vs $1/T$ curves; * temperature range 0–30 °C, ** temperature range 0–40 °C; $Q = \Delta(\Delta H^\circ) / 298 \times \Delta(\Delta S^\circ)$

Figure 1

trans-(-)-paroxetine

trans-(+)-paroxetine

Figure 2

CSP	QN or QD	C-8	C-9	ACHSA	Label	Remarks
CSP 1	QN	<i>S</i>	<i>R</i>	1'' <i>S</i> , 2'' <i>S</i>	ZWIX(+) TM	pseudo enantiomers
CSP 3	QN	<i>S</i>	<i>R</i>	1'' <i>R</i> , 2'' <i>R</i>	ZWIX(+A)	
CSP 2	QD	<i>R</i>	<i>S</i>	1'' <i>R</i> , 2'' <i>R</i>	ZWIX(-) TM	
CSP 4	QD	<i>R</i>	<i>S</i>	1'' <i>S</i> , 2'' <i>S</i>	ZWIX(-A)	

Figure 3

ZWIX(-)TM

ZWIX(+)TM

ZWIX(+A)

ZWIX(-A)

Figure 4

Figure 5

Figure 6

Figure 7