Evaluation of Stereo and Chemical Stability of Chiral Compounds

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ABSTRACT A stopped-flow bidimensional recycle HPLC (sf-BD-rHPLC) configuration has been used to investigate simultaneously the stereo and chemical stability of labile chiral compounds. The single enantiomers of a racemate can be separated on chiral column (first dimension) and each one can be trapped in the achiral column (second dimension) that works as reactor. By filling the achiral column with the appropriate aqueous buffers it is possible to evaluate the stability of the trapped enantiomer toward aqueous buffer itself. It was possible to recycle the reaction products formed in the chiral column (first dimension) where they are separated by a second six valve port. The reaction rate constants were calculated for the different processes occurred in the achiral column by means of corresponding peak areas. The method was applied to a pharmacological active compound: (±)7-chloro-5-ethyl-3-methyl-3,4-dihydro-2H-benzo[1,2,4]thiadiazine 1,1-dioxide ((±)-**1**) to evaluate enantiostability and hydrolysis in conditions similar to those of biological fluid. A classical batchwise kinetic method was used to calculate rate constants of hydrolysis and enantiomerization at the same temperature and in the same solvents used in sf-BD-rHPLC. The good agreement of the results obtained validate the novel procedure developed. Furthermore, the results generated off-line were used to determine the influence of solvents on the racemization of (±)-**1**. *Chirality 23:851–859, 2011.*

KEY WORDS: stopped-flow bidimensional recycle HPLC; chiral; hydrolysis; benzothiadiazines

INTRODUCTION

The evidence is that frequently only one enantiomer is pharmacologically active, whereas the other may be inactive or toxic has lead, over the past two decades, to an even more stringent demand for enantiopure drugs. Since recently national and international organizations such as food and drug administration require extensive stereochemical information on chiral drugs like their enantiomeric stability and enantiomeric purity, different methods have been developed to evaluate configurational and conformational lability of chiral compounds.[1,2]

Literature reports different approaches to evaluate stereochemical integrity like spectroscopic, chirooptical, and chromatographic methods.[3–11] Since several chiral stationary phases (CSPs) are recently commercially available, chromatographic techniques are currently widely used. Three experimental approaches have been exploited to study interconversion process by chromatography: classical batchwise methods combined with enantioselective separations, stopped-flow methods, and dynamic chromatography methods. The first approach is time consuming and cumbersome because it requires isolation of single pure enantiomer followed by an examination of batchwise racemization kinetics. Because dynamic and stopped-flow chromatographic methods do not require single pure enantiomers, they are widely used. If enantiomerization process occurs at the same time-scale of enantioseparation, it is possible to observe a characteristic plateau between the two peaks corresponding to the enantiomers. The height of plateau is proportionally to enantiomerization rate, and by dynamic HPLC (DHPLC), it is possible to evaluate the kinetic parameters of enantiomerization by several methods like the theoretical plate model, the stochastic model, the continuous flow model, peak deconvolution method, approximation function, and unified equation of chromatography.[3–18]

If enantiomerization process does not occur during chromatographic run, it is possible to apply stopped-flow methods in which separated enantiomers are interconverted for a certain time at the desired temperature by stopping the mobile phase flow in the chiral column. Original and interconverted enantiomers are then separated and enantiomerization rate constants are calculated from the corresponding peak areas.[11]

In both dynamic and stopped-flow methods, enantiomerization occurs in the chiral environment of CSP that could exerts stereoselective perturbing effects on the interconversion process. It is important to note that rate constants calculated from dynamic and stopped-flow chromatographic investigations are apparent rate constants ($k_1^{app} \neq k_{-1}^{app}$), which are weighted means of the different enantiomerization rates in the mobile phase and stationary phase.[12,19,20] Efforts have been done to combine the advantages of the on-column chromatographic methods with the option to perform enantiomerization in an achiral environment that does not influence interconversion process rate.

Multidimensional liquid chromatography (MDLC), that uses different column in series, is a high performance separation system that recently is gaining popularity for the separation of complex samples.

Recently, we have developed a stopped-flow multidimensional chromatographic system (sf-MDHPLC) that permits to

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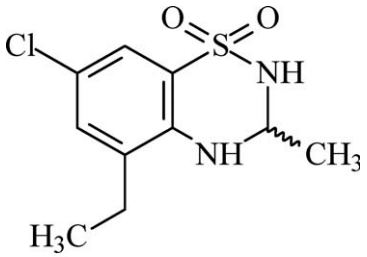


(1)

Fig. 1. Compound investigated.

perform enantiomerization in an achiral environment by using three columns in series: the first and the third column are chiral, and the second column is achiral.[19] After complete separation of the enantiomers in the first chiral column, at conditions where enantiomerization is suppressed, one enantiomer is introduced into the second column using a heart-cut technique. Then, the flow is stopped and enantiomerization is performed at a chosen temperature for selected time. Afterward, the resumed flow of the mobile phase introduces the enantiomers to the third column where they are separated. By this way, it is possible to perform enantiomerization in an achiral environment that does not exert stereoselective perturbing effects. Anyway, sf-MDHPLC method constrains to evaluate enantiomerization rate constants in mobile phase that could exert some influence on process rate, whereas from a pharmaceutical point of view it is important to evaluate stereointegrity of drugs in conditions similar to that of physiological fluids.[14,21,22]

More recently, we have developed an on-column stopped-flow bidimensional recycling HPLC (sf-BD-rHPLC) procedure to obtain an enantiomeric enrichment starting from a racemic mixture.[23,24] The method developed was applied to two chiral compounds of pharmaceutical interest, (±)(*R,S*)-2,3,3a,4-tetrahydro-1*H*-pyrrolo[2,1-c][1,2,4]benzothiadiazine 5,5-dioxide and (±)-7-chloro-3-methyl-3,4-dihydro-2H-1,2,4-benzothiadiazine 1,1-dioxide ((±)IDRA21), because the pharmacological activity of the two benzothiadiazine derivatives investigated has been ascribed to only one enantiomer. Starting from a racemic mixture, it was possible to obtain about 95% of pure enantiomer.

In this work, we have applied configuration system of on-column sf-BD-rHPLC procedure to the aim to conduct enantiomerization not only in achiral environment but also in aqueous solution at different pH similar to physiological conditions using a chiral and an achiral columns in series.

Recently, (±)7-chloro-5-ethyl-3-methyl-3,4-dihydro-2H-benzo[1,2,4]thiadiazine 1,1-dioxide ((±)-**1**) (Figure 1) has been attracted particular attention for its activity as positive allosteric modulators of 2-amino-2,3-dihydro-5-methyl-3-oxo-4-isoxazolepropanoic acid (AMPA) receptor indicating that it could be useful in the treatment of neurodegenerative disorders such as cognitive disorders, schizophrenia, depression, and Parkinson's disease.[25] Moreover, because it has been recently suggested that only *S*-enantiomer of (±)-**1** is the active one on AMPA receptor, it is relevant to develop chromatographic methods to separate the two enantiomers to investigate their enantiomeric stability.[26] Since previous studies have indicated that benzothiadiazine type compounds undergo to rapid enantiomerization in aqueous solutions and hydrolysis in acidic medium, it is important to evaluate stereointegrity and chemical stability of (±)-**1**.[27–31]

Preliminary studies conducted by dynamic chromatography have been demonstrated that (±)-**1** undergoes rapid enantiomerization in aqueous solvent and hydrolysis in acidic mobile phase. sf-BD-rHPLC method was applied to compound (±)-**1** to evaluate enantiomerization and hydrolysis rate constants in conditions similar to physiological fluids.

Moreover, the enantiomerization and hydrolysis kinetic parameters were calculated by off-column method on single pure enantiomer to evaluate matrix effect exerted by achiral stationary phases on both enantiomerization and hydrolysis processes.

EXPERIMENTAL

Instrumentation

The chromatographic apparatus was a Shimadzu LC-10AD Pump (Shimadzu Italia, Milano), a Merck Hitachi L-6200A Pump (Merck KGaA, Darmstadt, Germany), a Rheodyne 7725 manual injector equipped with a 50 μl sample loop. 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. Column temperature regulation was obtained with a Jasco CO-2067 column oven (Jasco Europe, Italy, Milan). The columns used were Chiralcel OD-RH [cellulose tris(3,5-dimethylphenylcarbamate); 150 mm × 4.6 mm I.D.; 5 μm] purchased from Daicel chemical industries, IllKirch, France, Chiraspher NT [poly(*N*-acryloyl-*S*-phenylalanineethylester); 250 × 10 mm I.D.; 5 μm] purchased from Merck, German, Supelcosil LC-18 (250 mm × 4.6 mm I.D.; 5 μm) purchased from Supelco Italy, Milan, and Supelcosil LC-8 (250 mm × 4.6 mm I.D.; 5 μm) purchased from Supelco 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 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. The IR spectra were recorded with an FT-IR spectrophotometer Digilab Scimitar Series FTS 2000. All pH measurements were made using Orion Research EA940 pH-meter.

Synthesis

(±)7-Chloro-5-ethyl-3-methyl-3,4-dihydro-2H-benzo[1,2,4]thiadiazine 1,1-dioxide ((±)-1). The compound was synthesized as previously described by Philips et al.[25]

Yield 70.6%, mp 180–183°C, FTIR 3399, 3223, 2952, 1491, 1437, 1304, 1282 cm^{-1}. ^{1}H NMR (DMSO-d_6) δ = 1.13 (t, 3H, *J* = 7.4 Hz), 1.48 (d, 3H, *J* = 7.1 Hz), 2.52 (m, 2H), 4.80 (m, 1H), 6.24 (s, 1H), 7.21 (d, 1H, *J* = 2.2 Hz), 7.35 (d, 1H, *J* = 2.2 Hz), 7.70 (d, 1H, *J* = 11.8 Hz).

Chromatography

Analytical enantioseparation. Separation of enantiomers of (±)-**1** was carried out isocratically at 0°C on Chiralcel OD-RH column using water:acetonitrile (60:40, v/v) as mobile phase. The compound was dissolved in ethanol and subsequently diluted 1:10 (v/v) with mobile phase at final concentration of 100 μg/ml. The injection volume was 50 μl. The detector was set at 254 nm. HPLC-grade acetonitrile and ethanol were obtained from Baker.

Preparative enantioseparation. Pure (+) and (−) enantiomers were obtained by semipreparative HPLC on Chiraspher NT column with fraction collection of the respective peaks. The mobile phase consisted of *n*-hexane and tetrahydrofuran (THF) 70:30 (v/v). The compound was dissolved in THF at final concentration of 2 mg/ml. The injection volume was 500 μl. The detectors was set at 254 nm. The collected fractions cor-

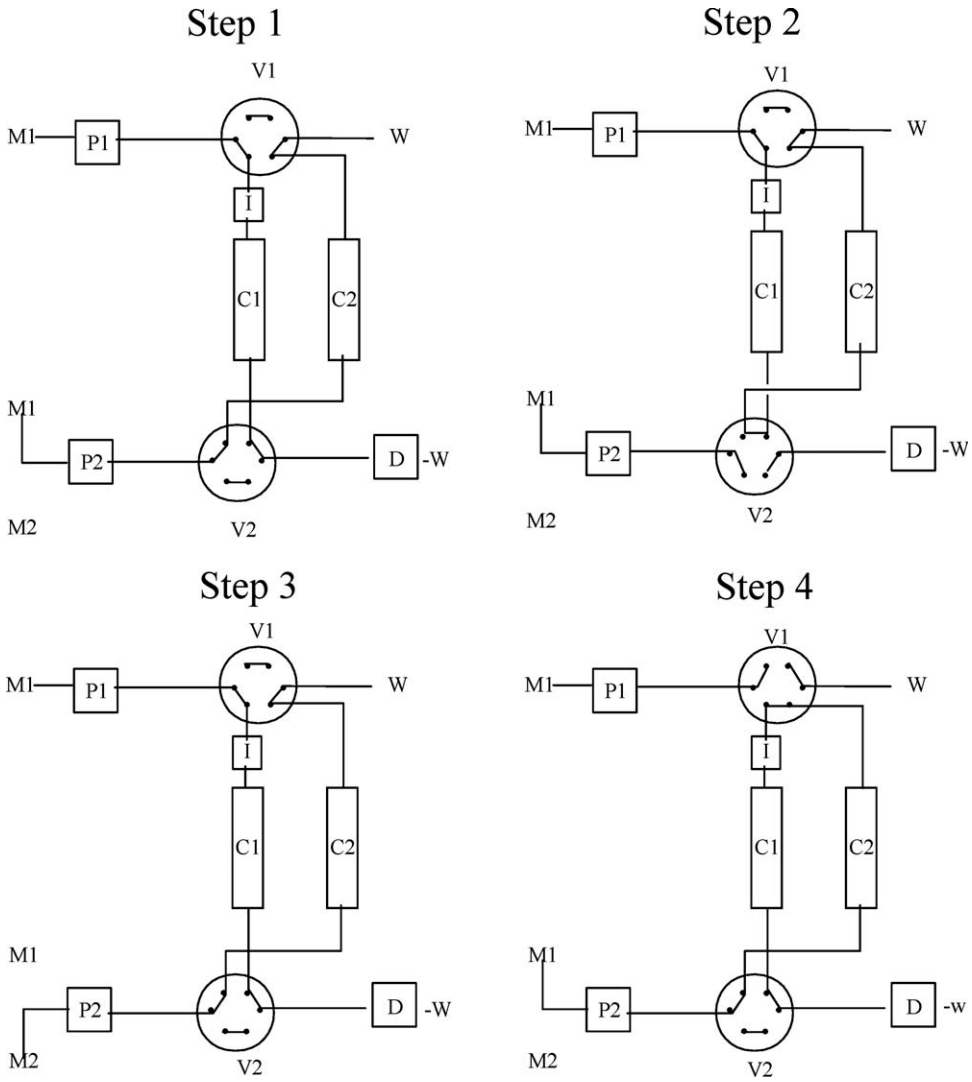


Fig. 2. Schematic represententation of the stopped-flow bidimensional HPLC system. M, mobile phase; P, pump; V1, valve 1; V2, valve 2; I, injector; C1, chiral column; C2, achiral column; W, waste; D, detector. Step 1: Racemic mixture is injected and enantioseparated on the chiral column C1. Step 2: One of the two eluted enantiomers was trapped into the achiral C_{18} column by switching valve 2. Step 3: C2 is filled with different pH buffers by pump 2 and enantiomerization and hydrolysis were performed at different temperatures for a set period of time. Step 4: By switching valve 1, the original mobile phase is reinforced in the achiral column and the enantiomers and hydrolysis product were separated in the first chiral column.

responding 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.

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.

Stopped-Flow Recycling Bidimensional HPLC

The HPLC procedure developed is described in Figure 2. The racemic mixture was injected, and the individual enantiomers were separated on the first chiral column in conditions where hydrolysis and enantiomerization do not take place (Step 1). In this first step, one of the two enantiomers is trapped in the achiral column by switching valve at appropriate time (Step 2). By pump 2, it is possible to fill the achiral column with a selected solvent in which hydrolysis and racemization occur in stopped flow conditions (Step 3). The achiral column and solvent were previously warmed to the desired temperature. After an appropriate time, the original mobile phase was reinforced in column 2 by using pump 2. The mixture originated, consisting in both the two enantiomers and in the hydrolysis product was separated in the chiral column 1 (Step 4).

Off-Column Method

Single enantiomers of (±)-**1**, obtained by semipreparative chromatography, were used to calculate enantiomerization and hydrolysis rates in different solvents. Enriched enantiomers were dissolved in ethanol and subsequently diluted 1:100 (v/v) with buffer (1 ml) at different pH (pH = 1.2, 2.2, 4.2, and 7.4) at final concentration of 10 μg/ml and warmed at 37°C.

Racemization was monitored by chromatography on the Chiralcel OD-RH column by using water:acetonitrile 60:40 (v/v) as mobile phase. Hydrolysis was monitored by chromatography on a supelcosil LC-18 column by using water:acetonitrile (50:50, v/v) as mobile phase. Four repeats of each experiment were made at different times. The peak corresponding to hydrolysis product of (±)-**1** was identified by injecting authentic 3-ethyl-2-aminobenzensulfonamide.

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. Chloroacetate 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 7.40 was prepared by mixing 6.54 ml of 0.02 M KH_2PO_4 with 28.97 ml of 0.01 Na_2HPO_4 and diluting with water to 100 ml.

Evaluation of Kinetic Rate Constants and Free Energy Barriers of Enantiomerization

The kinetic rate constants can be calculated by fitting the data obtained to Eq. 1

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

where k is the rate constant of forward or backward enantiomerization (sec^{-1}), a_o 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 (Eq. 2):

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

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

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 (sec^{-1}), A_o the peak of the (±)-**1** before hydrolysis (=100%), A_t 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).

RESULTS AND DISCUSSION

Chromatography

Enantioseparation of (±)-**1** was conducted with Chiralcel OD-RH column in reversed phase condition using water:acetonitrile (60:40, v/v) as mobile phase. Baseline enantiomeric resolution was obtained performing enantioseparation at 0°C (k_1 = 5.21, k_2 = 6.35, α = 1.22, R_s = 2.23) (Fig. 3). The observed plateau between the two peaks corresponding to the two enantiomers of (±)-**1** at temperatures above 25°C in reversed phase conditions indicates a rapid chiral interconversion during the chromatographic analysis (Fig. 3).

By changing the pH of the mobile phase (buffer pH 2.20:ACN 60:40 (v/v)), a third peak showed up in the chromatogram before the first eluted enantiomer (Fig. 3c).

Because we have recently reported that (±)-7-chloro-3-methyl-3,4-dihydro-2H-1,2,4-benzothiadiazine 1,1-dioxide ((±)IDRA21), a benzothiadiazine structurally related to (±)-**1**, undergoes to rapid enantiomerization and hydrolysis to 2-amino-5-chlorobenzensulfonamide and acetaldehyde in aqueous acidic solvents, it is plausible that (±)-**1** hydrolyzed in 2-amino-7chloro-5-ethylbenzensulfonamide and acetaldehyde in acidic aqueous mobile phase.[30]

Authentic 7-chloro-2-amino-5-ethylbenzensulfonamide was prepared and analyzed under identical experimental conditions used for enantioseparation of (±)-**1**. The retention times of unknown peak and pure 2-amino-7-chloro-5-ethylbenzensulfonamide were identical confirming the identity of hydrolysis product (Scheme 1). Recently, we have proposed a mechanism to explain enantiomerization and hydrolysis of chiral benzothiadiazine type compounds that involves a stereochemical inversion at chiral carbon atom via an imine achiral intermediate that could evolve toward hydrolysis acid catalyzed of imine bond to give the corresponding benzensulfonamide and aldehyde (Scheme 1) (Cannazza et al., submitted).

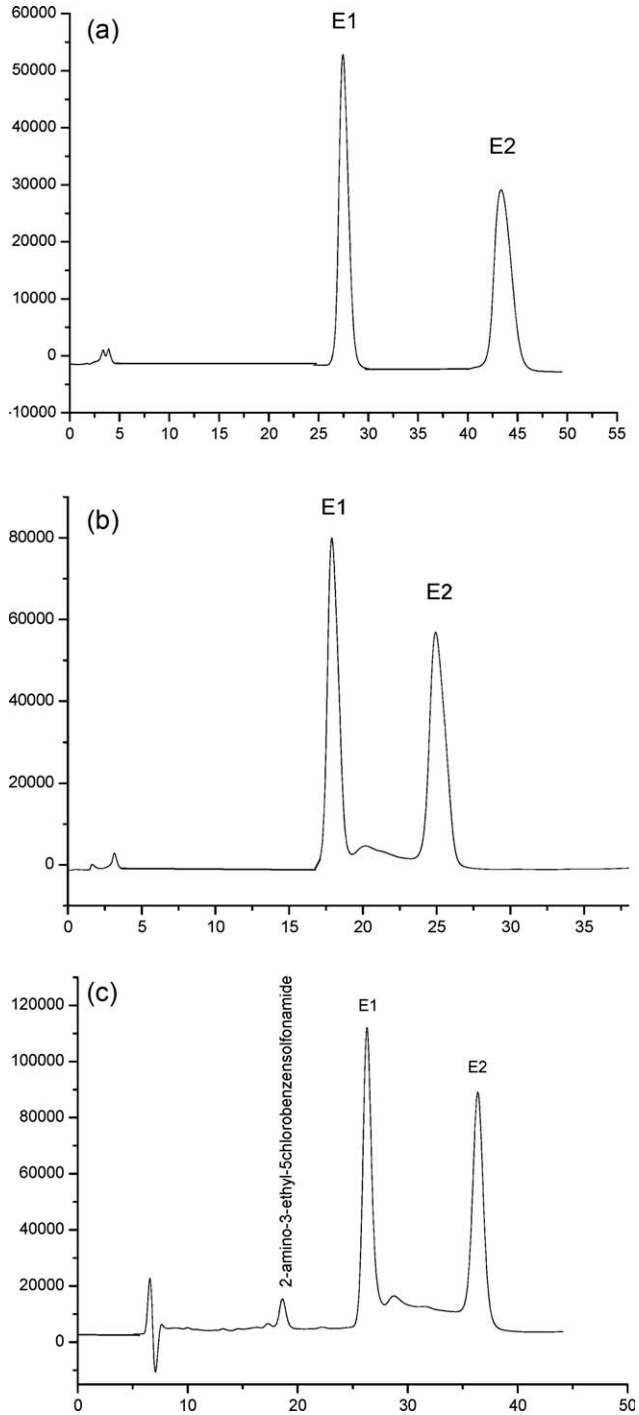
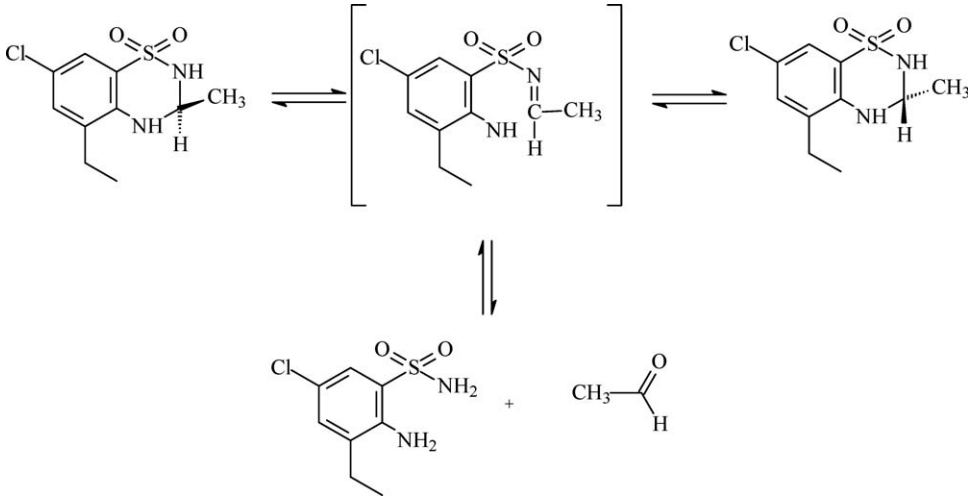


Fig. 3. On-column enantiomerization and hydrolysis of (±)1; flow rate: 0.5 ml/min, Column: Chiracel OD-RH (150 mm × 4.6 mm I.D.; 5 μm); (**a**) water:acetonitrile 60:40 (v/v); T = 0°C, (**b**) water:acetonitrile 60:40 (v/v); T = 37°C, (**c**) buffer pH 2.20 (0.05 M $NaClO_4/HClO_4$): acetonitrile 60:40 (v/v); T = 37°C.



Scheme 1. Proposed enantiomerization and hydrolysis mechanism.

Dynamic Chromatography

By dynamic chromatography, it is possible to investigate kinetics of enantiomerization and hydrolysis of chiral labile compounds like benzothiadiazine type compounds.

Recently, Trapp et al. have been developed the useful software DCXplorer to calculate kinetic constants of first-order processes that occur during chromatographic run by unified equation of chromatography.[16–18,32–38]

The software was herein used to calculate enantiomerization rate constants of (±)-**1** on column Chiralcel OD-RH and water:acetonitrile 60:40 (v/v) or buffer pH 2.20:ACN 60:40 (v/v) as mobile phases at temperature of 15°C and 37°C (Tables 1 and 2).

Moreover, the same software has permitted to determine hydrolysis rate constants of (±)-**1** on C_{18} column. By using water:acetonitrile 60:40 (v/v) or 80:20 (v/v) as mobile phases at temperatures of 15°C and 37°C, it was observed that no hydrolysis of (±)-**1** takes place during chromatographic run. By using buffer pH 2.20:ACN 60:40 (v/v) as mobile phase, the plateau between the hydrolysis peak and (±)-**1** peak is too low to be determine by DCXplorer, suggesting that the presence of organic modifier and/or C_{18} stationary phase influence hydrolysis process rate. Accordingly, hydrolysis rate constant was studied on C_{18} column and buffer pH 2.20:ACN 80:20 (v/v) as mobile phase at temperature of 37°C. In these conditions, it was possible to determine by DCXplorer the kinetic rate constant of hydrolysis ($k_i = 5.5 \times 10^{-4}$ sec^{-1}) indicating that hydrolysis process rate has been influenced by the presence of organic modifier, decreasing with the increasing of percentage of acetonitrile (Table 3).

Because dynamic chromatographic methods constrain calculation of kinetic constants in the mobile phase that usually contains organic modifier in different percentage, the reaction rates obtained could be different from those in pure solvent calculated by off-column methods.

Moreover, the rate constants of enantiomerization obtained by dynamic chromatography investigations are apparent rate constants, which are weighted means of the different enantiomerization rates in mobile phase and stationary phase. If enantioseparation of a labile compound can be accomplished, the forward (k_1^s) and backward (k_{-1}^s) rate constants of enantiomerization may be different in a chiral environment or in the presence of a CSP, respectively, whereas the forward (k_1^m) and backward (k_{-1}^m) rate constants of enantiomerization should be the same in achiral environment of the mobile phase. If k^m is known from independent measurements, k_1^s and k_{-1}^s can be calculated according to the following equations:

$$k_1^{app} = \frac{1}{1 + k^{enant(-)}} k^m + \frac{k^{enant(-)}}{1 + k^{enant(-)}} k_1^s \quad (4)$$

$$k_1^{app} = \frac{1}{1 + k^{enant(+)}} k^m + \frac{k^{enant(+)}}{1 + k^{enant(+)}} k_{-1}^s \quad (5)$$

where $k_{(-)}^{enant}$ and $k_{(+)}^{enant}$ are the retention factors of the (−) and (+) enantiomers, respectively.[14,20]

The rate of (±)-1 enantiomerization in buffer pH 2.20:acetonitrile 60:40 (v/v) and at temperature of 37°C was determined independently by off-column method ($k^m = 2.88 \pm 0.69 \times 10^{-4}$ sec^{-1}) using pure enantiomers collected by semipreparative chromatography on Chiraspher NT.

The k^m value obtained have been used to calculate, by eqs. 4 and 5, k_1^s and k_{-1}^s ($k_1^s = 2.36 \pm 0.23 \times 10^{-4}$ sec^{-1} and $k_{-1}^s = 2.43 \pm 0.15 \times 10^{-4}$ sec^{-1}).

The results confirm that the CSP stabilized the initial state of enantiomers being k^m greater than k^s.

Stopped-Flow Bidimensional HPLC

MDLC is an high performance separation system that is gaining popularity for the separation of complex samples. Two-dimensional liquid chromatography are currently widely using to separate enantiomers of complexed biological samples by coupling achiral-chiral columns. Previously, we have developed an on-column sf-BD-rHPLC procedure to obtain an enantiomeric enrichment starting from a racemic mixture.[24] The configuration system has been used to calculate enantiomerization and hydrolysis rate constants of (±)-**1**.

Figure 2 shows a schematic representation of the sf-BD-rHPLC, in which column C_1 is the column containing the CSP and C_2 is the achiral column that works as reactor. At the beginning of the experiment, the racemic mixture was injected and the individual enantiomers were separated on the first chiral column in conditions where hydrolysis and racemization do not take place. The switching valve 2 located

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TABLE 1. Enantiomerization of (±)-1 performed with sf-BD-rHPLC, off-column, and DCXplorer methods at 37°C

Enantiomerization method	Solvent	k_1^{aapp} (sec^{-1})	k_{-1}^{aapp} (sec^{-1})	k^{m} (sec^{-1})	$k^{c\ app}$ (sec^{-1})	$\Delta G_1^{\ddagger aapp}$ (K J mol^{-1})	$\Delta G_{-1}^{\ddagger aapp}$ (K J mol^{-1})	$\Delta G^{\#m}$ (K J mol^{-1})	$\Delta G^{\#c\ app}$ (K J mol^{-1})
Sf-BD-rHPLC	Buffer pH 2.20	$3.55 \pm 0.35 \times 10^{-3}$[a]	$3.70 \pm 0.01 \times 10^{-3}$[a]			88.97 ± 0.24[b]	88.86 ± 0.07[b]		
	Buffer pH 7.40	$1.42 \pm 0.15 \times 10^{-3}$[a]	$1.93 \pm 0.10 \times 10^{-3}$[a]			91.34 ± 0.26[b]	90.54 ± 0.13[b]		
Off-column	Buffer pH 2.20			$6.92 \pm 0.48 \times 10^{-3}$[c]				87.24 ± 0.18[d]	
	Buffer pH 7.40			$3.42 \pm 0.45 \times 10^{-3}$[c]				89.07 ± 0.32[d]	
DCXplorer	Buffer pH 2.20:ACN (60:40, (v/v))				$0.25 \pm 0.01 \times 10^{-3}$[e]				95.83 ± 0.01[f]
	H_2O:ACN (60:40, (v/v))				$0.19 \pm 0.04 \times 10^{-3}$[e]				96.83 ± 0.01[f]

Columns: Chiralcel OD-RH, Supelcosil LC-18. Column operation temperature 0°C. $n = 4$. Eluent (pump 1): water:acetonitrile 60:40 (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 7.40. Time intervals for enantiomerization at 37.5°C = 5′, 10′, 15′, 20′, 30′.
[a]Apparent rate constants on C_{18} SP (k_1^{aapp}, k_{-1}^{aapp}).
[b]Apparent free energy barriers on C_{18} SP ($\Delta G_1^{\ddagger aapp}$, $\Delta G_{-1}^{\ddagger aapp}$).
[c]Rate constants in mobile phase (k_1^{m}, k_{-1}^{m}).
[d]Free energy barriers ($\Delta G_1^{\ddagger m}$, $\Delta G_{-1}^{\ddagger m}$).
[e]Apparent rate constants on OD-RH CSP ($k^{c\ app}$).
[f]Apparent free energy barriers on OD-RH CSP ($\Delta G^{\#c\ app}$).

TABLE 2. Enantiomerization of (±)-1 performed with sf-BD-rHPLC, off-column, and DCXplorer methods at 15°C

Enantiomerization method	Solvent	k_1^{aapp} (sec^{-1})	k_{-1}^{aapp} (sec^{-1})	k^{m} (sec^{-1})	$k^{c\ app}$(sec^{-1})	$\Delta G_1^{\ddagger aapp}$ (K J mol^{-1})	$\Delta G_{-1}^{\ddagger aapp}$ (K J mol^{-1})	$\Delta G_1^{\ddagger m}$ (K J mol^{-1})	$\Delta G^{\#c\ app}$ (K J mol^{-1})
Sf-BD-rHPLC	Buffer pH 2.20	$3.63 \pm 0.20 \times 10^{-4}$[a]	$4.75 \pm 0.30 \times 10^{-4}$[a]			89.06 ± 0.13[b]	88.41 ± 0.15[b]		
	Buffer pH 7.40	$1.04 \pm 0.05 \times 10^{-4}$[a]	$2.39 \pm 0.19 \times 10^{-4}$[a]			92.09 ± 0.11[b]	90.08 ± 0.19[b]		
Off-column	Buffer pH 2.20			$7.34 \pm 0.69 \times 10^{-4}$[c]				87.35 ± 0.22[d]	
	Buffer pH 7.40			$5.98 \pm 0.23 \times 10^{-4}$[c]				87.85 ± 0.11[d]	
DCXplorer	Buffer pH 2.20:ACN (60:40 (v/v))				$0.62 \pm 0.01 \times 10^{-4}$[e]				93.37 ± 0.02[f]
	H_2O:ACN (60:40 (v/v))				$0.50 \pm 0.05 \times 10^{-4}$[e]				93.87 ± 0.23[f]

Columns: Chiralcel OD-RH, Supelcosil LC-18. Column operation temperature 0°C. $n = 4$. Eluent (pump 1): water:acetonitrile 60:40 (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 7.40. Time intervals for enantiomerization at 15°C = 5′, 10′, 15′, 20′, 30′, 45′, 60′, and 90′.
[a]Apparent rate constants on C_{18} SP (k_1^{aapp}, k_{-1}^{aapp}).
[b]Apparent free energy barriers on C_{18} SP ($\Delta G_1^{\ddagger aapp}$, $\Delta G_{-1}^{\ddagger aapp}$).
[c]Rate constants in mobile phase (k_1^{m}, k_{-1}^{m}).
[d]Free energy barriers ($\Delta G_1^{\ddagger m}$, $\Delta G_{-1}^{\ddagger m}$).
[e]Apparent rate constants on OD CSP ($k^{c\ app}$).
[f]Apparent free energy barriers on OD CSP ($\Delta G_1^{\ddagger capp}$).

TABLE 3. Hydrolysis of (±)-1 performed with sf-BD-rHPLC, off-column, and DCXplorer methods at 37°C

Hydrolysis method	Solvent	k_{E1i}^{aapp} (sec^{-1})	k_{E2i}^{aapp} (sec^{-1})	k_i^m (sec^{-1})	k_i^{capp} (sec^{-1})
Sf-BD-rHPLC	Buffer pH 2.20	$2.92 \pm 0.67 \times 10^{-4a}$	$2.80 \pm 0.44 \times 10^{-4a}$		
	Buffer pH 7.40	$8.01 \pm 0.81 \times 10^{-6a}$	$7.76 \pm 0.96 \times 10^{-6a}$		
Off-column	Buffer pH 1.20			$2.29 \pm 0.11 \times 10^{-3a}$	
	Buffer pH 2.20			$5.15 \pm 0.06 \times 10^{-4a}$	
	Buffer pH 4.20			$1.78 \pm 0.04 \times 10^{-5a}$	
	Buffer pH 7.40			$8.79 \pm 0.07 \times 10^{-6a}$	
DCXplorer	Buffer pH 2.20:ACN (80:20 (v/v))				$5.5 \pm 0.01 \times 10^{-4a,*}$
	H_2O:ACN (80:20 (v/v))				**

Columns Chiralcel OD-RH, Supelcosil LC-18. Column operation temperature at 0°C. $n = 4$. Time intervals for hydrolysis at 37.5°C = 5′, 10′, 15′, 20′, 30′.
[a]Rate constants in C18 column.
*, mobile phase buffer pH 2.20:ACN 80:20 (v/v).
**, any hydrolysis occurred during chromatographic run.

between the two columns is initially set to direct the flow from column 1 through a detector to waste. At the appropriate time by switching the valve two, one of the two enantiomers was trapped into the achiral column that was filled with the selected aqueous buffer by pump 2 (Step 2). Because the aqueous buffer selected as racemization medium has a low eluitropic force, it was possible to retain on C_{18} column the trapped enantiomer. The enantiomerization and hydrolysis were affected by heating at selected temperature column 2 in conditions of stopped-flow. Afterward, the original mobile phase was resumed in column 2 by pump 2 and the valve 1 was switched in position (b), allowing to the mobile phase to run through the chiral column 1. By this way, the enantiomers and hydrolyzed product were introduced into the chiral column where they are separated.

The chromatogram reported in Figure 4 refers to a representative example of sf-BD-rHPLC of compound (±)-**1**: peak 1° arises from the hydrolysis process and peaks 2° and 3° corresponding to the enantiomers of analytes injected, one arise from enantiomer trapped in the achiral column and the other one corresponding to its interconverted product. The elution order was validate by calculating retention times of single enantiomers and hydrolysis product on both column.

The kinetic parameters for both enantiomers (apparent rate constants of pseudo first-order of enantiomerization $^1k_1^{aapp}$ and $^1k_{-1}^{aapp}$ and apparent free energy barrier of forward $\Delta G_1^{\#aapp}$ and backward $\Delta G_{-1}^{\#aapp}$ enantiomerization) were calculated from the corresponding peak areas, from the enantiomerization time and from the enantiomerization temperature as described in the experimental part by fitting the data to eqs. 1 and 2. For both enantiomers, the corresponding plots gave straight lines with reasonable correlation coefficients.

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.[39–41]

By sf-BD-rHPLC, it was possible to study enantiomerization and hydrolysis processes, because the two reactions occur simultaneously in the achiral column under the same stopped-flow conditions.

The hydrolysis apparent rate constants of pseudo first-order for both enantiomers ($^1k_{E1i}^{aapp}$ and $^1k_{E2i}^{aapp}$) were calculated from the residual concentration of the parent racemate and by fitting the time-residual concentration data to a first-order exponential decay (Eq. 3), as reported in the experimental part. For both enantiomers, the corresponding plots gave straight lines with reasonable correlation coefficients.

The enantiomerization and hydrolysis of (±)-**1** were investigated at pH 2.20 and 7.40 and at temperatures of 15°C and 37°C (Tables 1–4).

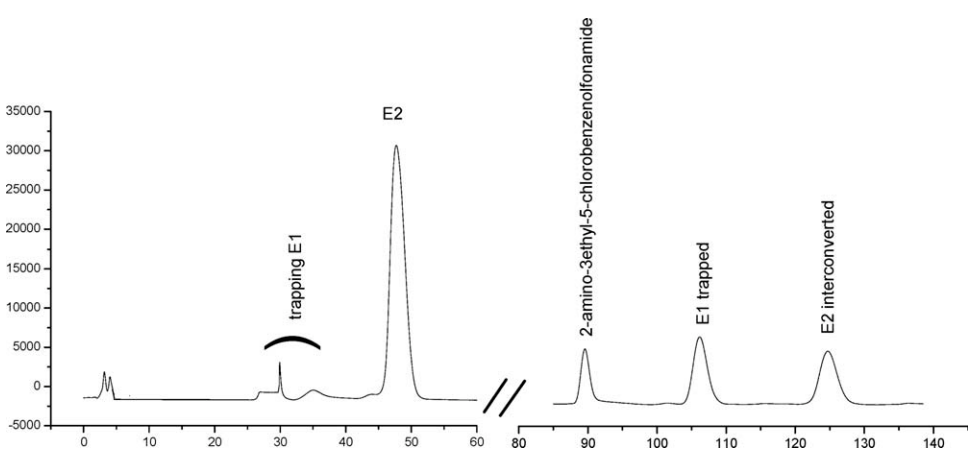

Fig. 4. Chromatogram of enantiomerization and hydrolysis experiment of (±)-1 performed by sf-BD-rHPLC procedure: peak 1° hydrolysis product, peak 2°and 3° corresponding to enantiomers of (±)-1. Columns: chiracel OD-RH (150 mm × 4.6 mm I.D.; 5 μm); Supelcosil LC-18 (250 mm × 4.6 mm ID; 5 μm). Mobile phases: water:acetonitrile 60:40 (v/v); chloroacetate buffer at pH 2.20. Time interval for enantiomerization/hydrolysis at 37.5°C = 20 min. Flow: 0.5 ml/min.

TABLE 4. Hydrolysis of (±)-1 performed with sf-BD-rHPLC and DCXplorer methods at 15°C

Hydrolysis method	Solvent	k_1^{aapp} (sec^{-1})	k_{-1}^{aapp} (sec^{-1})	$k^{c\ app}$ (sec^{-1})
Sf-BD-rHPLC	Buffer pH 2.20	$2.85 \pm 0.31 \times 10^{-5a}$	$2.82 \pm 0.20 \times 10^{-5a}$	
	Buffer pH 7.40	*	*	
DCXplorer	Buffer pH 2.20:ACN (80:20 (v/v))			**
	H_2O:ACN (80:20 (v/v))			**

Columns: Chiralcel OD-RH, Supelcosil LC-18. Column operation temperature 0°C. $n = 4$. Time intervals for hydrolysis at 37.5°C = 5′, 10′, 15′, 20′, 30′, 60′, and 120′.

[a]Rate constants in C18 column.

*, any hydrolysis occurred in 6 h.

**, any hydrolysis occurred during chromatographic run.

The sf-BD-rHPLC method allowed to determine kinetic rate constants of enantiomerization and hydrolysis in an achiral environment, permitting to suppress the perturbing effect of CSP originated from the diastereomeric nature of the transient adduct formed by interaction of the enantiomers with the chiral selector.

As shown in Tables 1 and 2, the apparent kinetic rate constants of pseudo first-order of the forward and backward enantiomerization are quite similar as expected since any stereospecific interactions occurs with stationary phase.

By using a C_{18} column as enantiomerization and hydrolysis reactor, it was possible to change the mobile phase solvent with the desired buffers because aqueous solvent are unable to elute the trapped enantiomer. For pharmaceutical compounds became important to evaluate stereo and chemical-stability in conditions similar to those they will meet in biological fluids when administered in vivo.

Anyway, enantiomerization and hydrolysis occur in C_{18} stationary phase that might not be inert in principle, it can act as promoter or inhibitor agent, increasing or decreasing the enantiomerization and hydrolysis rate constants of the studied compound.

To the aim to evaluate this matrix effect, enantiomerization and hydrolysis rate constants were calculated by classical batchwise off-column method in the same buffers and at the same temperatures used for sf-BD-rHPLC (Tables 1–4).

Comparisons of the kinetic rate constants of enantiomerization and hydrolysis obtained by sf-BD-rHPLC and off-column methods indicate that the processes were significantly faster in mobile phase than in C_{18} column, indicating that stationary phase could exerts an inhibitory effects. The enantiomerization and hydrolysis rate constants determined by sf-BD-rHPLC were performed in the hydrophobic conditions of C_{18} stationary phase that could be decrease the rates of both processes. The influence of polarity on enantiomerization and hydrolysis rate constants have been evaluated by using the less apolar C_8 stationary phase instead of C_{18} stationary phase as achiral column in sf-BD-rHPLC. The rate constants obtained on C_8 column (pH 2.20 and $T = 15$°C) were $5.16 \pm 0.19 \times 10^{-4}$ sec^{-1} and $3.89 \pm 0.20 \times 10^{-5}$ sec^{-1} for enantiomerization and hydrolysis, respectively, that are slight higher than that calculated in the same conditions on C_{18} column indicating that polarity of stationary phase could exert some effects on enantiomerization and hydrolysis rates.

CONCLUSIONS

sf-BD-rHPLC has been successfully applied to calculate enantiomerization and hydrolysis rate constants of stereo- and chemical-labile chiral compounds. It was possible to calculate enantiomerization and hydrolysis rate constants in achiral environment and in selected solvents. The method was applied to the pharmacological active compound (±)-**1** to evaluate its enantio- and chemical-stability in conditions similar to that of body fluid. The data obtained by off-column method were in good agreement with those obtained by sf-BD-rHPLC validating the accuracy and applicability of the novel method developed.

The sf-BD-rHPLC method offers different advantages respect previously developed stopped-flow HPLC system. It allows to determine kinetic rate constants of enantiomerization and hydrolysis in an achiral environment, permitting to suppress the perturbing effect of CSP originated from the diastereomeric nature of the transient adduct formed by interaction of the enantiomers with the chiral selector. It is possible to apply sf-BD-rHPLC method to racemic mixture that is only partial enantioresolved by cutting the peaks at appropriate times. sf-BD-rHPLC system requires only one chiral column.

sf-BD-rHPLC method together with dynamic methods could be a valuable tool in the investigations of stereo and chemical integrity of chiral compounds particularly in the pharmaceutical field where the knowledge of chemical stability is an important prerequisite for the further drug development process.

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