CHIRALITY 22:389–397 (2010)

Simultaneous Determination of Enantiomerization and Hydrolysis Kinetic Parameters of Chiral *N*-Alkylbenzothiadiazine Derivatives

MARINA M. CARROZZO, GIUSEPPE CANNAZZA,* UMBERTO BATTISTI, DANIELA BRAGHIROLI, AND CARLO PARENTI
Dipartimento di Scienze Farmaceutiche, Università degli Studi di Modena e Reggio Emilia, Modena, Italy

ABSTRACT On-column stopped flow multidimensional HPLC (sfMDHPLC) and dynamic high-performance liquid chromatography were applied to investigate the influence of alkyl substituents at the sulfonamidic and amino moieties of benzothiadiazine 1,1-dioxide derivatives on hydrolysis and enantiomerization rate constants. The data obtained indicate the presence of pyrrolo substituent at the 3,4 positions on benzothiadiazine rings inhibits the hydrolysis, whereas the enantiomerization occurs in acidic medium. Hydrolysis rates are quite similar for the two benzothiadiazines methyl substituted to nitrogen at 2- and 4-positions. Conversely, enantiomerization rate of 4-*N*-methyl substituted is significantly higher than 2-*N*-methyl substituted. *Chirality 22:389–397, 2010.*

KEY WORDS: *N*-alkylbenzothiadiazines; stopped-flow multidimensional HPLC; dynamic HPLC; enantiomerization; hydrolysis

INTRODUCTION

Previous studies on memory and cognition-enhancing drugs implicate AMPA (2-amino-2,3-dihydro-5-methyl-3-oxo-4-isoxazolepropanoic acid) type receptors as their target of action. These compounds [i.e., (**1**) diazoxide, (**2**) cyclothiazide, (**3**) IDRA21, (**4**) S18986, (**5**) 7-chloro-5-ethyl-3-methyl-3,4-dihydro-2*H*-benzo[1,2,4]thiadiazine 1,1 dioxide, and (**6**) 4-ethyl-7-fluoro-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxide] are thought to work by potentiating glutamate synaptic currents through removal of AMPA receptor desensitization (Fig. 1).[1–4] As it has been recently suggested that these compounds are useful in the treatment of neurodegenerative disorders such as cognitive disorders, schizophrenia, depression, and Parkinson' s disease,[5–7] they constitute potent innovative therapeutic agents. More recently, it has been reported that a novel *N*-alkylbenzothiadiazine 1,1-dioxide (**6**) (Fig. 1), structurally close to IDRA21, showed an interesting AMPA modulator activity.[8]

Previously, we have studied the stability of several benzothiadiazine derivatives and it turned out a rapid interconversion of the enantiomers (enantiomerization) in aqueous solution and a rapid hydrolysis in acidic medium.[9–13]

Rate constants and free energy barriers of enantiomerization of configurationally labile chiral compounds were calculated by several methods, such as dynamic NMR[14–16] (DNMR), 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] dynamic capillary electrophoresis (DCE),[21] and stopped-flow HPLC[22](sfHPLC)].

As on-column chromatographic methods required minute amounts of the unresolved racemic sample, they are widely used.[22]

By dynamic methods (DHPLC) it is possible to study enantiomerization of labile chiral compounds when the interconversion takes place at the time scale of the separation process.[20,21,23–26] As kinetic parameters of enantiomerization are calculated in the presence of chiral stationary phase (CSP), rate constants of enantiomerization could be enhanced or depressed by the presence of chiral environment. Efforts have been made to combine the advantages of the on-column approach with the option to perform enantiomerization in an achiral and inert environment.[20] With this aim, we have recently developed a multidimensional stopped-flow HPLC method (sfMDHPLC), in which enantiomerization is performed in an achiral environment. The technique employs three columns in series: 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 was performed in the second achiral column and then, the flow of the mobile phase introduces the enantiomers to the third chiral column, in which they are separated.[12,13]

The sfMDHPLC was successfully applied for the determination of the chiral inversion of (±)-2,3,3a,4-tetrahydro-1*H*-pyrrolo[2,1-c][1,2,4]benzothiadiazine 5,5-dioxide and IDRA21.[12,13]

Additional Supporting Information may be found in the online version of this article.

Contract grant sponsor: MIUR (Ministero dell'Istruzione, dell'Università e della Ricerca, Italy).

*Correspondence to: Giuseppe Cannazza, Dipartimento di Scienze Farmaceutiche, Università degli Studi di Modena e Reggio Emilia, Via Campi 183, 4100 Modena, Italy. E-mail: giuseppe.cannazza@unimore.it

Received for publication 21 January 2009; accepted in revised form 5 May 2009; Accepted 13 May 2009
DOI: 10.1002/chir.20751
Published online 2 July 2009 in Wiley InterScience (www.interscience.wiley.com).

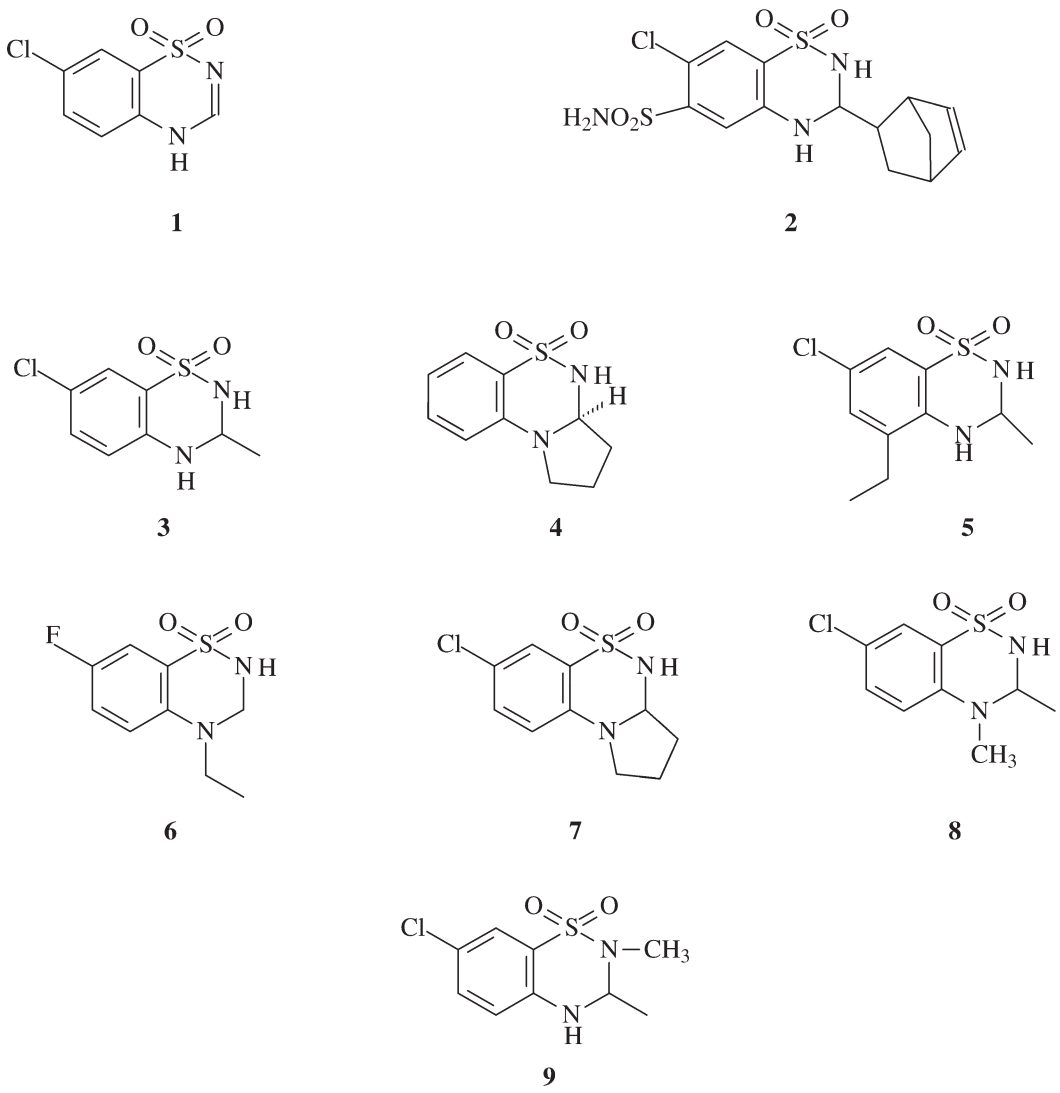

Fig. 1. Structures of benzothiadiazine derivatives.

To gain further insight in understanding the enantiomerization and hydrolysis mechanisms of chiral 3,4-dihydro-1,2,4-benzothiadiazine 1,1-dioxide type compounds, the sfMDHPLC and DHPLC procedures were applied to study enantiomerization and hydrolysis of three selected chiral compounds: 7-chloro-2,3,3a,4-tetrahydro-1*H*-pyrrolo[2,1-c][1,2,4]-benzothiadiazine 5,5-dioxide ((±)-**7**), 7-chloro-3,4-dimethyl-2*H*-1,2,4-benzothiadiazine 1,1-dioxide ((±)-**8**), and 7-chloro-2,3-dimethyl-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxide ((±)-**9**).

The results obtained were used to investigate the influence of substitution at the nitrogen at 2- and 4-positions of the benzothiadiazine ring on hydrolysis and enantiomerization kinetic constants.

EXPERIMENTAL

Instrumentation

The chromatographic apparatus consisted of a Shimadzu LC-10AD Pump, a Merck Hitachi L-6200A Pump, a Rheodyne 7125 manual injector equipped with a 50 μl sample loop. A Merck Hitachi L-7400UV was used as detector. Chromatograms were recorded with a Jasco J-700 program. A Rheodyne 7010 valve was installed after the first chiral column 1 and permitted the trapping of the individual enantiomers of (±)-**7** or (±)-**9** (Fig. 2). Column temperature regulation was achieved with a Haake F3 thermostated water bath.

The columns used were Chiralcel OD-RH [tris(3,5-dimethylphenylcarbamate); 150 mm × 4.6 mm ID; 5 μm] purchased from Daicel, and Supelcosil LC-18 (250 mm × 4.6 mm ID; 5 μm) purchased from Supelco. 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 consisted of 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

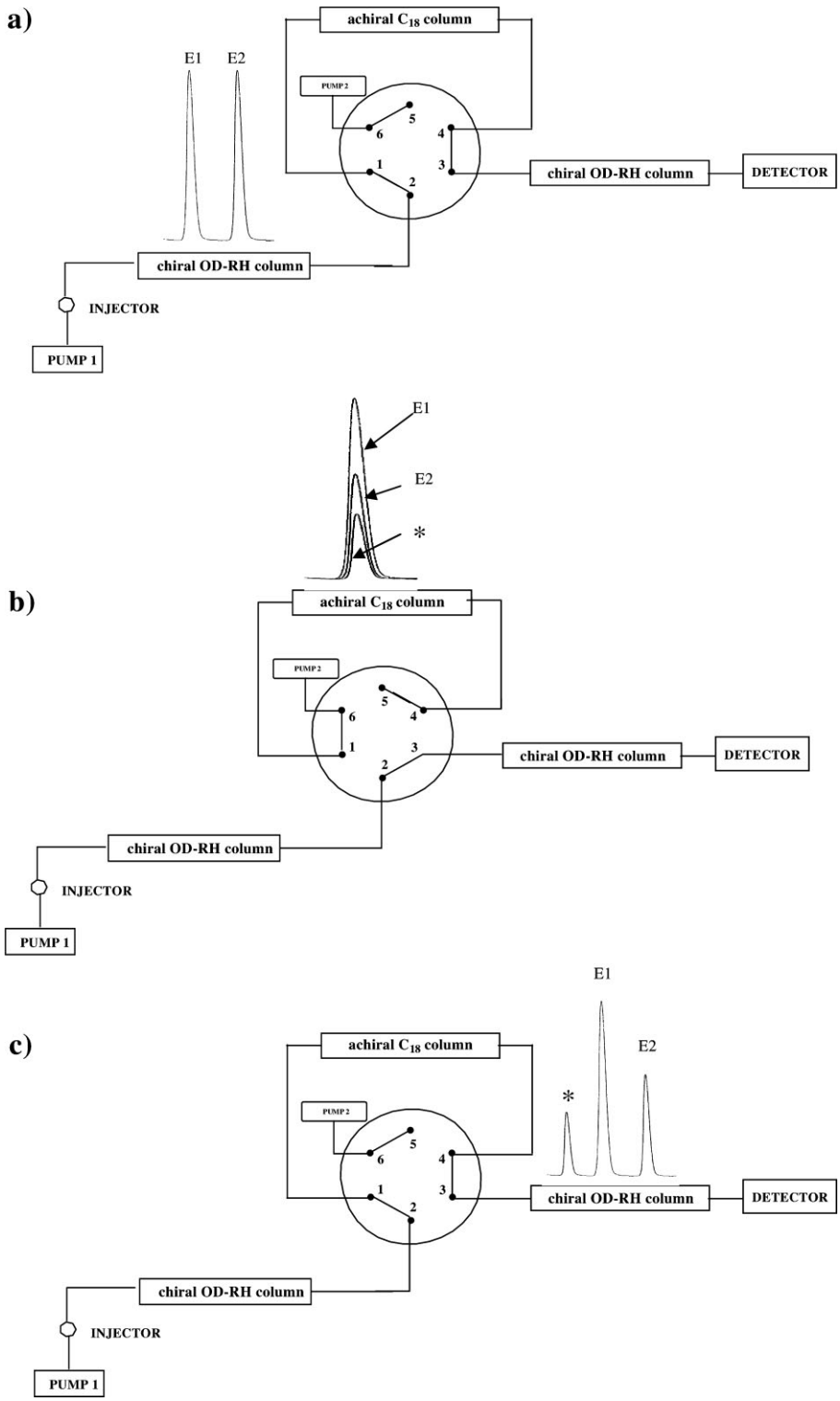

Fig. 2. Schematic representation of stopped-flow multidimensional HPLC (sfMDHPLC). Step 1: Racemic mixture was injected and enantioseparated on the first chiral OD-RH column at low temperature conditions. Step 2: One of the two eluted enantiomers was trapped into the second achiral C18 column and enantiomerization and hydrolysis were performed at different pH buffers at 37°C for a set time interval. Step 3: The enantiomers and hydrolysis product were separated in the third chiral OD-RH column at low temperature conditions. E1 and E2 corresponded to the first and second eluted enantiomers. (*) corresponded to hydrolysis product.

follows: s, singlet; d, doublet; dd, double doublet; 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.

Synthesis

(±)7-Chloro-2,3,3a,4-tetrahydro-1H-pyrrolo[2,1-c][1,2,4]-benzothiadiazine 5,5-dioxide ((±)-7). The compound was synthesized as previously described by Cameroni et al.[27]

Yield 56.4%, mp 212–214°C, FT-IR 3205 cm^{-1} (ν NH), 1323 cm^{-1} (ν SO_2 as), 1151 cm^{-1} (ν SO_2 sim). ^1H NMR (DMSO-d_6) δ = 1.78–2.09 (m, 3H), δ = 2.28–2.37 (m, 1H), δ = 3.12–3.25 (m, 1H), δ = 3.50–3.3.59 (m, 1H), δ = 4.97–5.06 (m, 1H), δ = 6.77 (d, 1H, J = 8.98), δ = 7.44 (dd, 1H, J = 2.55, J = 8.94), δ = 7.53 (d, 1H, J = 2.53). The microanalyses results were within ±0.4%.

(±)7-Chloro-3,4-dimethyl-2H-1,2,4-benzothiadiazine 1,1-dioxide ((±)-8). The compound was synthesized as previously described by Francotte et al.[8]

Yield 86%, mp 129–130°C, FT-IR 3205 cm^{-1} (ν NH), 1377 cm^{-1} (ν SO_2 as), 1154 cm^{-1} (ν SO_2 sim). 1H NMR(DMSO-d_6) δ = 1.45 (d, 3H), δ = 2.87 (s, 3H), δ = 4.75–4.80 (m, 1H), δ = 6.89 (d, 1H, J = 8.97), δ = 7.39–7.42 (dd, 1H, J = 2.64, J = 8.96), δ = 7.45 (d, 1H, J = 2.17). The microanalyses results were within ±0.4%.

(±)7-Chloro-2,3-dimethyl-3,4-dihydro-2H-1,2,4-benzothiadiazine 1,1-dioxide ((±)-9). The compound was synthesized as previously described by Francotte et al.[8]

Yield 73%, mp 74–75°C, FT-IR 3341 cm^{-1} (ν NH), 1321 cm^{-1} (ν SO_2 as), 1152 cm^{-1} (ν SO_2 sim). ^1H NMR (DMSO-d_6) δ = 1.44 (d, 3H), δ = 2.46 (s, 3H), δ = 5.21–5.26 (m, 1H), δ = 6.86 (d, 1H, J = 8.93), δ = 7.35–7.39 (dd, 1H, J = 2.24, J = 8.87), δ = 7.49 (d, 1H, J = 2.32). The microanalyses results were within ±0.4%.

Chromatography

Separation of enantiomers of (±)-**7**, (±)-**8**, and (±)-**9** were carried out isocratically at different temperatures on Chiralcel OD-RH column. The mobile phase consisted of water: acetonitrile at different percentage. Separation of compounds (±)-**8** and (±)-**9** from their hydrolysis products [5-chloro-2-(methylamino)benzensulfonamide (**11**) and 2-amino-5-chloro-*N*-methylbenzensulfonamide (**12**)] were carried out in the same conditions. 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 50 μl. The detectors were set at 254 nm.

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 enantiomer. The dead time of the columns (t_0) was determined by injection of 1,3,5-tri-*tert*-butylbenzene.

Stopped-Flow Multidimensional HPLC (sfMDHPLC)

Enantiomerization and hydrolysis of (±)-**7** and (±)-**9** were studied by the sfMDHPLC method described herein.[12,13] Figure 2 shows a schematic representation of the method used. In the first step, racemic compound was injected and enantioseparated on column 1. At the appropriate time, the individual enantiomers were cut off by switching the valve into position (b) and trapped into the second achiral column 2. In the second step, the achiral column was filled with the buffer at the desired pH and temperature by pump 2. The enantiomerization and hydrolysis were affected by heating at 37.5°C whereas no mobile phase passed through the column 2 (stopped-flow) for a set period of time. In the last step, the original mobile phase was resumed to permit the separation of the products originated in the second step by the trapping enantiomer.

The outcome of this protocol resulted in the presence of three peaks: peak 1° arises from the hydrolysis, peaks 2° and 3° corresponding to the enantiomers of analytes injected, one arises from enantiomer trapped in the achiral column and the other one corresponding to its interconverted product.

The elution order was validated by the use of a circular dichroism spectroscopy detector: whereas the second and the third peaks have opposite cotton effect, the first peak was not detected by CD detection because benzensulfonamide is an achiral compound.

The kinetic parameters (rate constants of enantiomerization k) and the enantiomerization barriers ($\Delta G^{\#}$) were calculated from the corresponding peak areas, from the enantiomerization time, and from the enantiomerization temperature as described in "Calculation of kinetic rate constants and free energy barriers of enantiomerization" section. It is assumed that the precision of $\Delta G^{\#}$ is not influenced within the experimental standard deviation by the warm-up times on the achiral column 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.[28–30]

Off-Column Hydrolysis

Pure (±)-**8** was dissolved in acetonitrile and subsequently diluted 1:10 (v/v) with individual solvent (1 ml) at final concentration of 100 μg/ml and kept thermostated at 37°C. The hydrolysis was monitored by chromatography on the Chiralcel OD-RH column using a mobile phase water and acetonitrile 60:40 (v/v). Four repeats of each experiment were made. The two peaks, corresponding to (±)-**8** and hydrolysis product (5-chloro-2-(methylamino)-benzensulfonamide (11)), were identified by injecting pure compounds in the same experimental conditions.

Buffer solution at pH 1.20 was prepared by mixing 407 ml of 0.2 M hydrochloric acid with 93 ml of 0.2 M potassium chloride and diluting with water to one liter. Chloroa-

cetate buffer solution at pH 2.20 was prepared by mixing 50.99 ml of 0.1 M chloroacetic acid with 2.76 ml of 0.1 M KOH and diluting with water to 100 ml. Acetate buffer solution at pH 4.20 was prepared by mixing 43.03 ml of 0.1 M acetic acid with 9.93 ml of 0.1 M KOH and diluting with water to 100 ml. Phosphate buffer solution at pH 6.40 was prepared by mixing 30.21 ml of 0.02 M KH_2PO_4 with 13.19 ml of 0.01 Na_2HPO_4 and diluting with water to 100 ml.

Calculation of Kinetic Rate Constants and Free Energy Barriers of Enantiomerization

Kinetic rate constants and free energy barriers of enantiomerization of (±)-**7** and (±)-**9** were calculated by sfMDHPLC.

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_o is the peak of the initial enantiomer before enantiomerization [= 100%], a_t is 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:

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

where k is the kinetic rate constant, k_B is the Boltzmann constant ($k_B = 1.380662 \times 10^{-23}$ J K^{-1}), h is the Planck's constant ($h = 6.626176 \times 10^{-34}$ J s), R is 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).

Kinetic rate constants and free energy barriers of enantiomerization of (±)-**8** were calculated by DCXplorer software, developed by Trapp (DCXplorer software is available from Trapp as executable programs, running under Microsoft Windows 2000, XP, and Vista).[31]

Calculation of Kinetic Rate Constants of Hydrolysis

The kinetic rate constants can be calculated by fitting the data to eq. 3:

$$A_t = A_0 \exp(-k_i t_i) \quad (3)$$

where k_i is the rate constant of hydrolysis [s^{-1}], A_o is the peak of the (±)-**8** and (±)-**9** before hydrolysis [= 100%], A_t is the relative peak area of the injected compound remaining after hydrolyzation time t_i [%] (i.e., a_t = 100-conversion), and t_i the hydrolysis time(s).

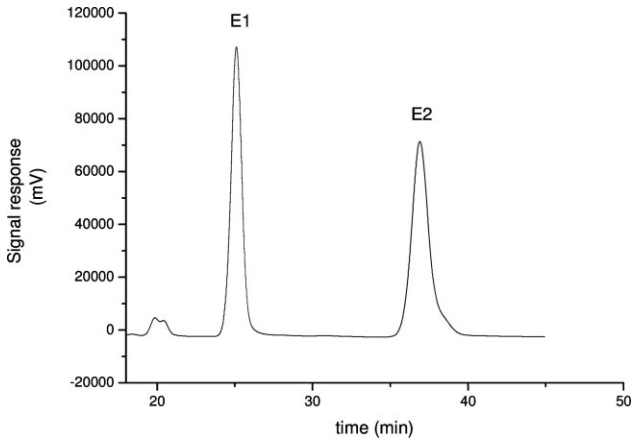

Fig. 3. Enantioresolution of (±)-**7**. Column: Chiralcel OD-RH (15 × 0.46 I.D., 5 μm); mobile phase: water:acetonitrile 60:40 (v/v); temperature: 25°C.

RESULTS AND DISCUSSION

Chromatography

Chromatographic analysis of (±)-**7** were performed on OD-RH column with a mobile phase of water:acetonitrile 60:40 (v/v). The enantioseparation was conducted at different temperatures between −10°C and 37°C. As expected the retention times and enantioseparation increase with the decreasing of temperature analysis (−10°C: $k'_1 = 7.02$, $k'_2 = 10.98$, $\alpha = 1.56$, $R_s = 3.70$; 0°C: $k'_1 = 6.17$, $k'_2 = 10.24$, $\alpha = 1.66$, $R_s = 2.97$; 25°C: $k'_1 = 3.67$, $k'_2 = 5.8$, $\alpha = 1.58$, and $R_s = 2.90$; 37°C: $k'_1 = 2.93$, $k'_2 = 4.50$, $\alpha = 1.53$, $R_s = 2.85$). Baseline resolution of (±)-**7** with acceptable analysis time was obtained at 25°C (Fig. 3). These results indicate that hardly any enantiomerization of the two enantiomers of (±)-**7** occurred during chromatographic enantioseparation at temperature between −10°C and 37°C using water:acetonitrile as mobile phase.

Chromatographic analysis of (±)-**8** was conducted on OD-RH column and with a mobile phase of water:acetonitrile 60:40(v/v) at different temperatures between −10°C and 25°C. Chromatograms in Figure 4 show that at temperature of 0°C a pronounced plateau was observed between the two peaks corresponding to enantiomers. A single peak was observed at 25°C indicating the coalescence of the two enantiomers. Chromatogram corresponding to the enantioresolution in the same chromatographic conditions [column OD-RH, mobile phase water:acetonitrile 60:40 (v/v)] but at temperatures of −10°C indicate an inhibition of enantiomerization process with a dramatic reduction of plateau between the peaks (Fig. 4). These results indicate that a rapid thermal enantiomerization of (±)-**8** occurred in water:acetonitrile with $\Delta G^{\#}$ barriers lower than 90 kJ mol^{-1}.[32] The free energy barrier of enantiomerization of (±)-**8** is not high enough to prevent racemization during chromatographic enantioresolution, at 0° and 25°C, and thereby does not allow isolation of single enantiomers. Consequently, the temperature has been lowered (−10°C) to decrease the rate of enantiomerization.

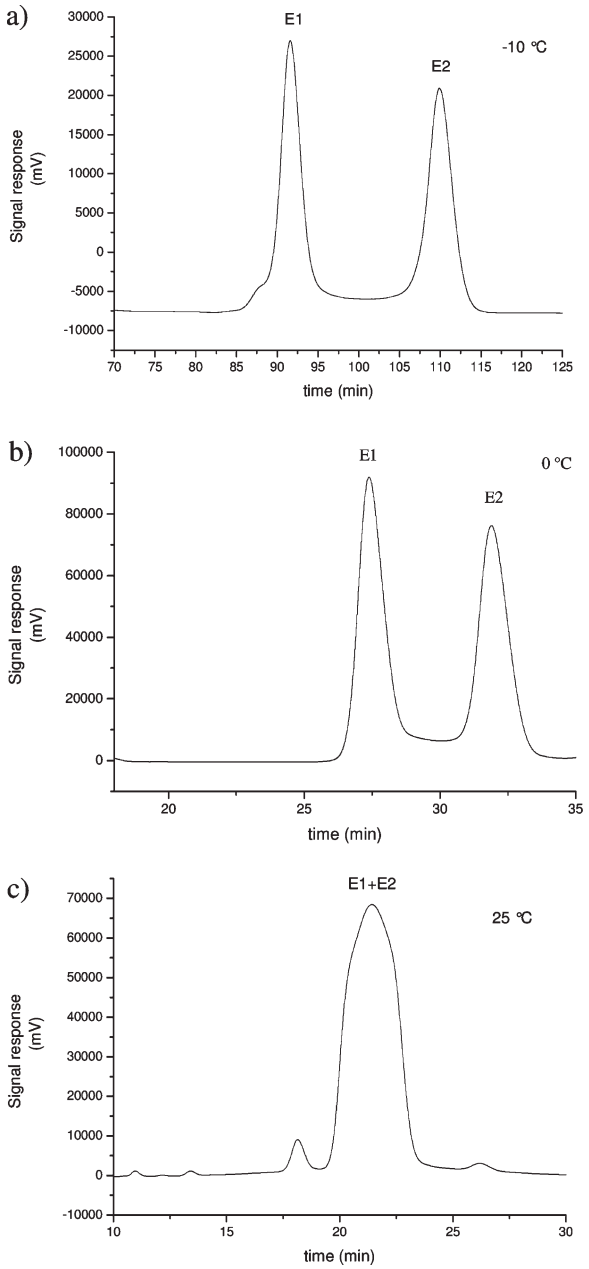

Fig. 4. Enantioresolution of (±)-**8**. Column: Chiralcel OD-RH (15 × 0.46 I.D., 5 μm); mobile phase: water:acetonitrile 60:40 (v/v); temperature: (**a**) −10°C, (**b**) 0°C, (**c**) 25°C.

Chromatographic analysis of the racemic (±)-**9** was performed on OD-RH column with water:acetonitrile at different percentage compositions and at different temperatures. No baseline enantiomeric resolution was obtained in chromatographic conditions used. Partial enantioresolution was obtained using OD-RH column with water:acetonitrile 70:30 (v/v) at 0°C (k'_1 = 16.2, k'_2 = 17, α = 1.05, R_s = 1.28; Fig. 5). The poor enantioresolution obtained for the compound (±)-**9** indicates that hydrogen bonding between the nitrogen atom of the sulfonamidic moiety adjacent to the chiral center has a great influence on chiral recognition of the OD-RH stationary phase. Indeed, a good enantioresolution has been obtained for (±)-**8** with nitrogen atom of the sulfonamidic moiety not substituted, whereas *N*-methylated (±)-**9** exhibits a partial optical resolution with α = 1.05.

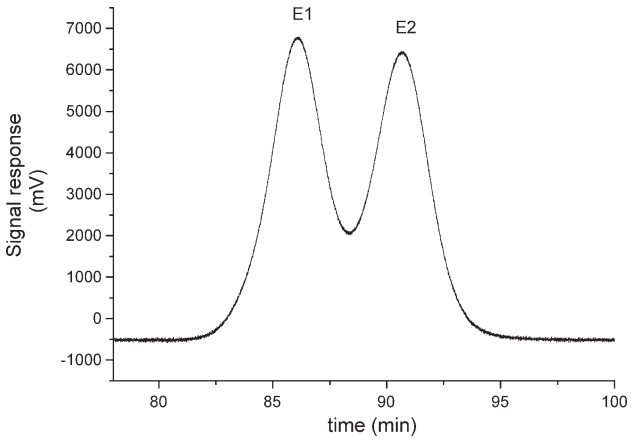

Fig. 5. Enantioresolution of (±)-**9**. Column: Chiralcel OD-RH (15 × 0.46 I.D., 5 μm); mobile phase: water:acetonitrile 70:30 (v/v); temperature : 0°C.

Enantiomerization and Hydrolysis of (±)-7

Enantiomerization and hydrolysis of (±)-**7** were studied by sfMDHPLC method (see experimental part for details) at 37°C and in the pH range over 1.20–6.40.

The sfMDHPLC method consists in trapping each enantiomer, previously separated by a chiral column, in a C18 achiral column that is subsequently filled with the selected buffers and left to react for certain time. Afterward, the products of enantiomerization and hydrolysis of the trapped enantiomer will be separated by a chiral column. From the peak areas it is possible to calculate kinetics of enantiomerization and hydrolysis simultaneously.

The results indicate that any hydrolysis of compound (±)-**7** occurred for at least 2 h at 37°C and at pH between 1.20 and 6.40.

The kinetic rate constants and Gibbs free energies of activation of enantiomerization of (±)-**7** at 37°C and at pH 1.20, 2.20, 4.20, and 6.40 were reported in Table 1.

The data obtained indicate that enantiomerization rates strongly depend on pH, increasing with the decreasing of pH (1.20–2.20), demonstrating that enantiomerization of (±)-**7** was an acid catalyzed process.

The results suggest that the pyrrolo substituent on the benzothiadiazine ring allowed enantiomerization in acidic medium whereas hydrolysis was inhibited.

Previous studies performed on S18986, structurally related to (±)-**7**, demonstrated that any hydrolysis occurred, whereas the enantiomerization is an acid catalyzed process.[12] The kinetic parameters obtained for (±)-**7** and S18986 are quite similar, indicating that the presence of chlorine atom at the 7-position of benzothiadiazine ring not significantly influence the rates of enantiomerization and hydrolysis.[12]

TABLE 1. Rate constants and free energy barriers of enantiomerization of (±)-7 determinated by sfMDHPLC

pH	k (sec^{-1})	$\Delta G^{\#}$ (kJ mol^{-1})
1.20	$10.50 \pm 1.44 \times 10^{-4}$[a]	92.12 ± 0.34[b]
2.20	$6.56 \pm 0.62 \times 10^{-4}$[a]	93.33 ± 0.23[b]
4.20	$0.22 \pm 0.05 \times 10^{-4}$[a]	102.09 ± 0.49[b]
6.40	[c]	[c]

Columns: Chiralcel OD-RH, Supelcosil LC-18. Column operation temperature: 0°C. Eluent (Pump 1): water:acetonitrile 60:40 (v/v). Buffers (Pump 2): buffer solution at pH 1.20, chloroacetate buffer solution at pH 2.20, acetate buffer at pH 4.20, and phosphate buffer at pH 6.40. Time intervals for enantiomerization at 37.5°C = 5′, 10′, 15′, 20′, 30′, 60′, 120′ ($n = 4$).
[a]Rate constants in C18 column.
[b]Free energy barriers in C18 column.
[c]No enantiomerization occurred in 2 h.

Enantiomerization and Hydrolysis of (±)-8

The chromatograms relative to the enantioseparation of (±)-**8** have been demonstrated that a rapid thermal enantiomerization occurs during chromatographic enantioresolution (Fig. 4). As the applicability of sfMDHPLC requires that the enantiomerization process is suppressed during the chromatographic separation (initial and final separation steps), it was not possible to apply the method to determinate the enantiomerization and hydrolysis kinetic parameters of (±)-**8**. Accordingly, the enantiomerization rate constants were calculated by dynamic chromatography experiments (DHPLC) using DCXplorer software.[31] The program employs the unified equation of chromatography that can evaluate reaction rate constants of all kinds of first order reactions taking place during a separation process.[33–41] The data obtained were reported in Tables 2 and 3.

The results indicate that enantiomerization rates depend poorly by temperature (−10°C: $k = 2.66 \times 10^{-5}$ sec^{-1} and $\Delta G^{\#} = 85.71$ kJ mol^{-1}, 0°C: $k = 9.63 \times 10^{-5}$ sec^{-1} and $\Delta G^{\#} = 86.13$ kJ mol^{-1}). Moreover, it has been observed that the pH does not significantly influence the enantiomerization rates, as only small differences were obtained between pH 1.20–6.40 (Table 2). Similarly, the variation of percentage of acetonitrile in the mobile phase causes only small changes in enantiomerization rate constants.

The rate constants of hydrolysis of (±)-**8** were calculated by the classical batch-wise kinetic method (off-column method) in a series of buffers over a pH range of 1.20–6.40 at 37°C as described in the experimental part.

The calculated kinetic rate constants of hydrolysis were reported in Table 4.

The data obtained show that hydrolysis rate constants depend strongly on pH, increasing with the decreasing of pH, indicating that hydrolysis of (±)-**8** was an acid catalyzed process.

TABLE 2. Rate constants and free energy barriers of enantiomerization of (±)-8 at pH 1.20–6.40 determinated in DHPLC by DCXplorer

Eluent	k (sec^{-1})	$\Delta G^{\#}$ (kJ mol^{-1})
Buffer 1.20:ACN	1.61×10^{-4}	84.96
Buffer 2.20:ACN	2.04×10^{-4}	84.42
Buffer 4.20:ACN	1.80×10^{-4}	84.70
Buffer 6.40:ACN	1.35×10^{-4}	85.36

Column: Chiralcel OD-RH; temperature: 0°C; eluent: buffer:acetonitrile 60:40 (v/v); buffers: chloride buffer pH 1.20, chloroacetate buffer pH 2.20, acetate buffer pH 4.20, and phosphate buffer pH 6.40.

TABLE 3. Rate constants and free energy barriers of enantiomerization of (±)-8 at different percentage of acetonitrile determinated in DHPLC by DCXplorer

Water:acetonitrile (v/v)	k (sec^{-1})	$\Delta G^{\#}$ (kJ mol^{-1})
60:40	9.63×10^{-5}	86.13
70:30	8.20×10^{-5}	86.49
75:25	6.81×10^{-5}	86.91

Column: Chiralcel OD-RH; temperature 0°C. Eluents:water:acetonitrile 60:40 (v/v), 70:30 (v/v), and 75:25 (v/v).

Enantiomerization and Hydrolysis of (±)-9

Chromatogram in Figure 5 of (±)-**9** indicates that only a partial enantioresolution has been obtained. As dynamic chromatography methods need at least a baseline resolution of the peaks corresponding to enantiomers, it was not possible to apply the DHPLC procedure to calculate enantiomerization kinetic parameters. The enantiomerization and hydrolysis of (±)-**9** were studied by sfMDHPLC procedure because it was possible to trap pure enantiomer by switching the valve at appropriate time in the achiral column, where occurs enantiomerization and hydrolysis processes (Fig. 6).

Single enantiomer of (±)-**9** hydrolyzed completely to 2-amino-5-chloro-*N*-methylbenzensulfonamide in 5 min at 37°C and at pH 1.20 and 2.20.

The kinetic rate constants of enantiomerization and hydrolysis at pH 4.20 and at 37°C were $1.66 \pm 0.11 \times 10^{-4}$ sec^{-1} ($\Delta G^{\#} = 96.88 \pm 0.09$ kJ mol^{-1}) and $4.15 \pm 0.33 \times 10^{-4}$ sec^{-1}, respectively.

Conversely, at pH 6.40 and at 37°C, any enantiomerization and hydrolysis were obtained for at least 2 h.

The data indicate that the enantiomerization and hydrolysis processes depend strongly by pH, increasing with the decreasing of pH.

TABLE 4. Rate constants of hydrolysis of (±)-8 at pH 1.20–6.40 determinated by off-column method

pH	k_i (sec^{-1})
1.20	(*)
2.20	$54.40 \pm 19.60 \times 10^{-4}$
4.20	$6.99 \pm 1.14 \times 10^{-4}$
6.40	$1.89 \pm 0.12 \times 10^{-4}$

Column: Supelcosil LC-18; flow 0.5 ml min^{-1}; temperature: 37°C; buffers: chloride buffer pH 1.20, chloroacetate buffer pH 2.20, acetate buffer pH 4.20, and phosphate buffer pH 6.40. Time intervals for off-column hydrolysis at 37.5°C = 5′, 10′,15′, 20′, 30′, 60′, and 120′ ($n = 4$).
(*) Hydrolysis too fast to be calculated.

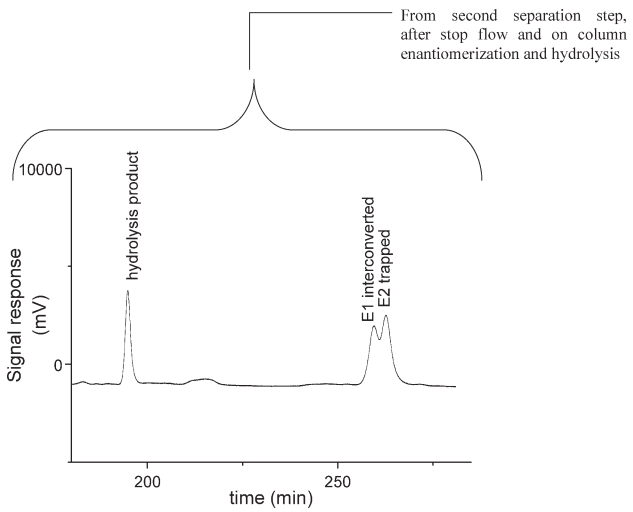

Fig. 6. Chromatogram of enantiomerization and hydrolysis experiments of (±)-**9** performed by sfMDHPLC procedure: peaks 1° (2-amino-5-chloro-*N*-methylbenzensulfonamide (**12**)) arises from hydrolysis process and peaks 2° and 3° corresponding to the enantiomers of (±)-**9**, one arise from enantiomer trapped in the achiral column and the other one corresponding to its interconverted product. Columns: Chiracel OD-RH (15 × 0.46 I.D., 5 μm) Supelcosil LC-18 (25 × 0.46 I.D.); mobile phase: water:acetonitrile 70:30 (v/v); time intervals 30 min.; temperature 37°C; buffer pH 4.20.

CONCLUSION

The sfMDHPLC is a novel method to calculate kinetic parameters of enantiomerization and hydrolysis processes of chiral labile compounds in an achiral and inert environment simultaneously.[12,13] Moreover, the method can be applied to partial enantioresolved compounds because it is possible to trap in the achiral column pure enantiomers by cutting the peaks at the appropriate time. The sfMDHPLC has been successfully used to calculate kinetic parameters of enantiomerization and hydrolysis of compounds (±)-**7** and (±)-**9**, demonstrating the applicability of the method developed.

Anyway, as sfMDHPLC method requires that enantiomerization is suppressed during the chromatographic separation process, the method cannot be applied to the determination of kinetic parameters of compounds that interconverted during chromatographic enantioresolution also at low temperature. Accordingly, the rate constants and free energy barriers of resolvable enantiomers that interconverted rapidly on the separation time scale can be calculated by DHPLC. As we were unable to suppress enantiomerization of (±)-**8** during chromatographic enantioresolution, its kinetic enantiomerization parameters were calculated by DHPLC with the novel software program DCXplorer that permits quick evaluation of elution profiles of interconverting stereoisomers, utilizing the unified equation of chromatography.[31,33–41]

The enantiomerization and hydrolysis of three benzothiadiazine different substituted to heterocyclic moiety has been studied by sfMDHPLC and DHPLC procedures.

The results indicate that the presence of pyrrolo substituent at the 3,4 positions on benzothiadiazine ring inhibits the hydrolysis, whereas the enantiomerization occurs in acidic medium. Hydrolysis rates are quite similar for the benzothiadiazines methyl substituted to N atom at 2- and 4-positions. Conversely, enantiomerization rate of 4-*N*-methyl substituted is significantly higher than 2-*N*-methyl substituted.

Recently, *N*-alkylbenzothiadiazine derivatives have attracted particular attention as they act as potent AMPA potentiators. The data obtained indicate that benzothiadiazine derivatives methyl-substituted to N atom at 2- and 4-positions rapidly hydrolyzed in acidic medium to the corresponding benzensulfonamide. Thus, it is possible that the structurally similar *N*-alkylbenzothiadiazine could be hydrolyzed when administered orally in the acidic medium of the stomach in pharmacological tests.

ACKNOWLEDGMENTS

The authors thank Ms. S. Lucchi for the technical helpful gave in enantiomerization experiments.

LITERATURE CITED

1. Lestage P, Danober L, Lockart B, Roger A, Lebrun C, Robin JL, Desos P, Cordi A. S18986, positive allosteric modulator of AMPA receptors as a novel cognition enhancer in rodents. Res Pract Alzheimer's Dis 2002;6:253–259.
2. Philips D, Sonnenberg J, Arai AC, Vaswani R, Krutzik PO, Kleisli T, Kessler M, Granger R, Lynch G, Chamberline AR. 5′-Alkyl-benzothiadiazides: a new subgroup of AMPA Receptor modulators with improved affinity. Bioorg Med Chem 2002;10:1229–1248.
3. Braghiroli D, Puia G, Cannazza G, Tait A, Parenti C, Losi G, Baraldi M. Synthesis of 3,4-Dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxide derivatives as potential allosteric modulators of AMPA/Kainate receptors. J Med Chem 2002;45:2355–2357.
4. Pirotte B, Podona T, Diouf O, de Tullio P, Lebrun P, Dupont L, Somers F, Delarge J, Morain P, Lestage P, Lepagnol J, Spedding M. 4*H*-1,2,4-pyridothiadiazine 1,1-dioxides and 2,3-dihydro-4*H*-1,2,4-pyridothiadiazine 1,1-dioxides chemically related to diazixide and cyclothiazide as powerful positive allosteric modulators of (*R/S*)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) propionic acid receptors: design, synthesis, phatmacology, and structure-activity relationships. J Med Chem 1998;41:2946–2959.
5. Graindorge E, Francotte P, Boverie S, de Tullio P, Pirotte B. New trends in the development of positive allosteric modulators. Curr Med Chem 2004;4:95–103.
6. Hashimoto K, Okamura N, Shimizu E, Iyo M. Glutamate hypothesis of schizophrenia and approach for possible therapeutic drugs. Curr Med Chem 2004;4:147–154.
7. Black MD. Therapeutic potential of positive AMPA modulators and their relationship to AMPA receptor subunits. A review of preclinical data. Psychopharmacology 2005;179:154–163.
8. Francotte P, de Tullio P, Goffin E, Dintilhac G, Graindgorge E, Fraikin P, Lestage P, Danober L, Thomas JY, Caignard DH, Pirotte B. Design, synthesis and pharmacology of novel 7-substituted 3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxide as positive allosteric modulators of AMPA receptors. J Med Chem 2007;50:3153–3157.
9. Cannazza G, Braghiroli D, Baraldi M, Parenti C. Chiral resolution of the enantiomers of 7-chloro-3-methyl-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxide using high performance liquid chromatography on cellulose-based chiral stationary phases. J Pharm Biomed Anal 2000;23:117–125.
10. Cannazza G, Braghiroli D, Tait A, Baraldi M, Parenti C, Lindner W. Studies of enantiomerization of chiral 3,4-dihydro-1,2,4-benzothiadiazine 1,1-dioxide type compounds. Chirality 2001;13:94–101.
11. Cannazza G, Braghiroli D, Iuliani P, Parenti C. Energy barrier determinationof enantiomerization of chiral 3,4-dihydro-1,2,4-benzothiadiazine 1,1-dioxide type compounds by enantioselective stopped-flow HPLC. Tetrahedron Asymmetry 2006;17:3158–3162.

12. Cannazza G, Carrozzo MM, Braghiroli D, Parenti C. 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. J Chromatogr B 2008;875:192–199.
13. Cannazza G, Carrozzo MM, Braghiroli D, Parenti C. Enantiomerization and hydrolysis of (±)-7-chloro-3-methyl-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxide by stopped-flow multidimensional high-performance liquid chromatography. J Chromatogr A 2008;1212:41–47.
14. Schurig V, Keller F, Reich S, Fluck M. Dynamic phenomena involving chiral dimethyl-2,3-pentadienedioate in enantioselective gas chromatography and NMR spectroscopy. Tetrahedron Asymmetry 1997;8:3475–3480.
15. Kiesswetter R, Burgemeister T, Mannschreck A. Chiral 2*H*-pyrans, 8.1′,3′,3′-trimethyl-6,8-dinitrospiro[2*H*-1-benzopyran-2,2′-indoline]: fast thermal enantiomerization and slow thermal equilibration with a ring-opened isomers. Enantiomer 1999;4:289–296.
16. Bringmann G, Heubes M, Breuning M, Gobel L, Ochse M, Schoner B, Schupp O. Atropisomerization barrier of configurationally unstable biaryl compounds, useful substrates for atroposelective conversionsa to axially chiral byiaryls. J Org Chem 2000;65:722–728.
17. Mannschreck A, Andert D, Eiglsperg E, Gmahl E, Buchner H. Chiroptical detection. Novel possibilities of its application to enantiomers. Chromatographia 1988;25:182–188.
18. Mannschreck A, Kiessel A. Enantiomerization during HPLC on an optically active sorbent. Deconvolution of experimental chromatograms. Chromatographia 1989;28:263–266.
19. Trapp O, Schoetz G, Schurig V. Determination of enantiomerization barriers by dynamic and stopped-flow chromatographic methods. Chirality 2001;13:403–414.
20. Krupcik J, Oswald P, Majek P, Sandra P, Armstrong DW. Determination of the interconversion energy barrier of enantiomers by separation methods. J Chromatogr A 2003;1000:779–800.
21. Wolf C. Stereolabile chiral compounds: analysis by dynamic chromatography and stopped-flow methods. Chem Soc Rev 2005;34:595–608.
22. Schurig V. Contributions to the thoery and practice of the chromatographic separation of enantiomers. Chirality 2005;17:205–226.
23. Mannschreck A, Zinner H, Pustet N. The significance of the HPLC time scale: an example of interconvertible enantiomers. Chimia 1989;43:165–166.
24. Veciana J, Crespo MI. Dynamic HPLC: a method for determination rate constants, energy barriers, and equilibration constants of molecular dynamic processes. Angew Chem Int Ed Engl 1991;30:74–77.
25. Jung M, Schurig V. Determination of enantiomerization barriers by computer simulation of interconversion profiles: enantiomerization of diaziridines during chiral inclusion gas chromatography. J Am Chem Soc 1992;114:529–534.
26. D'Acquarica I, Gasparrini F, Pierini M, Villani C, Zappia G. Dynamic HPLC on chiral stationary phases: a powerful tool for the investigation of stereosimulation processes. J Sep Sci 2006;29:1508–1516.
27. Cameroni R, Ferioli V, Bernabei MT. Sistemi eteropoliciclici-2,2-diidro-1*H*-pirrolo[2,1-*c*][1,2,4]benzotiadiazin-5,5-diossido e suoi derivati. Farmaco 1971;27:574–581.
28. Schurig V, Reich S. Determination of the rotational barriers of atropisomeric polychlorinated biphenyls (PCBs) by a novel stopped-flow multidimensional gas chromatographic technique. Chirality 1998; 10:316–320.
29. Reich S, Schurig V. Stopped-flow multidimensional gas chromatography: a new method for the determination of enantiomerization barriers. J Microcolumn Sep 1999;11:475–479.
30. Reich S, Trapp O, Schurig V. Enantioselective stopped-flow multidimensional gas chromatography: determination of interconversion barrier of 1-chloro-2,2-dimethylaziridine. J Chromatogr A 2000;892:487–498.
31. Trapp O. DCXplorer software. Available from http://www.mpi-muelheim.mpg.de/kofo/institut/arbeitsbereiche/trapp/downloads_e.html Accessed 6/26/2009.
32. Allenmark SG. Chromatographic enantioseparation. New York: Wiley; 1988. p 184.
33. Trapp O. A unified equation for fast and precise access to rate constants of first order reactions in dynamic and on-column reaction chromatography. Anal Chem 2006;78:189–198.
34. Trapp O. Fast and precise access to enantiomerization rate constants in dynamic chromatography. Chirality 2006;18:489–497.
35. Trapp O. The unified equation for the evaluation of first order reactions in dynamic electrophoresis. Electrophoresis 2006;27:534–541.
36. Trapp O. A dynamic molecular probe to investigate catalytic effects and Joule heating in enantioselective micellar electrokinetic chromatography. Electrophoresis 2007;28:691–696.
37. Trapp O, Schurig V. Stereointegrity of Troger's base: gas chromatographic determination of the enantiomerization barrier. J Am Chem Soc 2000;122:1424–1430.
38. Trapp O, Weber SK, Bauch S, Hofstadt W. High-throughput screening of catalysts by combining reactions and analysis. Angew Chem Int Ed Engl 2007;46:7307–7310.
39. Trapp O. Gas-chromatographic high-throughput screening techniques in catalysis (review article). J Chromatogr A 2008;1184:160–190.
40. Trapp O, Schurig V. ChromWin—a computer program for the determination of enantiomerization barriers in dynamic chromatography. Comput Chem 2001;25:187–195.
41. Trapp O. A novel software tool for high-throughput measurements of interconversion barriers: DCXplorer. J Chromatogr B 2008;875:42–47.