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Epimerization and hydrolysis of 3,6-dimethyl-2,3,5,6-tetrahydro[1,2,4]thiadiazino[6,5,4-*hi*]indole 1,1-dioxide

Marina M. Carrozzo [a], Giuseppe Cannazza [a,*], Umberto Battisti [a], Daniela Braghiroli [a], Luigino Troisi [b], Carlo Parenti [a]

[a] Dipartimento di Scienze Farmaceutiche, Università degli Studi di Modena e Reggio Emilia, Via Campi 183, 41125 Modena, Italy
[b] Dipartimento di Scienze e Tecnologie Biologiche ed Ambientali, University of Salento, via Prov.le Lecce-Monteroni, 73100 Lecce, Italy

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ABSTRACT

In this study, configurational and chemical stability of (R,R),(S,S),(R,S),(S,R)-3,6-dimethyl-2,3,5,6-tetrahydro[1,2,4]thiadiazino[6,5,4-*hi*]indole 1,1-dioxide (**1**) were investigated by dynamic and stopped-flow HPLC methods. Single epimeric mixtures (R,R),(R,S)-**1** and (S,S),(S,R)-**1** were obtained combining synthetic and chromatographic strategies. Separation of (R,R)-**1** and (R,S)-**1** was achieved by chiral chromatography and absolute configuration of eluted epimers has been assigned basing on molecular modelling calculations. Epimerization and hydrolysis of (R,R),(R,S)-**1** have been studied by classical off-column, dynamic HPLC and stopped-flow HPLC methods. The influence of different parameters, such as temperature, pH and dielectric constant was evaluated. The data obtained indicate that (R,R),(R,S)-**1** undergoes to a rapid epimerization in aqueous solvent and hydrolysis in acidic conditions. Moreover, epimerization and hydrolysis were investigated in presence of an artificial membrane and in physiological buffers (pH 2.2 and 7.0 at 37.5 °C) to simulate *in vivo* conditions.

1. Introduction

Recently chiral benzothiadiazine derivatives have been obtained particularly attention due to their pharmacological activity as α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor positive allosteric modulators [1–8]. Among them, (R,R),(S,S),(R,S),(S,R)-3,6-dimethyl-2,3,5,6-tetrahydro[1,2,4]thiadiazino[6,5,4-*hi*]indole 1,1-dioxide (**1**) has attracted particular attention because it is one of the most active benzothiadiazine derivatives as AMPAr modulator (Fig. 1) [9]. Molecular modelling studies have suggested that the desired pharmacological activity of **1** resides in the (S,S) enantiomer [9].

Since we have recently reported a rapid enantiomerization in aqueous solution of compounds structurally related to **1**, it becomes important to evaluate the configurational stability of its single stereoisomers [10–13].

Typically, stereoinversion of chiral labile compounds is studied by different techniques depending on their interconversion free energy barriers, such as dynamic NMR (DNMR) [14–16], chiro-optical methods [17,18], off-column and on-column chromatographic methods [i.e. dynamic gas chromatography (DCG) [19], dynamic high-performance liquid chromatography (DHPLC) [20,21], dynamic capillary electrophoresis (DCE) [22] and stopped-flow HPLC(sfHPLC) [23]].

A synthetic and chromatographic strategy has been developed to obtained single epimeric mixtures of **1** that were further employed for dynamic and stopped flow epimerization studies.

2. Experimental

2.1. Instrumentation

The chromatographic apparatus was a Shimadzu LC-10AD Pump (Shimadzu Italia, Milan), a Merck Hitachi L-6200A Pump (Merck KGaA, Darmstadt, Germany), a Rheodyne 7725 manual injector equipped with a 20 μl sample loop (Jasco Europe, Italy, Milan). As detector was used a Merck Hitachi L-7400UV (Merck KGaA, Darmstadt, Germany). Chromatograms were recorded with a Jasco J-700 program (Jasco Europe, Italy, Milan). Two Rheodyne 7000 valves were used to switch the mobile phase flow (Jasco Europe, Italy, Milan). Column temperature regulation was obtained with a Jasco CO-2067 column oven (Jasco Europe, Italy, Milan).

The columns used were Chiralcel OD-R [cellulose tris (3,5-dimethylphenylcarbamate); 250 mm × 4.6 mm I.D.; 10 μm], Chiralcel OD [cellulose tris (3,5-dimethylphenylcarbamate); 250 mm × 10 mm I.D.; 10 μm], Chiralcel OJ-RH [cellulose tris (4-methylbenzoate); 150 mm × 4.6 mm I.D.; 5 μm], Chiralcel OB-H [cellulose tribenzoate; 250 mm × 4.6 mm I.D.; 5 μm] purchased

* Corresponding author. Tel.: +39 059 2055013; fax: +39 059 2055750.
E-mail address: giuseppe.cannazza@unimore.it (G. Cannazza).

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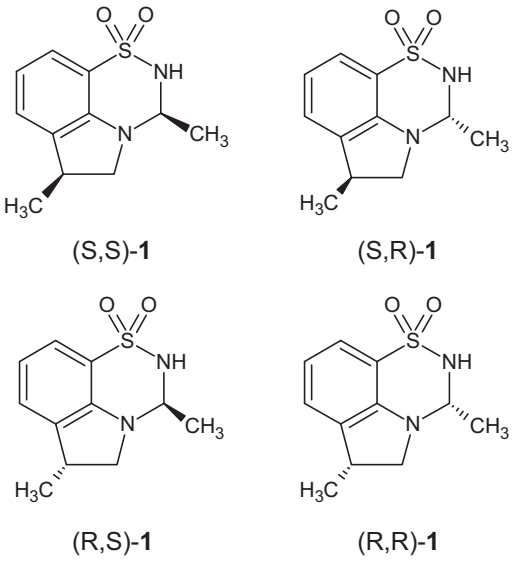

Fig. 1. (R,R),(S,S),(R,S),(S,R)-3,6-dimethyl-2,3,5,6-tetrahydro[1,2,4]thiadiazino [6,5,4-*hi*]indole 1,1-dioxide (**1**).

from Chiral Technologies Europe, Illkirch, France. Supelcosil LC-8 (250 mm × 4.6 mm I.D.; 5 μm) purchased from Supelco Italy, Milan, Lichrospher Si60 (250 mm × 10 mm I.D.; 10 μm), purchased from Merck KGaA, Darmstadt, Germany. Optical rotation (α) was measured with the P-2000 Digital Polarimeter (cell-length 100 mm, volume 1 ml) from Jasco Europe, Italy, Milan.

Melting points were determined with an electrothermal apparatus and they are uncorrected.

IR spectra were recorded on a PerkinElmer Model 1600 FT-IR spectrometer. ^{1}H NMR spectra were recorded with a Brucker DPX 200 spectrometer with 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; dt, double triplet; t, triplet; m, multiplet; apparent sextet. The electrospray ionization (HR-ESI-MS) experiments were carried out in a hybrid QqTOF mass spectrometer (PE SCIEX-QSTAR) equipped with an ion spray ionization source. MS (+) spectra were acquired by direct infusion (5 ml/min) of a solution containing the appropriate sample (10 pmol/ml), dissolved in a solution 0.1% acetic acid, methanol/water 50:50 at the optimum ion voltage of 4800 V.

LC–MS experiments were carried out on an Agilent 1200 series liquid chromatography and interfaced to an Agilent 6410 triple-quadrupole mass spectrometer equipped with an electrospray ionization source. All data were acquired and analyzed using Agilent MassHunter Quantitative Analyses version B.01.04 analyst data processing software.

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

HPLC-grade acetonitrile, n-hexane and 2-propanol were obtained from Sigma–Aldrich (Milan, Italy).

2.2. Synthesis

2.2.1. (R,R),(S,S),(R,S),(S,R)-3,6-dimethyl-2,3,5,6-tetrahydro[1,2,4]thiadiazino[6,5,4-hi]indole 1,1-dioxide (**1**)

1 was synthesized as previously described by Philips et al. [2].

Yield 25% (three steps), m.p. = 170–173 °C ^{1}H NMR (200 MHz, DMSO) δ = 1.2 (d, J = 6.9 Hz, 1H), 1.30 (d, J = 6.7 Hz, 2H), 1.36–1.46 (m, 3H), 2.82 (dd, J = 11.1 Hz, 8.4 Hz, 0.67H), 3.22 (dd, J = 8.0 Hz, 2.5 Hz, 0.33H), 3.30–3.40 (m, 1H), 3.48 (t, J = 8.7 Hz, 0,33H), 3.72 (t, J = 8.3 Hz, 0.67H), 4.56 (apparent sextet, J = 6.1 Hz, 0.67H), 4.66 (apparent sextet, J = 6.1 Hz, 0.33H), 6.71 (t, J = 7.2 Hz, 0.33H), 6.77 (t, J = 7.2 Hz, 0.67H), 7.18–7.30 (m, 2H), 7.82 (d, J = 11.4 Hz, 0.33H), 7.89 (d, J = 11.4 Hz, 0.67H).

HRMS-ESI: calcd. for $C_{11}H_{15}N_2O_2S$ $[M+H]^+$239.3134; found: 239.3136.

2.2.2. (R,R),(R,S)-**1** and (S,S),(S,R)-**1**

*2.2.2.1. 2,3-Dihydro-3-methyl-1H-indole (**3**).* The compound was synthesized as previously described by Gotor-Fernandez et al. starting from 3-methylindole (**2**) [24].

Yield 90%, ^{1}H NMR (200 MHz, $CDCl_3$) δ = 1.35 (d, J = 6.7 Hz, 3H), 3.13 (t, J = 8.5 Hz, 1H), 3.20 (s, broad, 1H), 3.38 (m, 1H), 3.72 (t, J = 8.5 Hz, 1H), 6.68 (d, J = 7.7 Hz, 1H), 6.77 (dt, J = 7.4 Hz, 1.0 Hz, 1H); 7.03 (dt J = 7.7 Hz, 1 Hz, 1H), 7.10 (d, J = 7.4, 1H).

HRMS-ESI: calcd. for $C_9H_{12}N$ $[M+H]^+$134.1977; found: 134.1979.

*2.2.2.2. 2,3-Dihydro-3-methyl-1H-indole-1-carboxylic acid 1,1-dimethyl ethyl ester (**4**).* Under nitrogen atmosphere, $(Boc)_2O$ (5 mmol) was added to a solution of 4-(N,N,-dimethylamino) pyridine (DMAP) (0.1 mmol) and 2,3-dihydro-3-methyl-1H-indole (4.5 mmol) in dry acetonitrile (5 ml) at room temperature. The reaction mixture was stirred overnight at room temperature, and then was evaporated under vacuum. Subsequently water was added and the resulting solution was extracted with ethyl acetate (EtOAc). The organic layer was dried over anhydrous Na_2SO_4 and evaporated. The resulting oil was purified by column chromatography (EtOAc/hexane = 1/10 (v/v)) to yield the pure compound as a colorless oil.

Yield 80%, ^{1}H NMR (200 MHz, $CDCl_3$) δ = 1.33 (d, J = 6.7 Hz, 3H), 1.50–1.62 (s, broad, 9H), 3.37 (m, 1H), 3.50 (s, broad, 1H), 4.14 (t, broad, J = 10.1 Hz, 1H), 6.68 (d, J = 7.7 Hz, 1H), 6.96 (dt, J = 7.4 Hz, 0.9 Hz, 1H); 7.13 (d J = 7.4 Hz, 1H), 7.16 (t, J = 7.8, 1H), 7.46–7.83 (s, broad, 1H).

HRMS-ESI: calcd. for $C_{14}H_{20}NO_2$ $[M+H]^+$234.3135; found: 234.3137.

*2.2.2.3. Semipreparative enantioseparation of 2,3-dihydro-3-methyl-1H-indole-1-carboxylic acid 1,1-dimethyl ethyl ester (**4**).* Pure (+)(S) and (−)(R) enantiomers of 2,3-dihydro-3-methyl-1H-indole-1-carboxylic acid 1,1-dimethyl ethyl ester were obtained by semipreparative HPLC on Chiralcel OD semipreparative column with fraction collection of the respective peaks. The mobile phase consisted of n-hexane and 2-propanol 98:2 (v/v). The compound was dissolved in n-hexane at final concentration of 400 mg/ml. The injection volume was 500 μl. The detector was 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.

*2.2.2.4. (R)-2,3-dihydro-3-methyl-1H-indol-7-sulfonamide ((R)-**5**) and (S)-2,3-dihydro-3-methyl-1H-indol-7-sulfonamide ((S)-**5**).* The compounds were synthesized following the procedure described by Philips et al. [2].

Yield 42%, m.p. 123–125 °C, ^{1}H NMR (200 MHz, $CDCl_3$) δ = 1.34 (d, J = 6.7 Hz, 3H), 3.25 (t, J = 8.2 Hz, 1H), 3.42 (m, 1H), 3.84 (t, J = 8.7, 1H), 4.90 (s, broad, 2H), 5.26 (s, broad, 1H), 6.71 (t, J = 7.3 Hz, 1H), 7.19 (dt, J = 7.1 Hz, 1.1 Hz, 1H), 7.44 (dt, J = 8.0 Hz, 0.9 Hz, 1H).

HRMS-ESI: calcd. for $C_9H_{13}N_2O_2S$ $[M+H]^+$213.2762; found: 213.2761.

*2.2.2.5. (R,R),(R,S)-**1** and (S,S),(S,R)-**1**.* A mixture of the (R)-2,3-dihydro-3-methyl-1H-indol-7-sulfonamide or (S)-2,3-dihydro-3-methyl-1H-indol-7-sulfonamide (4 mmol) and acetaldehyde (40 mmol) in 2-propanol (40 ml) supplemented with

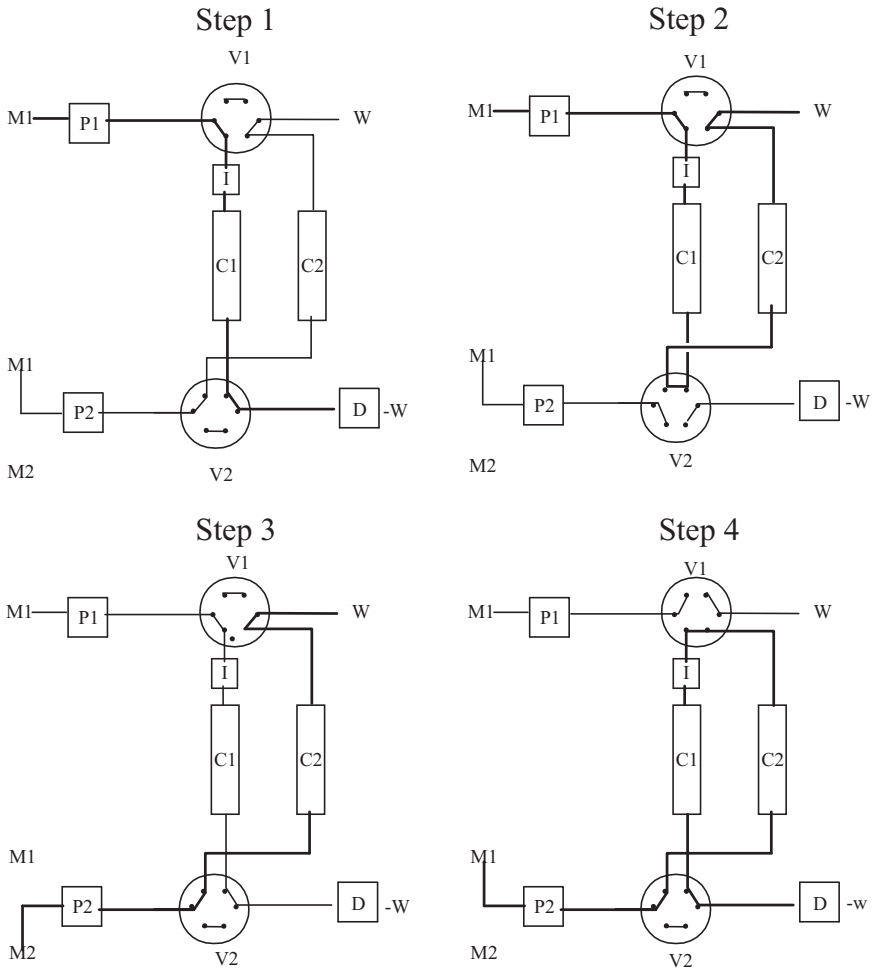

Fig. 2. Schematic representation of the stopped-flow bidimensional recycling HPLC system. M: mobile phase; P: pump; V1: valve 1; V2: valve 2; I: injector; C1: Chiralcel OD-R column; C2: Supelcosil LC-8 or IAM column; W: waste; D: detector. Step 1: (R,R),(R,S)-**1** were injected and quantitatively separated in the first chiral OD-R column at low temperature conditions. Step 2: one of the two eluted epimers was trapped into the second C8 (or IAM) column by switching valve 2. Step 3: C2 column is filled with a selected pH buffers by pump 2 and epimerization was performed at 37.5 °C for a set period of time. Step 4: the original mobile phase is reinforced in the second column and by switching valve 1, epimers were introduced and separated into the first column.

2 ml of ethyl acetate saturated with dry HCl was refluxed and stirred for 2 h at 50 °C. After cooling, the resulting suspension was concentrated to dryness under reduced pressure. The solid residue was purified by column chromatography on silica gel (elution solvent: ethyl acetate:petroleum ether (40–60 °C), 1:1 (v/v)).

Yield 98%, m.p. = 170–173 °C, ^{1}H NMR (200 MHz, DMSO) δ = 1.2 (d, J = 6.9 Hz, 1H), 1.30 (d, J = 6.7 Hz, 2H), 1.36–1.46 (m, 3H), 2.82 (dd, J = 11.1 Hz, 8.4 Hz, 0.67H), 3.22 (dd, J = 8.0 Hz, 2.5 Hz, 0.33H), 3.30–3.40 (m, 1H), 3.48 (t, J = 8.7 Hz, 0,33H), 3.72 (t, J = 8.3 Hz, 0.67H), 4.56 (apparent sextet, J = 6.1 Hz, 0.67H), 4.66 (apparent sextet,

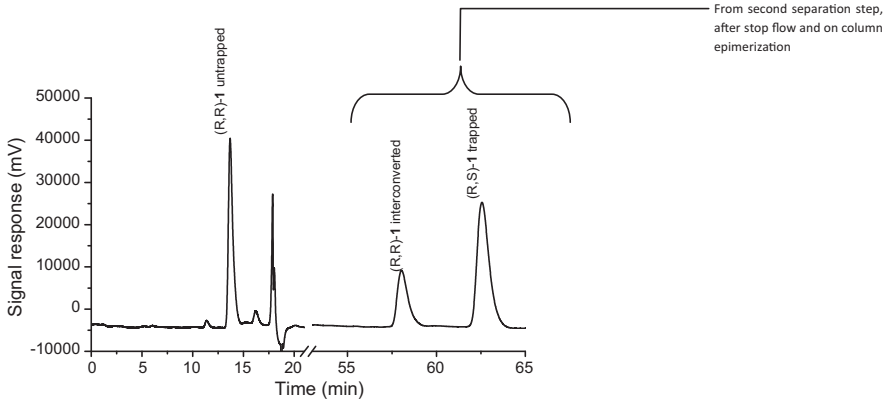

Fig. 3. Chromatogram of a typical epimerization experiment: peak 1° corresponded to untrapped epimer, peak 3° corresponded to epimer trapped in step 2 while peak 2° arises from interconverted epimer formed in step 3. Columns: Chiralcel OD-R (250 mm × 0.46 mm I.D., 10 μm); Supelcosil LC-8 (250 mm × 4.6 mm I.D., 5 μm). Mobile phase: water:acetonitrile 50:50 (v/v). Time interval for enantiomerization at 37.5 °C: 15′. Buffer: pH 7.00. Flow: 0.5 ml/min.

J = 6.1 Hz, 0.33H), 6.71 (t, J = 7.2 Hz, 0.33H), 6.77 (t, J = 7.2 Hz, 0.67H), 7.18–7.30 (m, 2H), 7.82 (d, J = 11.4 Hz, 0.33H), 7.89 (d, J = 11.4 Hz, 0.67H).

HRMS-ESI: calcd. for $C_{11}H_{15}N_2O_2S$ $[M+H]^+$239.3134; found: 239.3136.

2.3. Molecular modelling calculations

The whole molecular modelling calculations were performed by the software SPARTAN 08 (Wavefunction Inc., 18401 Von Karman Avenue, Suite 370 Irvine, CA 92612) running on a PC equipped with Intel Pentium 4, CPU 3.40 GHz, 2 GB of RAM and Windows XP Professional. Conformational search of 3,6-dimethyl-2,3,5,6-tetrahydro[1,2,4]thiadiazino[6,5,4-*hi*]indole 1,1-dioxide were carried as follow: MMFF force field; search by Montecarlo stochastic algorithm (all the rotatable bonds were explored); maximum number of conformers = 100. Structures generated were further optimized by Hartree–Fock ab initio method with 6-31G* basis set.

2.4. Chromatography

2.4.1. (R,R),(R,S)-**1** and (S,S),(S,R)-**1**

Separation of (R,R),(R,S)-**1** and (*S,S*),(*S,R*)-**1** were carried out isocratically at different temperatures on Chiralcel OD-R column. The mobile phase consisted of water:acetonitrile 50:50 (v/v).

The compounds were dissolved in acetonitrile and subsequently diluted 1:10 (v/v) with mobile phase at final concentration of 100 μg/ml. The injection volume was 20 μl. The detector was set at 254 nm.

2.4.2. (R,S)-2,3-dihydro-3-methyl-1H-indol-7-sulfonamide (**5**)

Separation of (R,S)-2,3-dihydro-3-methyl-1H-indol-7-sulfonamide from **1** was carried out isocratically on Chiralcel OD-R and/or Supelcosil LC-8 columns. The mobile phase was always water:acetonitrile 50:50 (v/v).

The compound was dissolved in acetonitrile and subsequently diluted 1:10 (v/v) with mobile phase at final concentration of 100 μg/ml. The injection volume was 20 μl. The detector was set at 254 nm.

2.5. 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 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.

2.6. Dynamic HPLC

An Agilent 1200 LC-system was connected to a triple quadrupole mass spectrometer (6410 Triple quadrupole) equipped with an electrospray ionization source (ESI). Data were processed by acquisition/processing Agilent MassHunter Quantitative software.

The mass spectrometer was operated in the positive scan mode between 200 and 300 m/z, gas temperature 350 °C, gas flow (N_2) 10 l/min, nebulizer 35 psi, capillary 3500 nA.

Epimerization kinetic parameters of (R,R),(R,S)-**1** have been investigated by dynamic chromatography experiments (DHPLC) by DCXplorer software developed by Trapp [25]. The program employs the unified equation of chromatography to directly evaluate elution profiles in a graphical user interface [25–34].

Extracted ion chromatograms (MH^+ 239 m/z) row data in ASCII have been opened with the DCxplorer software and the elution profiles have been evaluated by zooming into the area of the interconverting peaks. All chromatographic parameters have been directly determined by an integration method and have been used to calculate reaction rate constants [24–33].

2.7. Stopped-flow bidimensional recycling HPLC (sf-BD-rHPLC)

The HPLC procedure developed is described in Fig. 2. The epimeric mixture (R,R),(R,S)-**1** was injected on the first column (Chiralcel OD-R) and the individual epimers were separated quantitatively at 0 °C (step 1). At the appropriate time one of the two epimers is trapped into the second column by switching the valve 2 (step 2). By pump 2 it is possible to fill the second column (Supelcosil LC-8) with a selected solvent at desired pH and temperature (step 3). The epimerization was affected by heating at 37.5 °C while no mobile phase passed through the column 2 ("stopped-flow") for a set period of time. Afterwards the C8 column was cooled back to the previous low temperature (0 °C) and the valve 1 was switched. The original mobile phase was reinforced in column 2 by pump 2 to introduce the epimers into the first column (Chiralcel OD-R) where they were quantitatively separated (step 4).

Three peaks appears in chromatograms as expected (Fig. 3): peak 1° corresponds to untrapped epimer, peak 3° corresponds to epimer trapped in step 2 while peak 2° arises from interconverted epimer formed in step 3.

2.8. Off-column hydrolysis

1 was dissolved in acetonitrile and subsequently diluted 1:100 (v/v) with selected buffer (1 ml) at final concentration of 10 μg/ml. The solution was thermostated at 37.5 °C for a selected time and subsequently chromatographated on Supelcosil LC-8 column using water and acetonitrile 50:50 (v/v) as mobile phase. Four repeats of each experiment were made. Chloroacetate buffer solution of ionic strength of 0.01 at pH 2.20 was prepared by mixing 50.99 ml of chloroacetic acid 0.1 M with 12.76 ml of KOH 0.1 M and diluting with water to 100 ml. Phosphate buffer solution of ionic strength of 0.01 at pH 7.0 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.

2.9. Calculation of kinetic rate constants and free energy barriers of epimerization

The kinetic rate constants have been calculated by fitting the data to Eqs. (1)–(3):

$$\ln \frac{[A]_0 - [A]_{eq}}{[A]_t - [A]_{eq}} = (k_{A\text{-to-}B} + k_{B\text{-to-}A}) \times t = k_{A\text{-to-eq}} \times t \quad (1)$$

$$K_{eq} = \frac{k_{A\text{-to-}B}}{k_{B\text{-to-}A}} = \frac{[B]_{eq}}{[A]_{eq}} \quad (2)$$

$$k_{A\text{-to-eq}} = k_{A\text{-to-}B}\left(\frac{1+1}{K_{eq}}\right) = k_{B\text{-to-}A}(1 + K_{eq}) \quad (3)$$

where $k_{A\text{-to-}B}$ and $k_{B\text{-to-}A}$ are the rate constants of forward or backward epimerization [s^{-1}], $k_{A\text{-to-eq}}$ is the rate constant of formation of equilibrium mixture [s^{-1}], $[A]_0$ the concentration of decreasing stereoisomer (A) at time 0, $[A]_t$ the concentration of decreasing stereoisomer (A) at time t, $[A]_{eq}$ the concentration of decreasing stereoisomer (A) at equilibrium, $[B]_{eq}$ the concentration of increasing stereoisomer (B) at equilibrium and t the epimerization time(s).

From the kinetic rate constants, the corresponding activation energies of epimerization (rotational energy barriers) $\Delta G^{\#}(T)$ have

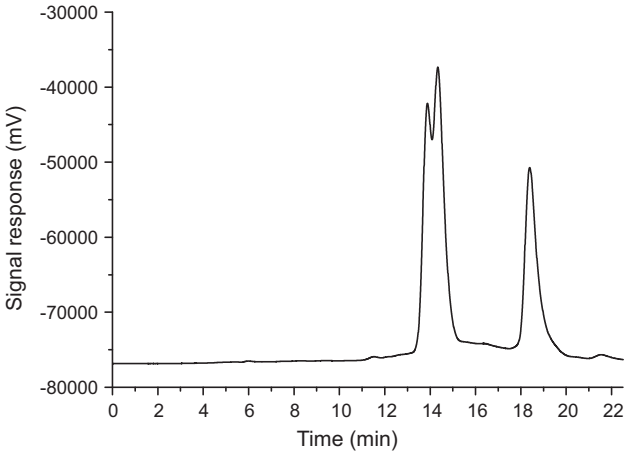

Fig. 4. Separation of **1**. Column: Chiralcel OD-R. Mobile phase: water:acetonitrile 50:50 (v/v). Flow: 0.5 ml/min.

been calculated by the Eyring equation:

$$\Delta G^{\#}(T) = -\mathrm{RT}\ln\left(\frac{kh}{\kappa k_B T}\right) \quad (4)$$

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

2.10. Calculation of kinetic rate constants of hydrolysis

The kinetic rate constants have been calculated by fitting the data to Eq. (5):

$$A_t = A_0 \exp(-k_i t_i) \quad (5)$$

where k_i is the rate constant of hydrolysis [s^{-1}], A_0 the peak of **1** before hydrolysis [=100%], A_t the relative peak area of **1** remaining after hydrolyzation time t_i [%] (i.e. $a_t = 100 -$ conversion) and t_i the hydrolysis time(s).

3. Results and discussion

3.1. Stereoisomer separation

Diastereomers of **1** were tentatively separated on silica column. No baseline resolution was obtained in chromatographic conditions tested. Partial separation was obtained by using hexane:2-propanol 70:30 (v/v) as mobile phase.

Moreover **1** was chromatographed on the following chiral stationary phases (CSPs): OJ, OB, OD. Two peaks were obtained in both normal and reverse phase mode employing all the above CSPs. The first eluted peak was higher, broader and split respect the second eluted one, suggesting a co-elution of multiple stereoisomers under the first peak (Fig. 4).

Since the CSPs tested were unable to separate stereoisomers of **1**, the two epimeric mixtures of **1** have been obtained following the synthetic/chromatographic strategy.

In the literature only few stereoselective methods to obtain single enantiomers of 2,3-dihydro-3-methyl-1H-indole (**3**) were present [24]. Moreover, only a partial enantioresolution of 2,3-dihydro-3-methyl-1H-indole was obtained on commercially available tested CSPs. In order to obtain single enantiomers of **3**, the racemic compound was derivatized with N-*tert*-butyl carbamate (BOC) and subsequently chromatographed on semipreparative

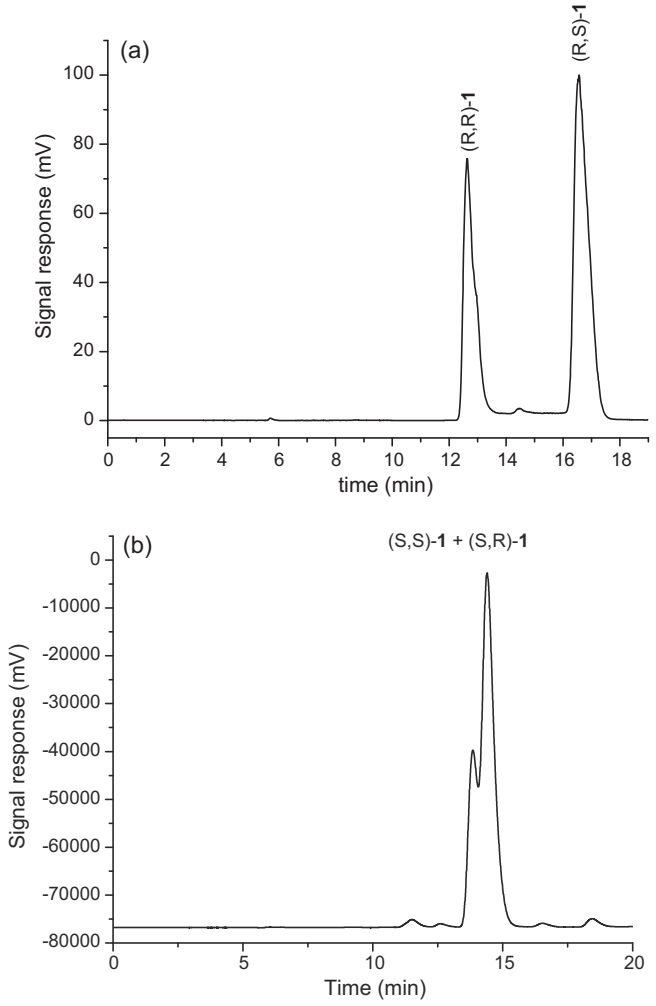

Fig. 5. Separation of epimeric mixtures (R,R),(R,S)-**1** (a) and (S,S),(S,R)-**1** (b). Column: Chiralcel OD-R. Mobile phase: water:acetonitrile 50:50 (v/v). Flow: 0.5 ml/min.

Chiralcel OD column obtaining a baseline separation. The collected fractions containing the single enantiomers show high enantiomeric excess values (e.e. >99%). Single BOC-derivatives enantiomers were deprotected and the specific rotation in chloroform of first and second eluted enantiomers were $[\alpha]_D = +35.32^\circ$ (4.53 mg/ml; chloroform; $t = 24\,^\circ$C) and $[\alpha]_D = -40.73^\circ$ (8.9 mg/ml, chloroform, $t = 24\,^\circ$C), respectively. The optical rotation values obtained are slightly different for the two enantiomers probably due to small difference in enantiomeric purity. Anyway, these values were in accordance with that reported in literature ((*S*)-enantiomer $[\alpha]_D^{20} = +30.2^\circ$ (c 0.25, chloroform) and (R)-enantiomer $[\alpha]_D^{20} = -30.2^\circ$ (c 0.25, chloroform)) [24]. From the signs of specific rotation obtained for single enantiomers of **3**, it was possible to assign absolute configurations: dextrorotatory enantiomer has (*S*) configuration and levorotatory enantiomer has (*R*) configuration [24].

Single (S)- and (R)-2,3-dihydro-3-methyl-1H-indole enantiomers were employed to prepare single enantiomers of (S)- and (R)-2,3-dihydro-3-methyl-1H-indol-7-sulfonamide. Single epimeric mixtures (R,R),(R,S)-**1** and (S,S),(S,R)-**1** were obtained by ring closure of single enantiomers of (R)-2,3-dihydro-3-methyl-1H-indol-7-sulfonamide and (S)-2,3-dihydro-3-methyl-1H-indol-7-sulfonamide with acetaldehyde (see Section 2).

Epimeric mixtures obtained were chromatographed on Chiralcel OD column in both normal and reverse phase mode. A baseline

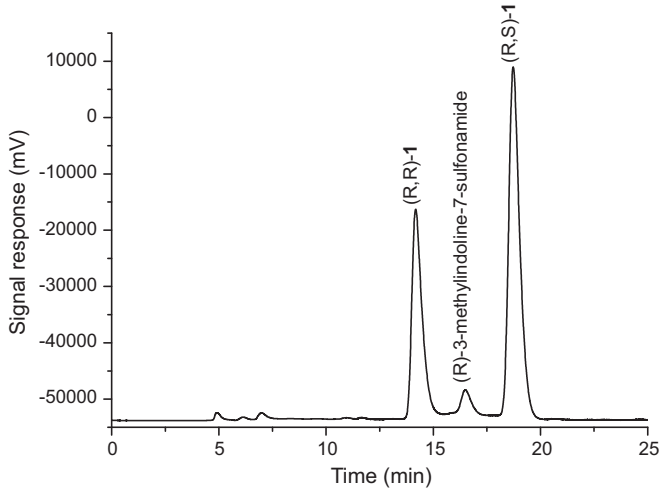

Fig. 6. Separation of (R,R),(R,S)-**1**. Column: Chiralcel OD-R. Mobile phase: water:acetonitrile 50:50. Flow: 0.5 ml/min. Temperature: 25 °C.

separation was obtained for (R,R),(R,S)-**1** epimers while a partial separation was achieved for (S,S),(S,R)-**1** epimers (Fig. 5).

When separation of (R,R),(R,S)-**1** epimers was conducted at temperatures above 25 °C in reverse phase mode, a plateau was observed between the two peaks corresponding to the epimers. Hardly no epimerization occurred during enantioseparation on Chiralcel OD column at temperatures between 4 and 50 °C using the nonaqueous solvents (hexane:2-propanol).

These data are in accordance with our previous studies that indicate a rapid enantiomerization in aqueous solvents at chiral carbon atom C3 of 3,4-diidrobenzothiadiazine type compounds structurally related to **1** [10–13].

Moreover a third peak was observed between the two peaks corresponding to the epimers (Fig. 6). It is known that benzothiadiazines, like IDRA21, structurally related to **1**, undergo hydrolysis in aqueous solutions to 2-amino-5-chlorobenzenesulfonamide and acetaldehyde [11]. Similarly **1**, in aqueous mediums could give (±)-2,3-dihydro-3-methyl-1H-indol-7-sulfonamide and acetaldehyde.

To confirm the identity of the unknown peak, pure (R)-2,3-dihydro-3-methyl-1H-indol-7-sulfonamide was analyzed under identical experimental conditions employed for the separation of epimeric mixture (R,R),(R,S)-**1**. The retention time of the hydrolyzed product of epimeric mixture corresponds to that of authentic (R)-2,3-dihydro-3-methyl-1H-indol-7-sulfonamide.

3.2. Configuration assignment

In order to study stereochemical and energetic properties of compound **1**, molecular modelling calculations with B3LYP method with 6-31G* basis set have been performed. As reported by Harpsøe et al. the flexibility of compound **1** resides only in the sulfonamidic ring [9]. Since two ring conformations are possible and the hydrogen atom on N2 can be located in an axial or in an equatorial position, there are four possible conformers for each stereoisomer (Fig. 7). The global energy minimum conformer for both enantiomer pairs was calculate to have the N2 hydrogen atom in an axial position and the methyl substituent at C3 in equatorial position. The calculated conformert was employed for subsequently calculation studies.

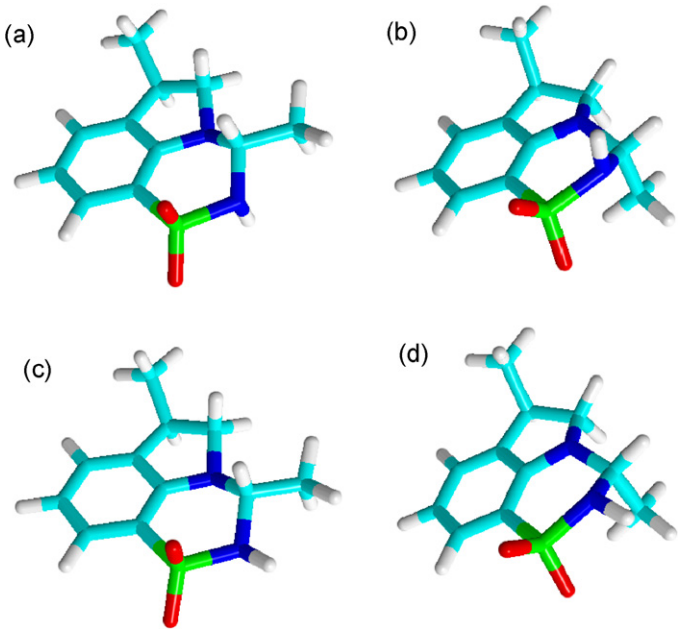

Fig. 7. The four possible conformers of (R,S)-**1**. (a) Global energy minimum, (b) +3.4 kcal/mol, (c) +4.3 kcal/mol and (d) +7.6 kcal/mol.

As reported in Table 1, (R,S)-**1** and (S,R)-**1** are the more stable forms both in gas and water phases with a Boltzmann population of 62% and 84% at 25 °C, respectively.

Molecular modelling results furnish a specific contribution to assign the absolute configuration of stereoisomers of epimeric mixture (R,R),(R,S)-**1**.

Previously it was reported that the peak areas ratio of the first and second elutedepimers of (R,R),(R,S)-**1** on Chiralcel OD-R column employing water:acetonitrile 50:50 (v/v) as mobile phase, was 37:63. Therefore, it is possible to assign the configuration (R,R)-**1** to the first eluted epimer and (R,S)-**1** to the second eluted one basing on Boltzmann population calculated by molecular modelling studies (Fig. 5a).

3.3. Dynamic chromatography

The epimeric kinetic parameters of epimeric mixture (R,R),(R,S)-**1** have been investigated by dynamic chromatography experiments (DHPLC) by using DCXplorer software developed by Trapp [25]. The program employs the unified chromatography equation to directly evaluate elution profiles in a graphical user interface [25–34]. The software calculates reaction rate constants by integration of the area of the interconverting peaks. It is quite difficult to calculate kinetic parameters of epimerization of (R,R),(R,S)-**1** by DCXplorer due to the presence of the peak corresponding to hydrolysis product on the plateau region of

Table 1
Molecular modelling calculations of diastereomerics forms of **1**.

Enantiomer	Energy[a] (gas phase) kcal mol^{-1}	Energy[a] (water) kcal mol^{-1}	Dipole Debye
R,S	0.0	0.0	7.49
R,R	0.29	1	7.43
S,R	0.0	0.0	7.49
S,S	0.29	1	7.43

[a] Relative energy related to the most stable enantiomer of **1**.

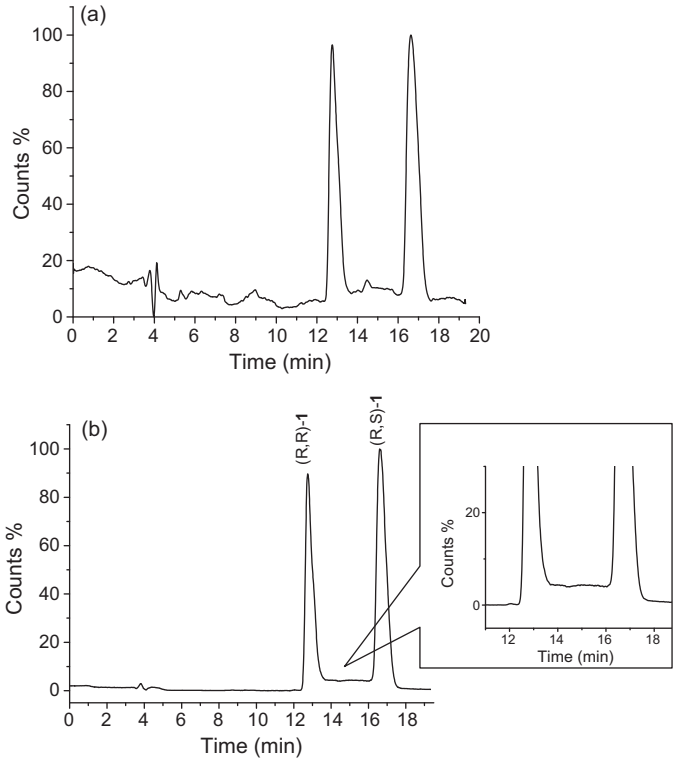

Fig. 8. "On-column" epimerization of (R,R),(R,S)-**1** performed by LC–MS. (a) Total ion chromatogram (TIC); (b) extracted ion chromatogram (EIC) of MH^+ 239 *m/z*. Column: Chiralcel OD-R. Mobile phase: water:acetonitrile 50:50 (v/v). Flow: 0.5 ml/min. Temperature 25 °C.

interconverting epimers in the chromatograms obtained by UV detector.

The separation of epimeric mixture was conducted employing a mass spectrometer detector instead of UV-detector. By this way, it was possible to extract chromatographic traces corresponding to molecular ion of compound **1** where the interfering peak corresponding to (R)-2,3-dihydro-3-methyl-1H-indol-7-sulfonamide was not present (Fig. 8).

Chromatographic row data of extracted ion chromatograms in ASCII format have been opened with DCXplorer software and elution profiles have been evaluated by zooming into the area of the interconverting peaks. All chromatographic parameters have been directly determined by integration and have been used to calculate reaction rate constants [25–34].

The analysis of (R,R),(R,S)-**1** were performed at different temperatures between 4 and 50 °C using a Chiralcel OD-R column and water:acetonitrile 50:50 (v/v) as mobile phase. The rate constants and free energy barriers of epimerization calculated by DCXplorer are reported in Table 2. The data obtained indicate that the epimerization rate increases with the increasing of temperature.

Moreover the separation of (R,R)(R,S)-**1** was conducted at different percentage of acetonitrile in the range between 50 and 65% to evaluate the influence of solvent on epimerization process. The data reported in Table 3 indicate that organic modifier slightly influence the epimerization rates, since only small differences were obtained.

3.4. Stopped-flow bidimensional recycling HPLC (sf-BD-rHPLC)

Dynamic chromatographic methods constrain calculation of epimerization kinetic constants of (R,R),(R,S)-**1** in the presence of CSP and mobile phase that could influence reaction rates. If epimers separation occurred, it means that stationary phase can stabilize one epimer respect the other one. Moreover, epimerization rates could be influenced by the presence of organic modifier or other components of mobile phase. In order to eliminate influences of stationary and mobile phases on the epimerization rate constants, the stopped-flow bidimensional recycling HPLC (sf-BD-rHPLC) method has been applied (Fig. 2) [10–12].

Briefly, it consists in a 4-steps experimental protocol: in step 1 the epimeric mixture was quantitatively separated on the first column (Chiralcel OD-R); in step 2 one of the two epimers is trapped into the second column (Supelcosil LC-8); in step 3 epimerization has been performed in the second column filled with the selected buffer and thermostated at 37.5 °C; in step 4 the original flow of mobile phase was resumed and the two epimers were separated in the first column.

Similarly to enantiomerization studies, it is possible to investigate epimerization rates by sf-BD-rHPLC without the influences of mobile and stationary phases. Since C8 column does not separate epimeric mixture, it has been chosen as reactor to conduct epimerization. Moreover, epimerization has been performed in buffers at pH 2.2 and pH 7.0 and at temperature of 37.5 °C in order to simulate physiological conditions.

The chromatogram reported in Fig. 3 refers to a representative example of sf-BD-rHPLC run of (R,R),(R,S)-**1**: peak 1° corresponds to untrapped epimer, peak 3° corresponds to epimer trapped in step 2 while peak 2° arises from interconverted epimer formed in step 3.

The kinetic parameters for both epimers, $k_{A\text{-to-eq}}$ and $k_{B\text{-to-eq}}$, were calculated from the corresponding peak areas, from the epimerization time and from the epimerization temperature as

Table 2
Epimerization of (R,R),(R,S)-**1** at different temperatures by DCXplorer.

Temperature (°C)	$k_{A\text{-to-B}}$ (s^{-1})	$k_{B\text{-to-A}}$ (s^{-1})	$k_{A\text{-to-eq}}$ (s^{-1})	$\Delta G^{\#}_{A\text{-to-eq}}$ (kJ/mol)
4	$0.16 \pm 0.09 \times 10^{-4}$	$0.09 \pm 0.06 \times 10^{-4}$	$0.25 \pm 0.01 \times 10^{-4}$	91.83 ± 0.13
25	$1.69 \pm 0.02 \times 10^{-4}$	$1.01 \pm 0.01 \times 10^{-4}$	$2.70 \pm 0.02 \times 10^{-4}$	92.94 ± 0.02
40	$4.47 \pm 0.29 \times 10^{-4}$	$2.95 \pm 0.11 \times 10^{-4}$	$7.42 \pm 0.41 \times 10^{-4}$	93.94 ± 0.14
50	$12.7 \pm 0.10 \times 10^{-4}$	$7.60 \pm 0.10 \times 10^{-4}$	$20.30 \pm 0.10 \times 10^{-4}$	94.31 ± 0.02

Column: Chiralcel OD-R; temperatures: 4, 25, 40 and 50 °C; eluents: water:acetonitrile 50:50 (v/v); flow rate: 0.5 ml/min.

Table 3
Epimerization of (R,R),(R,S)-**1** at different percentage of acetonitrile by DCXplorer.

Temperature (°C)	Eluente (H_2O:ACN)	$k_{A\text{-to-B}}$ (s^{-1})	$k_{B\text{-to-A}}$ (s^{-1})	$k_{A\text{-to-eq}}$ (s^{-1})	$\Delta G^{\#}_{A\text{-to-eq}}$ (kJ/mol)
40	35:65	$4.48 \pm 0.04 \times 10^{-4}$	$2.95 \pm 0.02 \times 10^{-4}$	$7.43 \pm 0.02 \times 10^{-4}$	93.93 ± 0.01
40	40:60	$5.32 \pm 0.15 \times 10^{-4}$	$3.36 \pm 0.06 \times 10^{-4}$	$8.68 \pm 0.20 \times 10^{-4}$	93.53 ± 0.06
50	35:65	$11.60 \pm 0.10 \times 10^{-4}$	$7.91 \pm 0.01 \times 10^{-4}$	$19.5 \pm 0.10 \times 10^{-4}$	94.41 ± 0.01
50	40:60	$9.80 \pm 0.12 \times 10^{-4}$	$6.10 \pm 0.08 \times 10^{-4}$	$15.9 \pm 0.19 \times 10^{-4}$	94.96 ± 0.30

Column: Chiralcel OD-R; Temperatures: 40 and 50 °C. Eluents: water:acetonitrile 40:60 (v/v) and 36:65 (v/v). Flow rate: 0.5 ml/min.

Table 4
Epimerization of (R,R),(R,S)-**1** by sf-BD-rHPLC in C8 column.

pH	$k_{A\text{-to-eq}}$ (s^{-1})	$k_{B\text{-to-eq}}$ (s^{-1})	$k_{A\text{-to-B}}$ (s^{-1})	$k_{B\text{-to-A}}$ (s^{-1})	$\Delta G^{\#}_{A\text{-to-eq}}$ (kJ/mol)	$\Delta G^{\#}_{A\text{-to-eq}}$ (kJ/mol)
2.2	$1.92 \pm 0.09 \times 10^{-3}$ [a]	$0.97 \pm 0.08 \times 10^{-3}$ [a]	$1.15 \pm 0.08 \times 10^{-3}$	$0.28 \pm 0.10 \times 10^{-3}$	90.57 ± 0.12 [b]	92.32 ± 0.21 [b]
7.0	$1.29 \pm 0.10 \times 10^{-3}$ [a]	$1.01 \pm 0.06 \times 10^{-3}$ [a]	$0.92 \pm 0.09 \times 10^{-3}$	$0.23 \pm 0.02 \times 10^{-3}$	91.41 ± 0.17 [b]	92.48 ± 0.20 [b]

Separative column: Chiralcel OD-R. Reactor column: Supelcosil LC-8. Column operation temperature at 0 °C $n = 4$. Eluent (pump 1): water:acetonitrile 50:50 (v/v). Buffers (pump 2): chloroacetate buffer solution of ionic strength of 0.01 at pH 2.20 and phosphate buffer solution of ionic strength of 0.01 at pH 7.00. Time intervals for enantiomerization at 37.5 °C = 5′, 10′, 15′, 20′, and 30′.

[a] Rate constants in C8 column.

[b] Free energy barriers in C8 column.

described in the experimental part by fitting the data to Eq. (1). For both epimers, the corresponding plots gave straight lines with reasonable correlation coefficients ($r^2 \geq 0.99$). The forward and reverse epimerization reaction rates ($k_{A\text{-to-B}}$ and $k_{B\text{-to-A}}$) were calculated by fitting data to Eqs. (2) and (3).

The data reported in Table 4 indicate that pH does not significantly influence the epimerization rates, since only small differences were obtained at pH 2.2 and 7.0. As expected, $k_{A\text{-to-B}}$ is higher than $k_{B\text{-to-A}}$. The epimerization rates ($k_{A\text{-to-eq}}$ and $k_{B\text{-to-eq}}$) are slightly different with $k_{A\text{-to-eq}}$ greater than $k_{B\text{-to-eq}}$, suggesting that interactions of single stereoisomers with stationary phase could influence epimerization kinetics.

Subsequently epimerization has been investigated in presence of a stationary phase that simulates physiological conditions.

Recently, an HPLC stationary phase material, called immobilized artificial membrane (IAM), has became commercially available [35–38]. The IAM stationary phase is comprised of monolayers of lecithin (phosphatidylcholine) wherein each lipid molecule is covalently bound to aminopropylsilica.

IAM chromatography is widely used to measure solute/membrane partition coefficients instead of classical partition coefficient octanol/water, since the results are more close to that *in vivo* [39–44].

With the aim to get accurate predictions of epimerization rates in biological systems, IAM stationary phase has been employed instead of C8 column in the sf-BD-rHPLC method.

The epimeric mixture (R,R),(R,S)-**1** has been injected on IAM column employing water:acetonitrile 50:50 (v/v) as mobile phase. No separation of epimeric mixture was obtained indicating that IAM stationary phase could not exert different perturbing effects on epimers.

Epimerization kinetics have been studied by sf-BD-rHPLC employing as first column a Chiralcel OD-R and as reactor column an IAM based one. The epimerization solvents employed were buffer at pH 2.2 and buffer at pH 7.0 with an epimerization temperature of 37.5 °C.

The data obtained are reported in Table 5 and are quite similar to that obtained previously in the presence of C8 stationary phase (Table 4). Moreover, differences between $k_{A\text{-to-eq}}$ and $k_{B\text{-to-eq}}$ have been obtained in the presence of IAM stationary phase, such as those obtained by using C8 stationary phase (Table 4). Anyway IAM stationary phase simulates the same conditions that single epimers of compound **1** could meet *in vivo*, suggesting that the same differences in epimerization rates could be occurred in presence of biological membranes.

3.5. Hydrolysis

Previous studies on chemical stability of (±)-7-chloro-3-methyl-3,4-dihydro-2H-1,2,4-benzothiadiazine 1,1-dioxide ((±)IDRA21), a compound structurally similar to **1**, have indicated a rapid hydrolysis in acidic solvents [11]. Since hydrolysis of (R,R),(R,S)-**1** occurred during chromatographic separation (Fig. 6), sf-BD-rHPLC method has been tentatively applied to evaluate hydrolysis kinetics of (R,R),(R,S)-**1**.

A Chiralcel OD-R has been employed as separative column and a C8 and IAM have been employed as reactor columns. Hydrolysis has been conducted in buffers at pH 2.2 and 7.0 and at temperature of 37.5 °C.

No hydrolysis product peak appears in the sf-BD-rHPLC chromatograms performed both on C8 and IAM columns at pH 7.0 after 90 min at 37.5 °C. A third peak identified as (R)-2,3-dihydro-3-methyl-1H-indol-7-sulfonamide is present in the sf-BD-rHPLC chromatograms performed in C8 or IAM columns at pH 2.2 after 90 min at 37.5 °C (Fig. 9). These results indicate that hydrolysis of (R,R),(R,S)-**1** is an acid catalyzed process since it occurs in acidic but not in neutral conditions. Anyway only 6% of compound (R,R),(R,S)-**1** undergoes hydrolysis after 90 min at pH 2.2 and 37.5 °C in both C8 and IAM columns as calculated from corresponding peak areas.

In order to validate the data obtained by sf-BD-rHPLC, an off-column method was applied to evaluate hydrolysis rates of (R,R),(R,S)-**1** at the same temperature (37.5 °C) and in the same buffers (pH 2.2 and 7.0) in absence of C8 and IAM stationary phases.

Samples of (R,R),(R,S)-**1**, incubated for a set times interval at 37.5 °C, were injected on Supelcosil LC-8 column and eluted with a mobile phase water:acetonitrile 50:50 (v/v). The two peaks, corresponding to (R,R),(R,S)-**1** and hydrolysis product ((R)-2,3-dihydro-3-methyl-1H-indol-7-sulfonamide), were identified by injecting pure compounds in the same experimental conditions. The rate constants of hydrolysis were calculated by plotting the natural logarithm of the decreasing integral of the peak corresponding to (R,R),(R,S)-**1** as a function of time. The corresponding plots gave straight lines with reasonable correlation coefficients ($r^2 \geq 0.998$). The kinetic rate constants of hydrolysis were obtained from the slope and were found to be $1.04 \pm 0.05 \times 10^{-4}$ (s^{-1}) and $1.15 \pm 0.08 \times 10^{-5}$ (s^{-1}) at pH 2.2 and 7.0, respectively.

Table 5
Epimerization of (R,R),(R,S)-**1** by sf-BD-rHPLC in IAM stationary phase.

pH	$k_{A\text{-to-eq}}$ (s^{-1})	$k_{B\text{-to-eq}}$ (s^{-1})	$k_{A\text{-to-B}}$ (s^{-1})	$k_{B\text{-to-A}}$ (s^{-1})	$\Delta G^{\#}_{A\text{-to-eq}}$ (kJ/mol)	$\Delta G^{\#}_{A\text{-to-eq}}$ (kJ/mol)
2.2	$2.80 \pm 0.19 \times 10^{-3}$ [a]	$1.45 \pm 0.12 \times 10^{-3}$ [a]	$1.70 \pm 0.40 \times 10^{-3}$	$0.42 \pm 0.10 \times 10^{-3}$	89.58 ± 0.17 [b]	91.28 ± 0.20 [b]
7.0	$0.89 \pm 0.10 \times 10^{-3}$ [a]	$0.99 \pm 0.06 \times 10^{-3}$ [a]	$0.75 \pm 0.05 \times 10^{-3}$	$0.19 \pm 0.01 \times 10^{-3}$	92.53 ± 0.28 [b]	92.26 ± 0.17 [b]

Separative column: Chiralcel OD-R. Reactor column: IAM based. Column operation temperature at 0 °C $n = 4$. Eluent (pump 1): water:acetonitrile 50:50 (v/v). Buffers (pump 2): chloroacetate buffer solution of ionic strength of 0.01 at pH 2.20 and phosphate buffer solution of ionic strength of 0.01 at pH 7.00. Time intervals for enantiomerization at 37.5 °C = 5′, 10′, 15′, 20′, and 30′.

[a] Rate constants in IAM column

[b] Free energy barriers in IAM column.

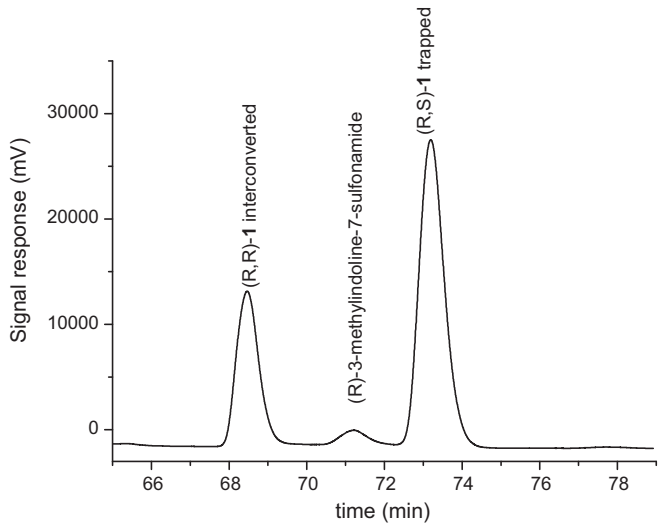

Fig. 9. Chromatogram of a typical epimerization and hydrolysis experiment by sf-BD-rHPLC. Columns: Chiralcel OD-R (250 mm × 0.46 mm I.D., 10 μm); Supelcosil LC-8 (250 mm × 4.6 mm I.D., 5 μm). Mobile phase: water:acetonitrile 50:50 (v/v). Time interval for enantiomerization at 37.5 °C: 90′. Buffer pH: 2.20. Flow: 0.5 ml/min.

The data obtained by off-column method confirm that hydrolysis is an acid catalyzed process being faster in acidic conditions than in neutral conditions. Anyway, hydrolysis rates of (R,R),(R,S)-**1** obtained by off-column method were greater than that obtained by sf-BD-rHPLC method, suggesting that IAM and C8 stationary phases could negatively influence hydrolysis rates.

Since IAM and C8 stationary phases possess alkyl chains that could interact with (R,R),(R,S)-**1**, it is possible that these lipophilic interaction influence hydrolysis process.

In order to evaluate the influence of solvent polarities on hydrolysis rates of (R,R),(R,S)-**1**, off-column experiments were conducted in solvents at different dielectric constants by adding different percentage of acetonitrile (5–25%) to buffer at pH 2.20. The addition of 5–25% of acetonitrile to the buffer at pH 2.2 leads to a significant decrease of the hydrolysis rates.

These data were further analyzed by plotting the $\log(k_{idr})$ *vs.* $1/D$, where D is the weighted dielectric constant of the solvent mixture and was calculated by using the dielectric constant and the density of 78.5 and 0.998 for water and 3.92 and 0.786 for acetonitrile at 20 °C, respectively. As shown in Fig. 10, plots of $\log(k_{idr})$ *vs.* $1/D$ at pH 2.2 shows linearity with negative slope.

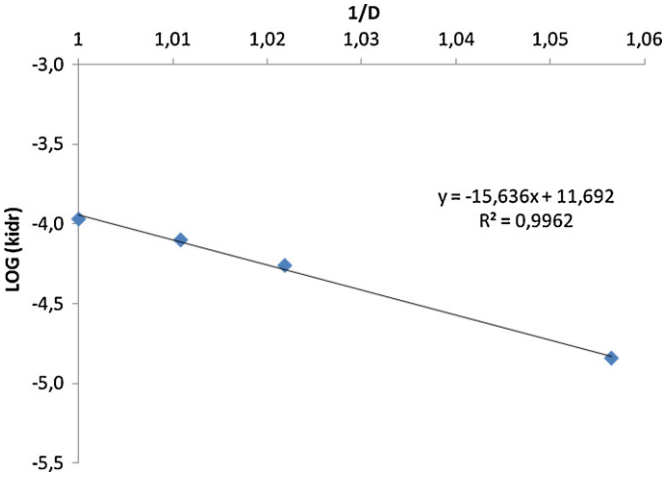

Fig. 10. Effect of dielectric constant on the hydrolysis rate constants of (R,R),(R,S)-**1** at pH 2.20 and 37.5 °C.

The hydrolysis rate constants tend to decrease with increasing acetonitrile concentration or decreasing dielectric constants in the medium, suggesting that the rate-controlling step of hydrolysis reaction may involve the formation of a polar transition state [45]. Apolar environment of IAM or C8 stationary phases can destabilize the polar transition state inhibiting hydrolysis of (R,R),(R,S)-**1**.

Since IAM stationary phase simulates the same microenvironment of biological membrane, it is possible to predict that hydrolysis rate of **1** will decrease *in vivo*.

4. Conclusion

Chemical and configurational stability of **1**, one of the most active positive allosteric modulator of AMPAr, have been investigated by dynamic and stopped-flow HPLC methods.

Epimeric mixture (R,R),(R,S)-**1** has been obtained combining synthetic and chromatographic strategies. Moreover (R,R)-**1** and (R,S)-**1** were separated on Chiralcel OD-R column and molecular modelling calculations were employed to assign (R,R) and (R,S) configuration to the first and second eluted epimers, respectively. Preliminary investigations concerning configurationally stability of (R,R),(R,S)-**1** by dynamic chromatography (DHPLC) have indicated epimerization during chromatographic runs in aqueous/acetonitrile solvents and in presence of CSP. Since it is important to evaluate configurationally stability of pharmaceutical compounds in conditions similar to those they will be meet *in vivo*, stopped-flow bidimensional recycling HPLC (sf-BD-rHPLC) method has been successfully applied to calculate epimerization rates. The study was performed on both C8 and immobilized artificial membrane (IAM) columns at pH 2.2 and 7.0 and at temperature of 37.5 °C. The data obtained indicate that (R,R),(R,S)-**1** undergoes a rapid epimerization in aqueous solvents at both pH 2.2 and 7.0.

sf-BD-rHPLC epimerization chromatograms performed in buffer at pH 2.2 show the presence of a third peak identified as the hydrolysis product of **1** ((R)-2,3-dihydro-3-methyl-1H-indol-7-sulfonamide). Hydrolysis studies have been performed by sf-BD-rHPLC and off-column methods. Comparisons of hydrolysis rate constants obtained by both methods in the same experimental conditions indicate that hydrophobic interactions between IAM or C8 stationary phases with (R,R),(R,S)-**1** negatively influence hydrolysis rates.

Since sf-BD-rHPLC method permits to select the appropriate stationary phase and solvents to simulate physiological conditions, hydrolysis rates constants obtained should be very close to those *in vivo*.

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