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Enantiomerization of chiral 2,3,3a,4-tetrahydro-1*H*-pyrrolo[2,1-*c*][1,2,4] benzothiadiazine 5,5-dioxide by stopped-flow multidimensional HPLC☆

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ABSTRACT

An on-column stopped-flow multidimensional HPLC (sfMDHPLC) procedure using two chiral stationary phases (CSPs) and one achiral C_{18} column was developed for the determination of rate constants and free energy barriers of enantiomerization of (±)(*R,S*)-2,3,3a,4-tetrahydro-1*H*-pyrrolo[2,1-*c*][1,2,4]benzothiadiazine 5,5-dioxide. Moreover, a stopped-flow HPLC (sfHPLC) method previously developed was applied to the determination of kinetic parameters of enantiomerization of the above compound in the presence of a CSP. The individual enantiomers of the studied compound were isolated in parallel by preparative HPLC and the rate constants and free energy barriers of enantiomerization were determined in different solvents (off-column method). The data obtained by sfMDHPLC, sfHPLC and off-column methods were compared. The (*S*) enantiomer of the studied compound (S18986) was prepared by asymmetric synthesis and subsequently purified by preparative HPLC, followed by the determination of rate constants and free energy barriers of enantiomerization in different buffer solutions at pH 2–9.3.

1. Introduction

Several benzothiadiazines have been shown to be potential drugs for memory deficits and cognition disorders, such as attention disorders in children and senile dementias, including early stage of Alzheimer diseases [1,2]. In particular, IDRA21 (±)-**1** [(±)-7-chloro-3-methyl-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxide] (Fig. 1) was shown to be a potent cognition enhancer in animals, suggesting the potential applicability of this drug as a nootropic agent [3–7]. These compounds are thought to work by potentiating glutamate synaptic currents through a positive allosteric modulation of AMPA receptor desensitization [1].

Among the compounds related to IDRA21, the racemic pyrrolo derivative 2,3,3a,4-tetrahydro-1*H*-pyrrolo[2,1-*c*][1,2,4]-benzothiadiazine 5,5-dioxide (±)-**2** (Fig. 1) was shown to be an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor positive modulator [8,9]. The individual enantiomers of (±)-**2** were isolated by chiral HPLC and it was demonstrated that the desired pharmacological activity resides in the (+)-(*S*) enantiomer (S18986) [8]. Recently S18986 has attracted particular attention since *in vivo* experiments demonstrated its cognition enhancing properties when it is administered orally at the dose of 1 mg/kg for 3 days [10,11].

Previously, we have reported the resolution of IDRA21 enantiomers by chiral chromatography and found that a rapid interconversion (enantiomerization) of the IDRA21 enantiomers occurred in aqueous solution [12]. A procedure for the determination of apparent rate constants and apparent free energy barriers of enantiomerization without the implementation of computer simulation has been developed [13–26]. The HPLC stopped-flow procedure developed was applied to study on-column enantiomerization of several chiral benzothiadiazines and it turned out a relatively low activation barrier of enantiomerization in aqueous solvents [13].

The U.S. Food and Drug Administration (FDA) requires extensive stereochemical informations on chiral drugs; consequently it is important to understand the stereochemical integrity of those drugs that are administered as pure enantiomers [27].

Since S18986 is structurally related to IDRA21, that enantiomerizes rapidly in aqueous solutions, it seems advisable to evaluate the enantiomeric stability of the S18986.

Rate constants and free energy barriers of enantiomerization of configurationally labile chiral compounds have been calculated by means of dynamic NMR (DNMR) [28–30], chirooptical methods [31,32], dynamic gas chromatography (DGC) [33], dynamic

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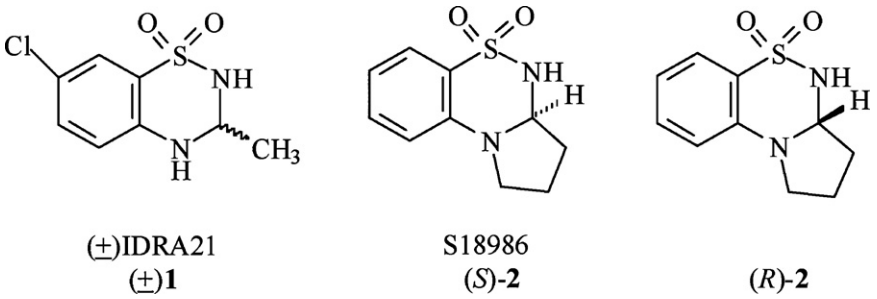

Fig. 1. Structures of analytes investigated.

high-performance liquid chromatography (DHPLC) [26], dynamic capillary electrophoresis (DCE) [34].

More recently it was developed a procedure for the determination of rotational barriers of configurationally labile compounds based on single column stopped-flow HPLC (sfHPLC) and stopped-flow GC (sfGC) where, in contrast to dynamic methods, computer simulation is not necessary [13–26]. Unfortunately, by using single column dynamic and stopped-flow methods, enantiomerization

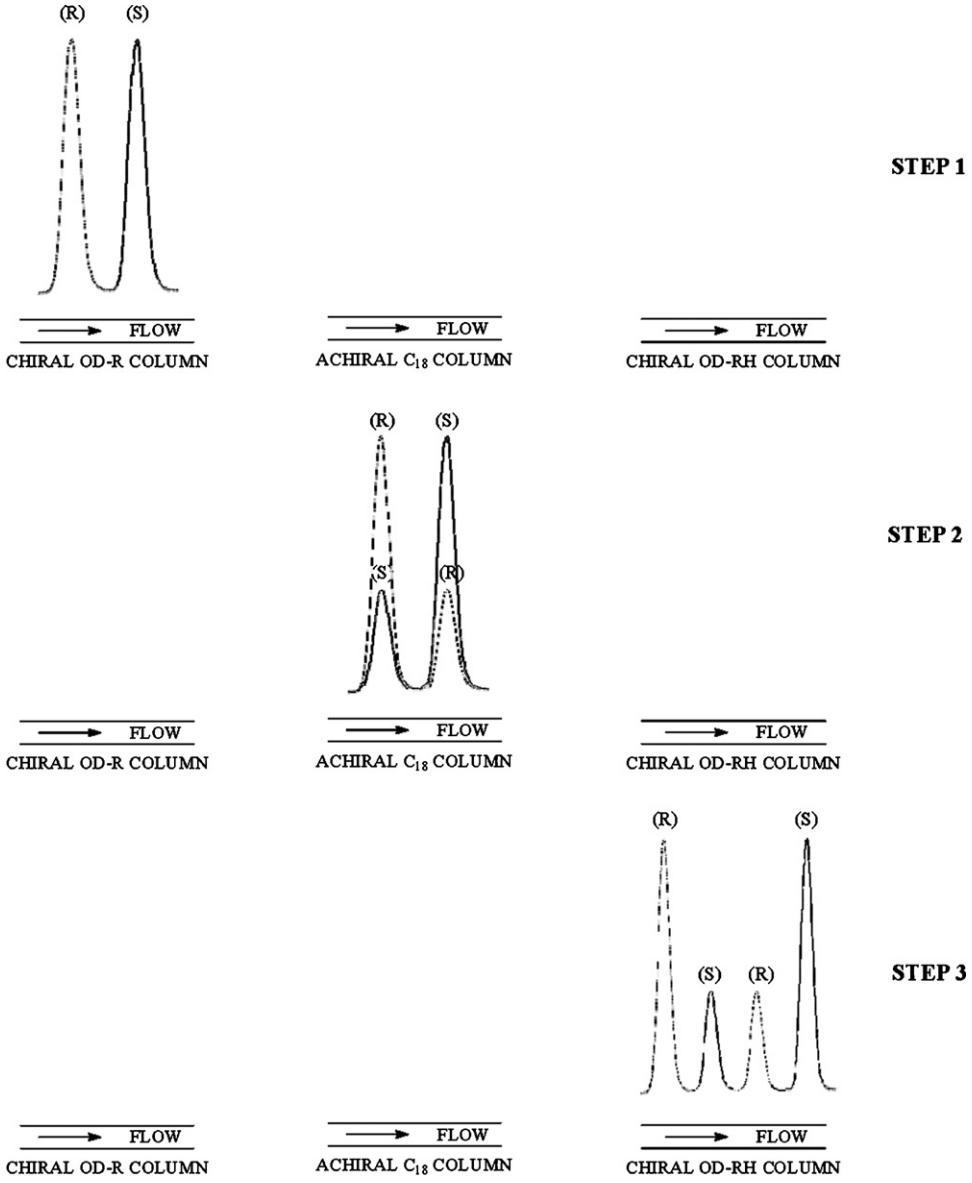

Fig. 2. Concept of stopped-flow multidimensional HPLC (sfMDHPLC) for enantiomerization of (±)(*R*,*S*)-**2**. Step 1: (±)(*R*,*S*)-**2** was injected and a quantitatively separation of enantiomers occurred in the first chiral OD-R column at low temperature conditions. Step 2: the second achiral C_{18} column was kept for a set time at 37 °C. Each enantiomers were interconverted in the other one in a time-dependent manner. Step 3: the enantiomers were separated in the third chiral OD-R column at low temperature conditions.

proceeds in the environment of the chiral stationary phase (CSP), which is required to separate the enantiomers on-line. The presence of the CSP could affect the enantiomerization barrier, thus efforts have been made to perform enantiomerization in an achiral environment [26].

At this aim, multidimensional techniques employing three columns in series in GC (sfMDGC) [35–37] and in CE (sfMDCE) [38,39] have been developed. Usually the first and the third column are chiral and the second column is achiral. In the first chiral column the enantiomers are quantitatively separated and the enantiomerization has been performed in the second achiral column. Afterwards, the flow of the mobile phase introduces the enantiomers to the third chiral column where they are separated.

In the present work, the apparent rate constants and apparent free energy barriers of S18986 enantiomerization are determined by the novel stopped-flow multidimensional HPLC (sfMDHPLC) chromatographic technique similar to sfMDGC and to sfMDCE.

In addition, the apparent rate constants and apparent free energy barriers of enantiomerization of S18986, obtained by asymmetric synthesis and subsequently purification by preparative chiral HPLC, were investigated by classical batchwise kinetic methods (off-column method).

The results obtained by sfMDHPLC and by conventional racemization kinetics were compared to validate the method developed.

2. Experimental

2.1. Instrumentation

The chromatographic apparatus consisted of a Shimadzu LC-10AD Pump, a Rheodyne 7125 manual injector equipped with a 50 or 500-μl sample loop. A Jasco Model J-710 spectropolarimeter or a Merck Hitachi L-7400UV were used as detectors. Chromatograms were recorded with a Jasco J-700 or a HSM Hitachi chromatography programs. A Rheodyne 7010 valve was installed post the first chiral column 1 and permitted the trapping of the individual enantiomers of (±)(*R*,*S*)-**2**. Column temperature regulation was achieved with a Haake F3 thermostated water bath.

The columns used were Chiralcel OD-R [tris(3,5-dimethylphenylcarbamate); 250 mm × 4.6 mm ID; 10 μm] purchased from Daicel, Chiralcel OD-RH [tris(3,5-dimethylphenylcarbamate); 150 mm × 4.6 mm ID; 5 μm] purchased from Daicel, Chiraspher NT [poly(*N*-acryloyl-*S*-phenylalanineethylester); 250 mm × 10 mm ID; 5 μm] purchased from Merck and Supelcosil LC-18 (250 mm × 4.6 mm ID; 5 μm) purchased from Supelco. Rotation angle (α) was measured with the P-2000 Digital Polarimeter (cell-length 100 mm, volume 1 ml) from Jasco. Melting points were determined with an electrothermal apparatus and they are uncorrected. IR spectra were recorded on a PerkinElmer Model 1600 FT-IR spectrometer and were consisted with the assigned structures. ^{1}H NMR spectra were recorded with a Brucker DPX 200 spectrometer using DMSO-d_6 as solvent and tetramethylsilane (TMS) as external standard. Chemical shifts (δ) are in part per million and coupling constant (J) in hertz. Multiplicities are abbreviated as follows: s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet.

Elemental analyses were performed on a Carlo Erba Analyzer Model 1106 apparatus.

All pH measurements were made using Orion Research EA940 pH meter.

2.2. Synthesis

2.2.1. (±)-2,3,3a,4-Tetrahydro-1H-pyrrolo[2,1-c][1,2,4]-benzothiadiazine 5,5-dioxide ((±)-**2**)

The compound was synthesized as previously described by Cameroni et al. [8].

Yield 56.4%, mp 226–227 °C, FT-IR 3222 cm^{-1} (ν NH), 1300 cm^{-1} (ν SO_2 as), 1160 cm^{-1} (ν SO_2 sim). ^{1}H NMR (DMSO-*d*6) δ = 1.87–2.75 (m, 4H), δ = 3.31 (t, 2H, J = 4), δ = 4.93–5.18 (m, 1H), δ = 6.70–6.95 (m, 2H), δ = 7.34–7.71 (m, 3H). The microanalyses results were within ±0.4%.

2.2.2. (+)(S)-2,3,3a,4-Tetrahydro-1H-pyrrolo[2,1-c][1,2,4]-benzothiadiazine 5,5-dioxide (S18986)

The compound was synthesized in our laboratory as described by Desos et al. [9] with minor modification. Briefly, an ether solution containing the (+)-(2*S*,3*R*)-4-dimethylamino-3-methyl-1,2-diphenyl-2-butanol (16.08 g) was added at 0 °C to a magnetically stirred solution of $LiAlH_4$ 1 M in ether (25.21 ml) diluted in 113.32 ml of ether. The reaction mixture was vigorously stirred at 0 °C during 15 min. The 2,3-dihydro-1*H*-pyrrolo[2,1-*c*][1,2,4]benzothiadiazine 5,5-dioxide (4 g) was added in several portions and the mixture was stirred 4 h at 0 °C. The reaction was quenched by addition of water and filtered by vacuum pump. The basic filtrate was acidified below to pH 5–6 by HCl 1.2 M. The white precipitate was collected (2.3 g; e.e. = 84%) and purified by HPLC (e.e. = 99.21%).

$[\alpha]_D = +214^\circ$ (0.758 mg/ml; 95% ethanol; 24 °C).

2.3. Chromatography

2.3.1. Analytical enantioseparation

On-column enantioseparation of (*S*)- and (*R*)-**2**, was carried out isocratically at different temperatures. The mobile phases for Chiralcel OD-R and Chiralcel OD-RH consisted of buffer $NaClO_4$ (pH 2) 0.05 M:acetonitrile 60:40 (v/v) or water and acetonitrile 60:40 (v/v). The compounds were dissolved in ethanol and subsequently diluted 1:100 (v/v) with mobile phase at final concentration of 18.6 μg/ml. The injection volume was 50 μl. The detectors were set at 254 nm. HPLC-grade ethanol 95%, acetonitrile were obtained from Baker; sodium perchlorate, perchloric acid was obtained from Fluka.

2.3.2. Preparative enantioseparation

Pure (+)(*S*) and (−)(*R*) enantiomers were obtained by preparative HPLC on Chiraspher NT column with fraction collection of the respective peaks. The mobile phase consisted of *n*-hexane and THF 70:30 (v/v). The compound was dissolved in THF at final concentration of 3.5 mg/ml. The injection volume was 500 μl. The detectors were set at 254 nm. The collected fractions corresponding to the enantiomers were analyzed by injection on the same column and in the same chromatographic conditions. HPLC-grade *n*-hexane and THF were obtained from Baker.

2.3.3. Chromatographic parameters

The separation factor (α) was calculated as k'_2/k'_1 and retention factors (k'_1 and k'_2) as $k'_1 = (t_1 - t_0)/t_0$ where t_1 and t_2 refer to the retention times of the first and second eluted enantiomers. The resolution factor (R_s) was calculated by the formula $R_s = 2(t_2 - t_1)/(w_1 + w_2)$ where w_1 and w_2 are the peak widths at base for the first and second eluted enantiomers. The dead time of the columns (t_0) was determined by injection of 1,3,5-tri-*tert*-butylbenzene.

2.4. On-column enantiomerization

A stopped-flow procedure previously developed was employed to study the on-column enantiomerization of the (*S*)- and (*R*)-**2** enantiomers [13]. The racemic mixture of (±)(*R*,*S*)-**2** was chromatographed at low temperature (0 °C) on the OD-R CSP.

After a specific time, the mobile phase flow was stopped and the column was brought up to 37.5 °C for a certain period of time to affect racemization of the separated individual enantiomers of (±)(*R*,*S*)-**2**. Afterwards, the column was cooled back to the previous low temperature and the original flow rate was resumed. Apparent enantiomerization rates (k_1^{app} and k_{-1}^{app}) were calculated using the peak areas of all four separated enantiomers.

2.5. Stopped-flow multidimensional HPLC enantiomerization (sfMDHPLC)

A schematic representation of the sfMDHPLC technique is shown in Figs. 2 and 3. At the beginning of the experiment, (±)(*R*,*S*)-**2** was injected on the Chiralcel OD-R column 1 and the individual enantiomers (*S*)- and (*R*)-**2** were initially separated quantitatively at 0 °C. At the appropriate time the individual enantiomers were cut off by switching the valve into position (b) (Fig. 3) and trapped into the second achiral column 2. The enantiomerization was effected by heating at 37.5 °C while no mobile phase passed through the column 2 ("stopped-flow") for a set period of time (t_{enant}). Afterwards the column 2 was cooled back to the previous low temperature (0 °C) and the valve was switched again in position (a), so that the mobile phase was allowed to run through the second achiral column and the enantiomers were introduced into the third chiral column and allowed to be separated quantitatively. On elution four peaks appeared in the chromatogram as expected (Fig. 2). Peaks 1° and 4° arise from the enantiomer separation in step 1 while the peaks 2° and 3° arise from interconverted sample formed in step 2 (Fig. 2).

2.6. Off-column racemization

Single enantiomers of (±)(*R*,*S*)-**2**, purified by preparative chromatography and solvent evaporation of the respective peak fractions, were used to study enantiomerization rates in different solvents. Pure enantiomers were dissolved in ethanol and subsequently diluted 1:100 (v/v) with individual solvent (1 ml) at final concentration of 18.6 μg/ml and kept thermostated. Racemization was monitored by chromatography on the OD-RH column. Four repeats of each experiment were made and enantiomeric ratios were calculated from the peak areas of the enantiomers. Perchlorate buffer solution was prepared by dissolving 3.51 g of sodium perchlorate in 500 ml of water and adjusting to pH 2 by perchloric acid. Chloroacetate buffer solution of ionic strength of 0.01 at pH 3 was prepared by mixing 16.21 ml of chloroacetic acid 0.1 M with 8.89 ml of KOH 0.1 M and diluting with water to 100 ml. Acetate buffer solution of ionic strength of 0.01 at pH 5 was prepared by mixing 15.34 ml of acetic acid 0.1 M with 9.99 ml of KOH 0.1 M and diluting with water to 100 ml. Phosphate buffer solution of ionic strength of 0.01 at pH 7 was prepared by mixing 13.96 ml of KH_2PO_4 0.02 M with 24.04 ml of Na_2HPO_4 0.01 M and diluting with water to 100 ml. Borate buffer solution of ionic strength of 0.01 at pH 9.3 was prepared by mixing 18.30 ml of $Na_2B_4O_7$ 0.025 M with 8.56 ml of KOH 0.01 M and diluting with water to 100 ml. Simulated gastric fluid (SGF) without pepsin was prepared according to USP 24.

2.7. Calculation of kinetic rate constants and free energy barriers of enantiomerization

The processes of enantiomerization follow reversible first-order reaction kinetics. The kinetic rate constants can be calculated by fitting the data to Eq. (1)

$$\ln\left(\frac{a_0}{2a_t - a_0}\right) = 2kt_{enant} \quad (1)$$

where k is the rate constant of forward or backward enantiomerization (s^{-1}), a_0 the peak of the initial enantiomer before enantiomerization (=100%), a_t the relative peak area of the enantiomer remaining after enantiomerization time t (%) (i.e. $a_t = 100 -$ conversion), and t_{enant} the enantiomerization time (s).

From the kinetic rate constants, the corresponding activation energies of enantiomerization (rotational energy barriers) $\Delta G^{\#}(T)$ can be calculated by the Eyring equation:

$$\Delta G^{\#}(T) = -RT \ln\left(\frac{kh}{\kappa k_B T}\right) \quad (2)$$

where k is kinetic rate constant, k_B the Boltzmann constant ($k_B = 1.380662 \times 10^{-23}$ J K^{-1}), h Planck's constant ($h = 6.626176 \times 10^{-34}$ J s), R the universal gas constant ($R = 8.31441$ J K mol^{-1}), κ the transmission coefficient ($\kappa = 0.5$ for the reversible microscopic interconversion) and T the temperature (K).

3. Results and discussion

3.1. Chromatography

Baseline enantiomeric resolution was obtained using OD-RH column with a mobile phase of water:acetonitrile 60:40 (v/v) ($k'_1 = 2.33$, $k'_2 = 3.33$, $\alpha = 1.43$, and $R_s = 2.67$). The enantioseparation was performed at different temperatures. Hardly any enantiomerization of (*S*)- and (*R*)-**2** occurred during enantioseparation at temperatures 0–37 °C using water:acetonitrile as mobile phase.

The change of pH of mobile phase (buffer $NaClO_4$ (pH 2) 0.05 M:acetonitrile 60:40, v/v) did not influence the chromatographic parameters but a plateau was observed between the peaks corresponding to the enantiomers, indicating that acidic condition catalyzed enantiomerization of (±)(*R*,*S*)-**2** (Fig. 4).

In order to prepare (*S*)-enantiomer (S18986) an asymmetric synthesis was performed [9]. The enantiomeric excess achieved in this reaction was reasonably high (84%), as calculated by HPLC analysis. S18986 was subsequently purified by semipreparative chiral chromatography on Chiraspher NT column. A loading of 1.7 mg of S18986 (e.e. 84%) dissolved in 500 μl of THF on the 1-cm ID Chiraspher NT column afforded complete enantioseparation, without peaks overlapping. The collected fractions containing the single enantiomers revealed high enantiomeric excess value (e.e. >99%).

A specific rotation in ethanol of first eluted enantiomer was $[\alpha]_D = +214°$ (0.758 mg/ml; 95% ethanol; 24 °C). This value was slightly lower than that reported in literature [9] for (*S*)-enantiomer $[\alpha]_D = +260°$ (0.5 mg/ml; 95% ethanol). From the sign of specific rotation it was possible to assign the absolute configuration: the first eluted dextrorotatory enantiomer had (*S*) configuration and the second levorotatory eluted enantiomer (*R*) configuration on Chiraspher NT column [9].

Injection of S18986 on OD-RH column, using the chromatographic conditions described above, turned out an inversion of elution orders of the enantiomers compared with Chiraspher NT column: the first eluted enantiomer had (*R*) configuration, the second eluted enantiomer had (*S*) enantiomer (Fig. 4).

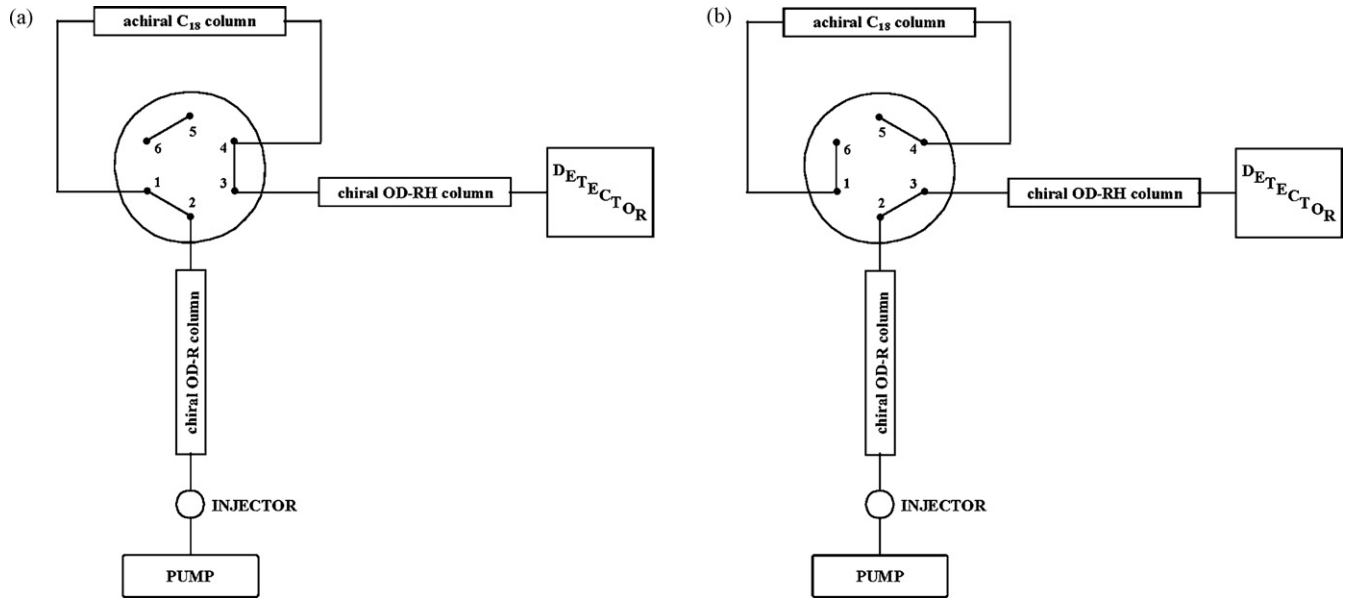

Fig. 3. Schematic representation of the stopped-flow multidimensional HPLC system.

3.2. Stopped-flow HPLC

The on-column HPLC stopped-flow procedure previously developed was used to study the enantiomerization of (±)(*R*,*S*)-**2** [13].

The resulted k_1^{app} and k_{-1}^{app} and associated free energies of enantiomerization ($\Delta G_1^{\#app}$ and $\Delta G_{-1}^{\#app}$) for the individual enantiomers of (±)(*R*,*S*)-**2** are listed in Table 1. The interconversion rate of the forward and reverse reaction of (±)(*R*,*S*)-**2** enantiomers on Chiralcel OD-RH were different.

Since $\Delta G^{\#app}$ of the conversion of the more strongly retained enantiomer (*S*)-**2** (S18986) into (*R*)-**2** was only slightly greater than that for the reverse reaction (*R*)-**2** into (*S*)-**2**, the interactions of the individual enantiomers with the CSP and the chiral environment appeared to have lower effect on enantiomerization.

3.3. Stopped-flow multidimensional HPLC

(±)(*R*,*S*)-**2** enantiomers undergo rapid enantiomerization during chromatography under reversed phase condition employing an acidic mobile phase. This process can be studied by the sfMDHPLC method described herein.

As shown in Fig. 2, a three-step experimental protocol was followed. In step 1, (±)(*R*,*S*)-**2** was injected and the enantiomers were quantitatively separated on the first chiral column 1. Subsequently, the two eluted enantiomers were trapped by column switching into the second achiral column 2, where enantiomerization was affected by heating quickly to 37 °C while no mobile phase passed through the column. Enantiomerization of the individual (±)(*R*,*S*)-**2** enantiomers occurred during column 2 heating at the desired temperature (37 °C) for a set time interval: (*R*)-enantiomer was partially converted into (*S*)-enantiomer, conversely (*S*)-enantiomer was also transformed into (*R*)-enantiomer. In step 3 the flow of the mobile phase introduced the enantiomers into the third chiral column 3, where they are separated.

The outcome of this protocol resulted in the presence of four peaks in the chromatograms (Fig. 5): peaks 1° and 4° arise from the enantiomer separation in step 1 while the peaks 2° and 3° arise from interconverted sample formed in step 2.

The trapping of individual enantiomer (*S*)- or (*R*)-**2** by column switching into the second achiral column 2 showed clearly that the second peak, (*S*), originated from the first eluted enantiomer (*R*), and the third peak (*R*) was the product of the partial enantiomerization of the last eluted enantiomer (*S*). The elution order in sfMDHPLC method was validated by the use of a circular dichroism spectroscopy detector.

The kinetic parameters (rate constants of enantiomerization k_1 and k_{-1}) and the enantiomerization barriers of both enantiomers (energy barrier of forward $\Delta G_1^{\#}$ and energy barrier of backward $\Delta G_{-1}^{\#}$) were calculated from the corresponding peak areas, from the enantiomerization time and from the enantiomerization temperature as described in the experimental part.

It is assumed that the precision of $\Delta G^{\#}$ is not influenced within the experimental standard deviation by the warm-up times on the column 2 for stopped-flow multidimensional experiments, in which separation is carried out at low temperature (0 °C) and the enantiomerization at higher temperature (37 °C). However, in case of very short enantiomerization times, this systematic error increases and it cannot be ignored [35–37].

Overall kinetic rate constants of forward (k_1) and backward (k_{-1}) interconversion can be calculated by fitting the data to Eq. (1). For both enantiomers, the corresponding plots gave straight lines with reasonable correlation coefficients. The kinetic rate constants k_1 and k_{-1} were obtained from the slope and were found to be $6.0 \pm 0.1 \times 10^{-4}$ and $6.4 \pm 0.1 \times 10^{-4}$ s^{-1} (correlation coefficient $r^2 > 0.99$) (Table 1).

Gibbs free energies of activation $\Delta G_1^{\#}$ and $\Delta G_{-1}^{\#}$, calculated from the kinetic rate constants by the Eyiring Eq. (2) are 93.63 ± 0.04 and 93.50 ± 0.03 kJ/mol at 37 °C (Table 1).

Since enantiomerization occurred in the achiral environment of the column 2, the kinetic rate constants and the free energies barriers of the forward and backward enantiomerization were practically the same.

3.4. Off-column method

The individual enantiomers (±)(*R*,*S*)-**2** obtained by semipreparative chromatography HPLC on Chiraspher NT column with *n*-hexane:THF 70:30 (v/v), were used to determine the enan-

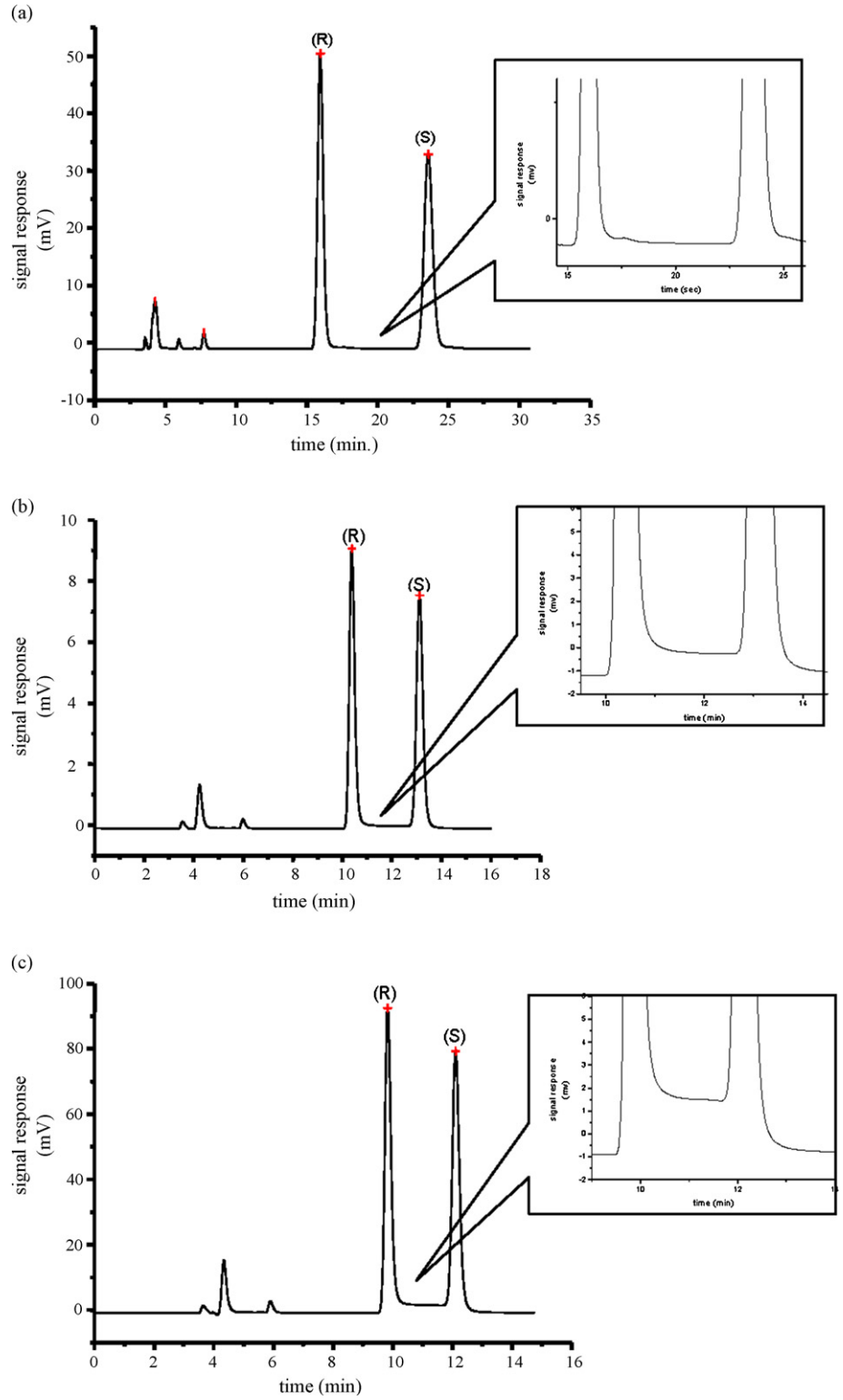

Fig. 4. "On-column" interconversion of enantiomers of (±)(*R*,*S*)-**2** at (a) 0 °C, (b) 37 °C and (c) 45 °C. Column: Chiralcel OD-RH (15 × 0.46 ID, 5 μm); mobile phase: buffer pH 2 ($NaClO_4$ 0.05 M/$HClO_4$):acetonitrile 60:40 (v/v); flow rate: 0.5 ml/min.

tiomerization rate constants k_1^m and k_{-1}^m and the associated energy barriers of enantiomerization ($\Delta G_1^{\#m}$ and $\Delta G_{-1}^{\#m}$) at the same temperature and in the same solvent (buffer $NaClO_4$ (pH 2) 0.05 M:acetonitrile 60:40, v/v), used for on-column enantiomerization. The results are listed in Table 1. As expected the enantiomerization rate constants k_1^m and k_{-1}^m and the associated energy barriers of enantiomerization ($\Delta G_1^{\#m}$ and $\Delta G_{-1}^{\#m}$) are quite similar.

It was possible to calculate the forward (k_1^s) and backward (k_{-1}^s) rate constants of enantiomerization and relative energy barriers of enantiomerization ($\Delta G_1^{\#s}$ and $\Delta G_{-1}^{\#s}$) for (±)(*R*,*S*)-**2** enantiomers in the chiral environment of CSP from k_1^{app}, k_{-1}^{app} and k^m according to

Table 1
Enantiomerization of (±)(*R*,*S*)-**2** enantiomers performed with sfHPLC, sfMDHPLC, off-column

Enantiomerization method	k_1 (s^{-1})	k_{-1} (s^{-1})	$\Delta G_1^{\#}$ (kJ/mol)	$\Delta G_{-1}^{\#}$ (kJ/mol)
sfHPLC	$1.60 \pm 0.01 \times 10^{-4}$ [a]	$1.21 \pm 0.01 \times 10^{-4}$ [a]	97.09 ± 0.02 [b]	97.83 ± 0.02 [b]
sfMDHPLC	$4.40 \pm 0.23 \times 10^{-4}$ [c]	$4.40 \pm 0.23 \times 10^{-4}$ [c]	94.47 ± 0.13 [d]	94.47 ± 0.13 [d]
Off-column	$3.38 \pm 0.03 \times 10^{-4}$ [e]	$4.60 \pm 0.10 \times 10^{-4}$ [e]	95.15 ± 0.02 [f]	94.35 ± 0.05 [f]

Columns: Chiralcel OD-RH, Chiralcel OD-R, and Supelcosil LC-18. Column operation: temperature at 0 °C, $n = 4$. Eluent: buffer pH 2 ($NaClO_4$ 0.05 M/$HClO_4$):acetonitrile 60:40 (v/v). Time intervals for enantiomerization at 37.5 °C = 5′, 10′, 15′, 20′, and 30′.

[a] Apparent rate constants (k_1^{app} and k_{-1}^{app}).

[b] Apparent free energy barriers ($\Delta G_1^{\#app}$ and $\Delta G_{-1}^{\#app}$).

[c] Rate constants in C_{18} column.

[d] Free energy barriers in C_{18} column.

[e] Rate constants in mobile phase (k_1^m and k_{-1}^m).

[f] Free energy barriers in mobile phase ($\Delta G_1^{\#m}$ and $\Delta G_{-1}^{\#m}$).

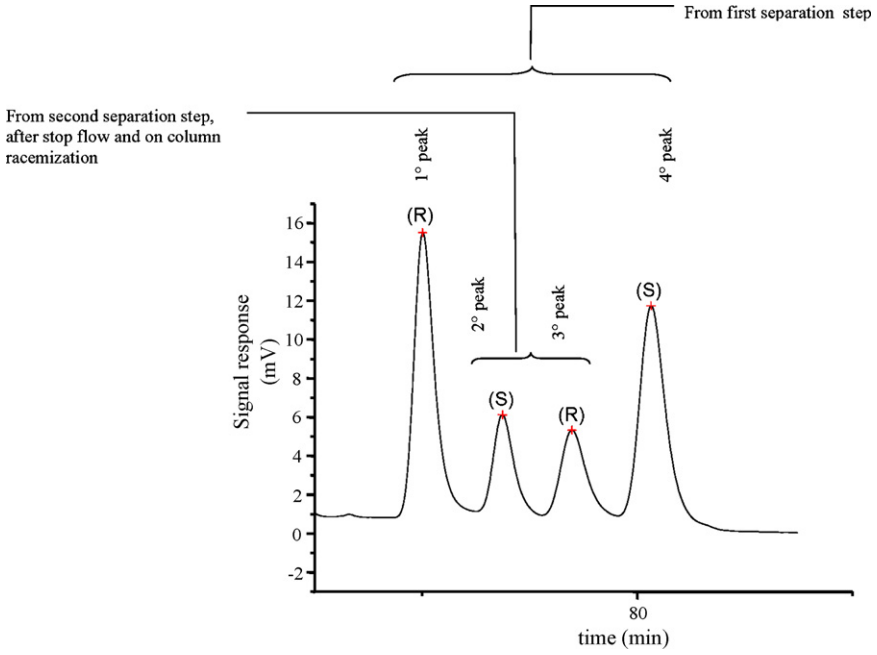

Fig. 5. Chromatogram of a typical enantiomerization experiment. Peaks 1° and 4° belong to the initially separated enantiomers of (**2**). Peaks 2° and 3° refer to the signals corresponding to the interconverted analytes. Columns: Chiralcel OD-R (25 × 0.46 ID, 10 μm); Supelcosil LC-18 (25 × 0.46 ID); Chiracel OD-RH (15 × 0.46 ID, 5 μm). Mobile phase: buffer pH 2 ($NaClO_4$ 0.05 M/$HClO_4$):acetonitrile 60:40 (v/v). Time interval for enantiomerization at 37.5 °C = 15′. Flow: 0.5 ml/min.

the following equations [13]:

$$k_1^{app} = \frac{1}{1 + k_{(-)}^{enant}} k^m + \frac{k_{(-)}^{enant}}{1 + k_{(-)}^{enant}} k_1^s \quad (3)$$

$$k_{-1}^{app} = \frac{1}{1 + k_{(+)}^{enant}} k^m + \frac{k_{(+)}^{enant}}{1 + k_{(+)}^{enant}} k_{-1}^s \quad (4)$$

where $k_{(-)}^{enant}$ and $k_{(+)}^{enant}$ are the retention factors of the (−) and (+) enantiomers, respectively.

The results (Table 2) showed that interactions of enantiomers with the CSP have an inhibitory effect on enantiomerization being $\Delta G_1^{\#s}$ and $\Delta G_{-1}^{\#s}$ greater than $\Delta G^{\#m}$.

Table 2
Calculated values of enantiomerization of (±)(*R*,*S*)-**2** on OD-CSP

k_1^s (s^{-1})	$0.84 \pm 0.01 \times 10^{-4}$
k_{-1}^s (s^{-1})	$0.19 \pm 0.01 \times 10^{-4}$
$\Delta G_1^{\#s}$ (kJ/mol)	98.75 ± 0.03
$\Delta G_{-1}^{\#s}$ (kJ/mol)	102.60 ± 0.13

k_1^s and k_{-1}^s was calculated according to Eqs. (3) and (4).

The finding that complexation of enantiomers with the CSP increased the enantiomerization barrier was expected, as interactions with the CSP may have to be broken to reach transition state.

The enantiomerization rate constants in the mobile phase (k_1^m and k_{-1}^m) were similar to those obtained by sfMDHPLC. The good agreement between the enantiomerization parameters obtained by the two independent methods, sfMDHPLC and off-column racemization, clearly validates the accuracy and applicability of multidimensional stopped-flow HPLC method.

To gain further insight in understanding the enantiomeric stability of S18986, the off-column procedure was applied to study enantiomerization rates in different solvents.

Enantiomerization hardly occurred in ethanol or acetonitrile at temperatures ranging between 20 and 40 °C for at least 3 h. Furthermore S18986 enantiomerization was faster in aqueous perchlorate 0.05 M buffer solution (pH 2) than in mobile phase (buffer $NaClO_4$ (pH 2) 0.05 M:acetonitrile 60:40, v/v), demonstrating the inhibitory effect of acetonitrile on enantiomerization rate.

In order to evaluate the influence of the pH on the enantiomerization rate of S18986, off-column enantiomerization experiments were performed in a series of 0.01 ionic strength buffers over a pH

Table 3
Off-column enantiomerization of (±)(*R*,*S*)-**2** enantiomers in diverse solvents at 37 °C

Solvents	$k^{\#}$ (s^{-1})	$\Delta G^{\#}$ (kJ/mol)
Buffer pH 2	$17.80 \pm 7.20 \times 10^{-4}$	90.86 ± 0.88
Buffer pH 3	$8.33 \pm 0.20 \times 10^{-4}$	92.82 ± 0.06
Buffer pH 5	$0.60 \pm 0.04 \times 10^{-4}$	99.62 ± 0.16
Buffer pH 7	$0.60 \pm 0.04 \times 10^{-4}$	99.62 ± 0.01
Buffer pH 9.3	$0.40 \pm 0.00 \times 10^{-4}$	100.67 ± 0.00
Simulated gastric fluid	$85.7 \pm 8.00 \times 10^{-4}$ [a]	86.80 ± 0.24[a]

Column: Chiralcel OD-RH; flow: 0.75 ml/min, column operation temperature: 0 °C, $n = 4$; time intervals for off-column enantiomerization at 37.5 °C = 5′, 10′, 15′, 20′, and 30′.

[a] Time intervals for off-column enantiomerization: 1′, 2.5′, 5′, and 7.5′.

range of 3–9.3 at 37 °C. The values for the first-order rate constants and Gibbs free energy of enantiomerization obtained are compiled in Table 3.

Enantiomerization rate strongly depends by pH being faster at pH 3 and only small increases were observed between pH 5 and 9.3, demonstrating that chiral inversion of S18986 was an acid catalyzed process.

Since one could be concerned with accelerated enantiomerization in the low pH environment of the stomach, as it has been reported that S18986 was administered orally in some pharmacological experiments [10,11], next we have studied the chiral interconversion rate constant in simulated gastric fluid at 37 °C. The rate constant of enantiomerization of S18986 in simulated gastric fluid was determined to be $85.70 \pm 80.00 \times 10^{-4}\ s^{-1}$ and the free energy barrier was calculated to be 86.80 ± 0.24 kJ/mol (Table 2). This result suggested that S18986 *in vitro* racemized in few minutes in acidic conditions similar to that of stomach.

4. Conclusion

The sfMDHPLC procedure developed is a simple and fast method to study interconversion phenomena and to determine rate constants and energy barriers in achiral environment. Only minute sample amounts of unpurified racemate are required. The novel method has been applied successfully to measurement of kinetics parameters of enantiomerization of (±)(*R*,*S*)-**2**.

Moreover, each enantiomers of (±)(*R*,*S*)-**2**, were isolated by preparative chromatography, and the enantiomerization rate constants and the associated energy barriers of enantiomerization were calculated (off-column enantiomerization). The good agreement between the two independent methods, sfMDHPLC and off-column, clearly validates the accuracy and applicability of stopped-flow MDHPLC, which to our knowledge has been tested for the first time for HPLC.

Furthermore enantiomerization studies conducted on each individual enantiomer of (±)(*R*,*S*)-**2**, in different buffers, demonstrated that enantiomerization strongly depends upon pH, being faster in acid conditions.

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