Degradation and configurational changes of thioridazine 2-sulfoxide

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Abstract

Thioridazine (THD) is a phenothiazine neuroleptic drug used for the treatment of psychiatric disorders. After oral administration THD is extensively biotransformed to thioridazine 2-sulfone (THD 2-SO$_2$), thioridazine 5-sulfoxide (THD 5-SO) and thioridazine 2-sulfoxide (THD 2-SO). THD 2-SO and THD 5-SO have two chiral centres and therefore exist as two diastereoisomeric pairs.

The degradation and epimerization of THD 2-SO in human plasma, buffer and methanolic solutions were studied using an enantioselective HPLC method. The samples were prepared by liquid–liquid extraction with diethyl ether and the chiral resolution of the enantiomers was carried out on a Chiralpak AD column using a mobile phase consisting of hexane:ethanol:2-propanol (90:7:3, v/v/v) containing 0.2% diethylamine. The method was validated and used to study the degradation and epimerization under different conditions of incubation. Our results showed that both enantiomers were stable at varying temperatures, pH and ionic strengths; however, solubility problems were observed, mainly at pH 8.5. The influence of light on stability was studied using methanolic solutions and degradation and epimerization of the THD 2-SO enantiomers were observed under UV light of 366 and 254 nm, respectively.
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1. Introduction

Thioridazine (THD), a phenothiazine neuroleptic drug used for the treatment of schizophrenia and other psychiatric disorders [1], is extensively metabolized by side chain oxidation resulting in the formation of thioridazine 2-sulfoxide (THD 2-SO) and thioridazine 2-sulfone (THD 2-SO$_2$) and by ring sulfoxidation leading to thioridazine 5-sulfoxide (THD 5-SO) [8] (Fig. 1). THD is a chiral drug due to an asymmetric carbon at position 2 in the piperidyl ring and the oxidation of ring or side chain sulphur atoms gives rise to an additional chiral centre, therefore, THD 5-SO and THD 2-SO exist in the form of two diastereoisomeric pairs of enantiomers, specified as fast eluting (FE) and slow eluting (SE) according to their chromatographic behavior [11].

Differences observed in the plasma concentrations of THD and its metabolite enantiomers can be explained due to the stereoselective protein binding, distribution volume and metabolism, the last one been the major factor contributing to the expression of stereoselectivity. Pharmacokinetic studies described in the literature show high enantioselectivity in the metabolism of THD and important interindividual variability [9,11]. The cited authors also observed that CYP2D6 is apparently involved in the formation of THD 2-SO and THD 5-SO. However, this enzyme is not responsible for THD 2-SO$_2$ formation [11]. In addition, Siccardi and Kamali [17] studied the in vitro stereoselectivity in THD metabolism, observing significant differences in the formation of THD metabolites when one of its enantiomers or racemic mixture was present in the incubation mixture.

Until recently only three methods have been described in the literature for the enantioselective analysis of THD and its metabolites, all of them based on an achiral sepa-

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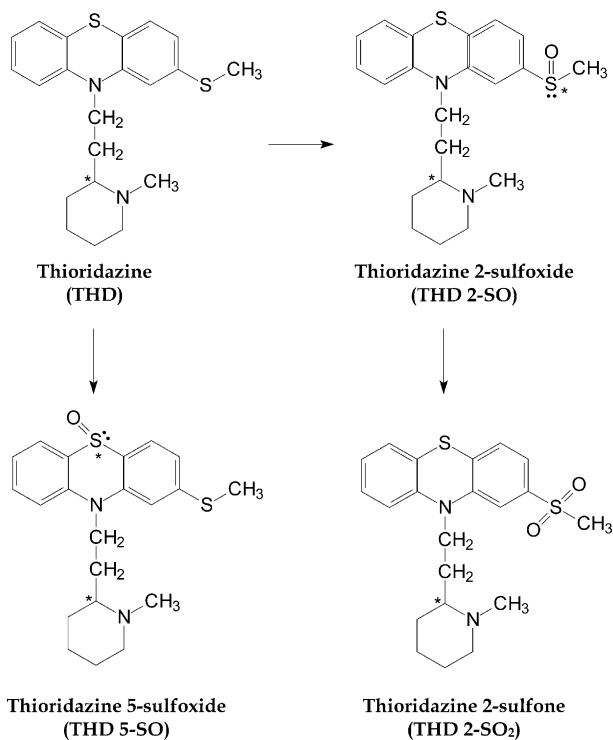*E-mail address:* psbonato@fcfrp.usp.br (P.S. Bonato).

Fig. 1. Thiroidazine metabolism (the chiral centers are indicated by an asterisk).

ration followed by a chiral separation [6,7,9]. One of the difficulties in the development and validation of these methods is the instability of this drug and metabolites under different conditions, mainly when exposed to light. In 1993, Eap et al. [5] studied the influence of indirect sunlight on the stability of THD and its metabolites under different conditions. The authors observed that the kinetics of degradation was very fast in acidic media for all compounds studied; however, degradation occurred also in neutral media for THD 2-SO.

Thus, we have conducted a series of studies to evaluate the influence of sample storage conditions on the degradation and racemization of THD and its metabolites. These studies were carried out for THD and THD 2-SO$_2$ [19] using an HPLC enantioselective method and for THD 5-SO using an enantioselective capillary electrophoresis method [20]. These studies showed decomposition and/or epimerization mainly when the stereoisomers of THD and THD 5-SO were exposed to UV light. Racemization and decomposition of THD 5-SO diastereoisomers when the samples were exposed to direct sunlight or to a UV lamp have also been reported by Eap et al. [3].

To conclude the study on the stability of enantiomers of THD and its metabolites, the present paper describes the development of a method for the analysis of THD 2-SO enantiomers in human plasma and the study of the stability of its stereoisomers under different incubation conditions.

2. Experimental

2.1. Standard solutions and chemicals

Thioridazine 2-sulfoxide was kindly supplied by Novartis Pharma AG. (Basel, Switzerland). Stock standard solutions were prepared in methanol in the concentration range of 2–200 μg/ml and stored at –20 °C in the absence of light.

The solvents used as mobile phase or in the extraction procedure were of HPLC grade (Merck, Darmstadt, Germany or EM Science, Gibbstown, NJ, USA). All other chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany) or Carlo Erba (Milan, Italy).

2.2. Instrumentation and chromatographic conditions

All experiments were performed using a Shimadzu (Kyoto, Japan) liquid chromatography apparatus consisting of an LC 10 AS model solvent pump, a Rheodyne model 7125 injector with a 50 μl loop, an SPD-10 A model variable-wavelength UV detector operating at 262 nm, and a CR6-A model integrator. The chiral resolution of the THD 2-SO enantiomers was carried out using the Chiralpak AD column (250 mm × 4.6 mm i.d., 10 μm particle size, Chiral Technologies, Exton, USA) protected with a 4 mm × 4 mm RP-8 endcapped guard column (Merck). The mobile phase was hexane:ethanol:2-propanol (90:7:3, v/v/v) plus 0.2% diethylamine, at a flow rate of 1 ml/min.

2.3. Extraction

Human plasma samples (1 ml) from healthy volunteers were spiked with standard solutions of THD 2-SO (FE) enantiomers (different concentrations depending on the study). After the addition of 200 μl 4 mol/l sodium hydroxide, 200 μl 4 mg/ml sodium metabisulfite and 4 ml diethyl ether (recently distilled and tested for peroxides) the extraction was performed in a mixer for 1 min. After centrifugation at 1800 × *g* for 5 min, exactly 3 ml of organic phases were recovered and the solvent was evaporated under a nitrogen flow; the residues were dissolved in 100 μl mobile phase, and 50 μl were injected into the HPLC system. The extraction procedure was carried out protecting the solutions from light sources and by maintaining the window blinds closed; in addition, room temperature was set at 20 °C in order to prevent solvent evaporation during sample preparation.

2.4. Calibration curves and method validation

In the stability assays carried out on plasma samples and buffer solutions, the calibration curves used were obtained by analyzing 1.0 ml drug-free human plasma spiked with 25 μl of *rac*-THD 2-SO (FE) standard solutions, prepared in methanol, at concentrations of 2.0, 4.0, 10.0 and 50.0 μg/ml, resulting in plasma concentrations of 25, 62.5, 125 and 625 ng/ml of each enantiomer, respectively. Plots of

plasma concentrations versus peak height were constructed and the linear regression lines were used for determination of enantiomer concentration in plasma samples.

In the evaluation of light stability, the calibrations curves were prepared by direct injection of *rac*-THD 2-SO (FE) solutions in the mobile phase, in the same concentration range.

Linearity of the method was obtained by preparing plasma calibration samples in the concentration range of 25–5000 ng/ml for each enantiomer. The efficiency of the extraction procedure was evaluated by analysing plasma samples ($n = 3$) containing THD 2-SO (FE) in the concentration range of 25–625 ng/ml of each enantiomer (25, 62.5, 125 and 625 ng/ml). The concentrations of these samples were determined on the basis of a calibration curve obtained with the data for the metabolite not submitted to extraction.

The precision and accuracy of the method were evaluated by within-day and between-day assays using plasma samples spiked with THD 2-SO (FE) at the concentrations of 62.5 and 625 ng/ml of each enantiomer and the results obtained were expressed as relative standard deviations (coefficient of variation, CV) and relative error.

2.5. Elution order

The elution order for the THD 2-SO (FE) enantiomers was established based on the method reported by Eap et al. [3,5]. Twenty-five microliters of standard methanolic solution of THD 2-SO were transferred to tubes, the solvent was evaporated under nitrogen and the residues were dissolved in the mobile phase and analysed by HPLC using a Chiralpak AS column and a mobile phase consisting of hexane:ethanol (81:19, v/v) containing 0.2% diethylamine, at a flow rate of 1.0 ml/min. The separated enantiomers of THD 2-SO (FE) were collected at the end of the column, the solvent was evaporated and the residues were analysed according to the conditions described in this paper i.e., the residues were analysed using the Chiralpak AD column and a mobile phase consisting of hexane:ethanol:2-propanol (90:7:3, v/v/v) and 0.2% diethylamine. Under these conditions, the first enantiomer collected corresponded to the (S)-THD 2-SO (FE) and the second enantiomer collected corresponded to the (R)-THD 2-SO (FE).

2.6. Stability studies

The stability studies were carried out using the isolated THD 2-SO (FE) enantiomers. The racemic mixture was re-solved using the Chiralpak AD column and mobile phase of hexane:ethanol:2-propanol (90:7:3, v/v/v) plus 0.2% diethylamine. After several injections and collections, the solvent was evaporated dry under nitrogen and the residues were dissolved in 2 ml methanol. These solutions were stored at –20 °C in the absence of light. The relative concentration of these solutions was determined using a calibration curve of racemic THD 2-SO (FE) not submitted to extraction.

The stability assays were carried out under different incubation conditions as shown in Table 1. In the temperature studies, drug-free human plasma was spiked with the isolated enantiomers of THD 2-SO (FE), whereas to investigate the stability of enantiomers at differents pH values and ionic strength, 2 ml of sodium phosphate buffer spiked with the isolated enantiomers was used.

After the established incubation time, 1 ml aliquots of the spiked plasma samples were removed and submitted to the extraction procedure. In the case of spiked buffer solutions, aliquots of 50 μl were removed and added to tubes containing 1 ml of drug-free human plasma and the extraction procedure for each experimental point was performed.

The plasma extraction procedure was performed in triplicate for each experimental time point and the concentration of these spiked samples was determined using a calibration curve also submitted to the extraction procedure.

The stability of the THD 2-SO (FE) enantiomers was also evaluated at two different wavelengths (254 and 366 nm) and using only visible light. The standard methanolic solution of the enantiomers were exposed to UV and visible light for several periods of time (energy source at 10 cm from the sample). After exposure to light, the solvent was evaporated dry under nitrogen, the residues were dissolved in 100 μl of the mobile phase and 50 μl were injected into the HPLC equipment. To determine the concentration of the enantiomers, a calibration curve was constructed by injecting standard solutions of the racemic mixture prepared in the mobile phase.

2.7. Statistical analysis

Statistical analysis of the effect of temperature, pH, ionic strength and light was performed by one-way ANOVA with the level of significance set at $\alpha = 0.01$. If statistically significant differences were found, Dunnet's Test was used for comparison to the control time ($\alpha = 0.05$) [15]. The difference between the theoretical concentrations used to fortify plasma or buffer samples with TDD-2SO and the real concentration

Table 1
Incubation conditions for the stability studies

	Temperature	pH	Ionic strength	UV and visible light
Concentration (ng/ml)	100.0	100.0	110.0	–
Matrix	Plasma	Sodium phosphate buffer	Sodium phosphate buffer	Methanol
Temperature (°C)	38, 4 and −20	4	4	25
pH	–	5.0, 7.0 and 8.5	7.0	–
Ionic strength (mol/l)	–	0.2	0.2, 0.5 and 1.0	–
Incubation time	0, 1, 2, 3, 7 and 10 days	0, 1, 2, 3, 7 and 10 days	0, 1, 2, 3, 7 and 10 days	0, 1, 2, 3, 4, 5, 6, 7 h

obtained after chromatographic analysis was assessed using Student's *t*-test at $\alpha = 0.01$.

3. Results and discussion

3.1. Method optimization

As already mentioned, THD 2-SO is a chiral compound with two chiral centers, and in this case a total of four stereoisomers is possible. The standard used in this study was a mixture of the FE (86%) and SE (14%) pairs of enantiomers, so we initially tried to resolve the four isomers simultaneanly.

Using the Ultron ES-OVM column, a protein-based chiral stationary phase, we observed the complete resolution of the four isomers of THD 2-SO; however, this column was not selected for our study due to the lack of reproducibility of the separation. Among the several chiral stationary phases based on polysaccharide derivatives, the amylose derived phases (Chiralpak AD and Chiralpak AS columns) resulted in better resolution for THD 2-SO enantiomers than cellulose derived phases [18] due to difference in higher-order structure: a left-handed 3/2 and 4/1 helical structure for cellulose and amylose derivatives, respectively [10].

Using the Chiralpak AD column we obtained only a resolution of the pair (S)-THD 2-SO (FE) and (R)-THD 2-SO (FE) whereas the other pair of enantiomers [(S)-THD 2-SO (SE) and (R)-THD 2-SO (SE)] eluted with the same retention time of (R)-THD 2-SO (FE) and (S)-THD 2-SO (FE), respectively.

Based on previous results obtained for THD 5-SO [20], we expected an epimerization of (R)-THD 2-SO (FE) to (R)-THD 2-SO (SE) and (S)-THD 2-SO (FE) to (S)-THD 2-SO (SE). As a consequence, the resolution of the four isomers was not necessary.

The liquid–liquid extraction was used to isolate THD 2-SO from the plasma matrix. Due to the basic character of THD 2-SO, 200 μl of 4.0 mol/l sodium hydroxide were added to the plasma samples. Among the several solvents evaluated during optimization of the method, higher extraction efficiency was obtained with diethyl ether. In addition, this solvent resulted in a plasma chromatogram free from endogenous interferents. During the extraction procedure, special care was taken with the sealing of tubes and with room temperature because diethyl ether is an extremely volatile solvent. Solvent evaporation during sample workup would introduce errors because we did not use an internal standard. In addition, re-
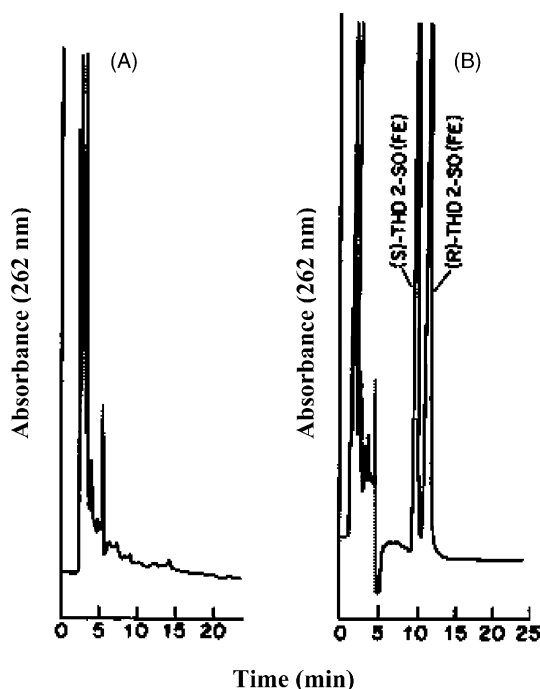

Fig. 2. Chromatogram for the enantioselective analysis of THD 2-SO in plasma: (A) drug-free plasma sample; (B) plasma sample spiked with 625 ng/ml of each enantiomer. Chromatographic conditions: Chiralpak AD column; hexane:ethanol:2propanol:diethylamine (909:7:3:0.2) as mobile phase; flow rate 1 ml/min; detection at 262 nm.

producibility in the extraction was only possible when sodium metabisulfite was added to prevent analyte oxidation. Typical chromatograms obtained after the extraction procedure of drug-free plasma and plasma spiked with 625 ng/ml of THD 2-SO (FE) enantiomers are shown in Fig. 2.

3.2. Method validation

Linear regression analyses were performed by plotting peak height (*y*) versus concentrations (*x*) of the (S)-THD 2-SO (FE) and (R)-THD 2-SO (FE) in the concentration range of 25–5000 ng/ml. Typical correlation coefficients were higher than 0.998 for both enantiomers.

Recoveries of about 100% were obtained for both enantiomers (Table 2). In addition, the coefficients of variation were lower than 10%, attesting to the good repeatability of the extraction procedure [2,12,14].

The analytical precision of the method was expressed as within- and between-day coefficient of variation (CV, %) and

Table 2
Recovery obtained for the developed method

Concentration (ng/ml)	(S)-THD 2-SO (FE)	CV (%)	(R)-THD 2-SO (FE)	CV (%)
25.0	96.4	5.7	93.6	8.3
62.5	99.0	4.5	96.6	3.9
125.0	92.6	0.2	90.6	0.9
625.0	107.0	3.5	106.8	6.4
Mean	98.7	6.2	96.9	7.3

CV: coefficient of variation.

Table 3
Precision and accuracy of the analysis of THD 2-SO (FE) enantiomers

Parameters	(S)-THD 2-SO (FE)		(R)-THD 2-SO (FE)	
Within-day precision				
Concentration (ng/ml)	62.47	643.88	63.11	682.75
n	10	10	10	10
CV (%)	4.1	2.9	5.3	3.0
Between-day precision				
Concentration (ng/ml)	64.73	641.47	65.55	644.54
n	3	3	3	3
CV (%)	3.1	6.5	3.6	8.9
Accuracy				
Within-day (%)	−0.05	+3.0	+0.1	+9.2
Between-day (%)	+3.6	+2.6	+4.9	+3.1

n: number of determination.

Table 4
Stability of THD 2-SO (FE) enantiomers as a function of pH

Time (days)	Concentration (S)-THD 2-SO FE (ng/ml)			Concentration (R)-THD 2-SO FE (ng/ml)		
	pH 5.0[a]	pH 7.0[a]	PH 8.5[a]	pH 5.0[a]	pH 7.0[a]	pH 8.5[a]
0	69.90	77.61	63.44	58.43	60.78	55.95
1	69.45	72.16	68.14	71.28	70.88	[b]
2	66.55	71.51	70.32	59.40	79.69	74.11[c]
3	71.68	77.19	72.10[c]	72.94	72.09	64.33
7	70.21	86.40	77.86[c]	71.93	73.23	73.85[c]
10	78.87	91.11	88.37[c]	83.00	86.68	80.82[c]

[a] Statistically significant difference between all values analysed and the theoretical concentration (100 ng/ml) (Student's t-test).
[b] Lost sample.
[c] Statistically significant difference between incubation and zero time (Dunnet's test).

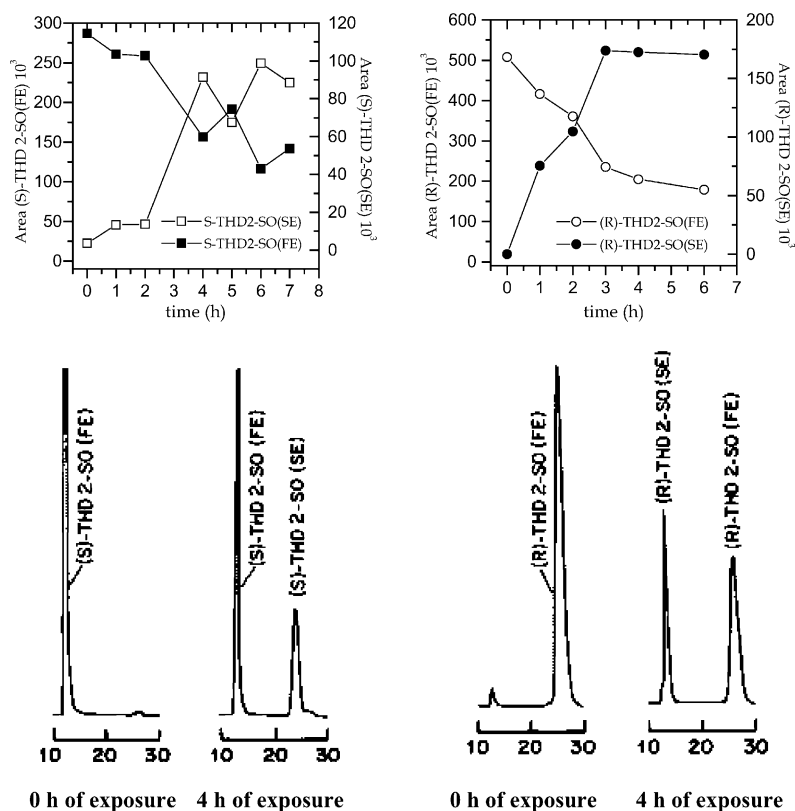0 h of exposure   4 h of exposure   0 h of exposure   4 h of exposure

Fig. 3. Configurational changes of THD 2-SO (FE) enantiomers at 254 nm wavelength. Chromatographic conditions were the same as in Fig. 2.
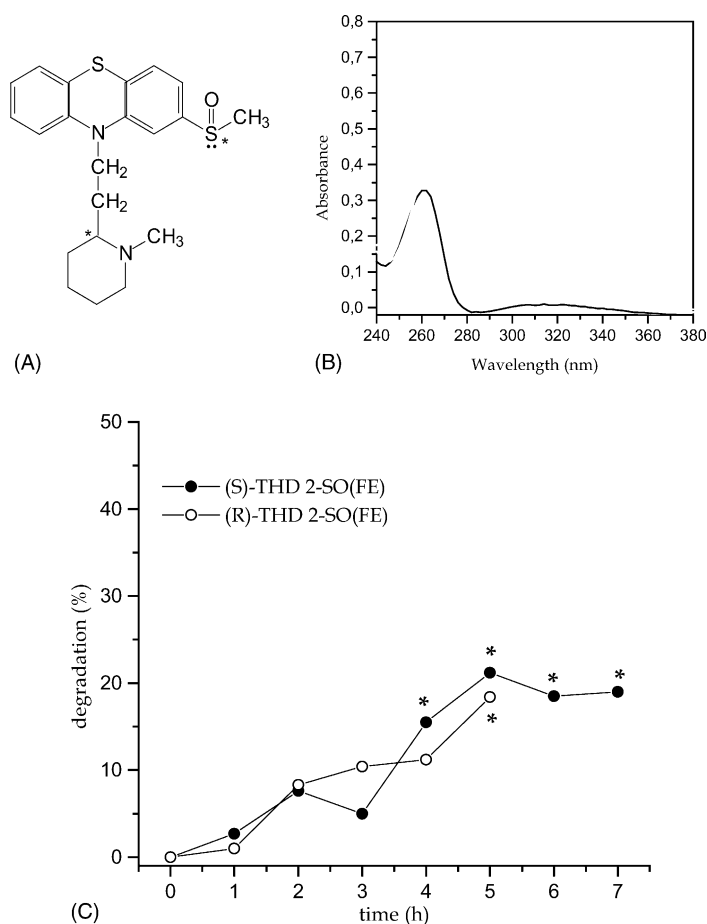
Fig. 4. (A) Molecular structure of thioridazine 2-sulfoxide (THD 2-SO) (the chiral centres are indicated by an asterisk); (B) Electronic spectrum of THD 2-SO in methanol; (C) Influence of UV light (366 nm) on stability of THD 2-SO (FE) enantiomers; (*) significant difference with respect to zero time for $P < \alpha = 0.01$. Chromatographic conditions were the same as in Fig. 2.

the accuracy of the method was expressed as % relative error. The within-day ($n = 10$) and between-day ($n = 3$) precision and accuracy of the assay (Table 3) were determined by analyzing the spiked plasma samples with THD 2-SO (FE) at concentrations of 62.5 and 625 ng/ml. At both concentration levels evaluated, the coefficients of variation and relative error were below 10% [4,14].

3.3. Stability studies

Table 4 shows the plasma concentrations for THD 2-SO (FE) enantiomers at the three pH values studied as a fuction of incubation time. Statistical analysis showed that there was significant difference between some values analysed at the different periods of time and zero time. In addition, comparison of these values with the theoretical concentration (100 ng/ml) showed a statistically significant difference, for $\alpha = 0.01$, for all pH values studied. These results confirm those obtained in a recent study reported by our group for THD [8]. In the previous investigation we studied the influence of some parameters on stability of THD and THD 2-SO$_2$ enantiomers and observed that both enantiomers of THD and THD 2-SO$_2$ were stable at varying temperatures, pH and ionic

strengths; however, solubility problems for THD and THD 2-SO$_2$ enantiomers were observed, mainly at pH 8.5. THD and its metabolites are basic compounds and, depending on the pH, they are either in the protonated form or not. When pH is increased from 5.0 to 8.5, the non-protonated form is more likely to be present, resulting in a further impairment of its solubility in the buffer solution.

No degradation or configurational changes of THD 2-SO were observed for the experiments carried out by changing the temperature and ionic strength (results not shown). In 1993, Eap et al. [5] observed a significantly reduction in the peak area for plasma samples spiked with THD 2-SO but in that study, the samples were exposed to indirect sunlight.

The stability and configurational changes of THD 2-SO enantiomers when exposed to UV (254 and 366 nm) and visible (fluorescent bulb) light were evaluated using standard solutions prepared in methanol. The samples were exposed to light for several periods of time and enantiomer concentration was determined based on the direct injection of standard racemic solutions of each analyte in the mobile phase.

Fig. 3 shows the degradation profiles and configurational changes of THD 2-SO (FE) when exposed to the action of UV light (254 nm). The degradation was inferred because the

sum of the peak areas obtained after light exposure was lower than the initial area of each enantiomer (i.e., area (S)-THD 2-SO (FE) + area (S)-THD 2-SO (SE) after exposure to UV light < area (S)-THD 2-SO (FE)). However, no extra peaks were observed in the chromatogram.

At 366 nm wavelength, only degradation of THD 2-SO (FE) enantiomers was observed (Fig. 4C). This behavior can be explained based on the structure of the molecule and absorption spectrum. Fig. 4B shows an intense band of absorption at 260 nm due to an aromatic ring (transition $\pi \rightarrow \pi^*$) and another less intense band at 315 nm due to the presence of sulfur and nitrogen atoms ($n \rightarrow \pi^*$ transition) [13]. Diaryl or alkyl aryl sulfoxides are known to have a pyramidal structure and to undergo racemization at about 200 °C (thermal pyramidal inversion) or photoinversion reaction. The reaction pathway of photoinversion has not been elucidated, but some mechanisms have been described [16]. In our case, since the molecule showed a peak of maximum absorption at 260 nm, when the molecule was excited at a wavelength near this value (254 nm), the energy absorbed was sufficient to promote the reaction of photoinversion. For the excitation wavelength of 366 nm, the absorptivity of the molecule was low, however sufficient to cause photodegradation. The photoinversion reaction occurred with higher efficiency than photolysis, probably due to the existence another reaction route for photoinversion [16].

As far as visible light is concerned, we did not observe degradation or configurational changes of THD 2-SO (FE) enantiomers, a fact that can be explained by the low energy intensity provided by visible light.

Based on these results, we suggest that all analyses of THD 2-SO should be performed protected from UV light sources, whereas other factors such as pH, temperature and ionic strength do not affect its stability.

4. Conclusions

In this paper we described a simple and reproducible method for the determination of THD 2-SO (FE) enantiomers in plasma. In addition, studies on the stability of THD 2-SO (FE) enantiomers were carried out by changing the incubation conditions as a function of time. Degradation or configurational changes were not observed in the study of temperature (38, 4 and −20 °C), pH (5.0, 7.0 and 8.5) and ionic strength (0.2, 0.5 and 1.0 mol/l), however, solubility problems at the pH values studied were observed. Degradation was observed when the enantiomers of THD 2-SO (FE) were exposed to 366 nm UV light and degradation and configurational changes were observed when the enantiomers were exposed to 254 nm UV light.

Acknowledgments

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