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Stereoselective analysis of thioridazine-2-sulfoxide and thioridazine-5-sulfoxide: An investigation of *rac*-thioridazine biotransformation by some endophytic fungi

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Abstract

The purpose of this study was to develop a method for the stereoselective analysis of thioridazine-2-sulfoxide (THD-2-SO) and thioridazine-5-sulfoxide (THD-5-SO) in culture medium and to study the biotransformation of *rac*-thioridazine (THD) by some endophytic fungi. The simultaneous resolution of THD-2-SO and THD-5-SO diastereoisomers was performed on a CHIRALPAK® AS column using a mobile phase of hexane:ethanol:methanol (92:6:2, v/v/v) + 0.5% diethylamine; UV detection was carried out at 262 nm. Diethyl ether was used as extractor solvent. The validated method was used to evaluate the biotransformation of THD by 12 endophytic fungi isolated from *Tithonia diversifolia*, *Viguiera arenaria* and *Viguiera robusta*. Among the 12 fungi evaluated, 4 of them deserve prominence for presenting an evidenced stereoselective biotransformation potential: *Phomopsis* sp. (TD2) presented greater mono-2-sulfoxidation to the form (S)-(SE) (12.1%); *Glomerella cingulata* (VA1) presented greater mono-5-sulfoxidation to the forms (S)-(SE) + (R)-(FE) (10.5%); *Diaporthe phaseolorum* (VR4) presented greater mono-2-sulfoxidation to the forms (S)-(SE) and (R)-(FE) (84.4% and 82.5%, respectively) and *Aspergillus fumigatus* (VR12) presented greater mono-2-sulfoxidation to the forms (S)-(SE) and (R)-(SE) (31.5% and 34.4%, respectively).

Keywords: Thioridazine; Thioridazine-2-sulfoxide; Thioridazine-5-sulfoxide; Biotransformation; Endophytic fungi

1. Introduction

Microorganisms have been used to produce chemicals, pharmaceuticals and perfumes for decades [1,2] and also for pollutants degradation and recovery of the environment contaminated by chemicals [3]. The use of microorganisms can simplify or in some instances even enable the production process of complex chemicals and drug intermediates. In addition, they can add stereospecificity to the process, eliminating the need for complicated separation and purification steps. However, the use of microorganisms by the industry to obtain interesting compounds is still modest, considering the great availability of useful microorganisms and the large scope of reactions that can be accomplished by them.

Another interesting use of microorganisms is for studying biotransformation of drugs and other chemicals such as pesticides. The microbial biotransformation system is very similar to the mammalian phase I metabolic reactions. Therefore, these *in vitro* models can be an attractive alternative for tests of new drugs, making possible the production of metabolites in high amounts, facilitating the structural elucidation and toxicological tests [4].

Smith and Rosazza, in the early 1970's, established the use of microbial models for mammalian metabolism [5,6]. It has been demonstrated that some fungi can generate a similar metabolic profile to mammalian and that the metabolism is also stereoselective [7]. Since them some studies have been reported

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describing the use of fungi for the biotransformation of drugs [8–10].

A special kind of fungi are the endophytic fungi, microorganisms that colonize and grow asymptomatically within internal plant tissues without causing harm to their host [11]. To present date, there are few studies using endophytic fungi in stereoselective biotransformation. Agusta et al. [12] reported the stereoselective oxidation at C-4 of flavans by the endophytic fungus *Diaporthe* sp. isolated from a tea plant *Camelia sinensis*.

Thioridazine (THD), a phenothiazine neuroleptic drug used for the treatment of schizophrenia and other psychiatric disorders, is commercially available as a racemic mixture of the enantiomers (−)-(S) and (+)-(R). It is extensively metabolized by *S*-oxidation to thioridazine-5-sulfoxide (THD-5-SO) and thioridazine-2-sulfoxide (THD-2-SO) which is further oxidized to thioridazine-2-sulfone (THD-2-SO_2) [13,14,15]. THD-2-SO and THD-2-SO_2 are metabolites considered pharmacologically active while THD-5-SO contributes to the cardio toxicity of the drug [16,17]. A summary of *rac*-thioridazine's metabolic profile in humans is demonstrated in Fig. 1. THD-2-SO and THD-5-SO exist as two diastereoisomeric pairs, referred to fast-eluting (FE) THD-2-SO and slow-eluting (SE) THD-2-SO, and fast-eluting THD-5-SO and slow-eluting THD-5-SO, respectively, based on their chromatography properties [18,19]. The (R) and (S) configurations of THD and their metabolites are related to the chiral carbon at position 2 in the piperidyl ring [19].

Considering that THD metabolites are also therapeutically active, but with undesirable side effects that can be attributed to specific stereoisomers, the study of its metabolism by endophytic fungi can be useful to obtain THD metabolites in the enantiomerically pure form and further accomplishment of clinical and/or toxicological studies.

Therefore, the aim of the present work was to develop a method for the stereoselective analysis of THD-2-SO and THD-5-SO in culture medium and to study the biotransformation of *rac*-thioridazine (THD) by some endophytic fungi. Although the stereoselective analyses of THD and/or its metabolites have already been described in the literature, none of the methods reported allows the simultaneous resolution of THD-2-SO and THD-5-SO stereoisomers [18,20–22].

2. Experimental

2.1. Standard solutions and chemicals

rac-thioridazine, thioridazine-2-sulfoxide and thioridazine-5-sulfoxide were kindly supplied by Novartis Pharma AG (Basel, Switzerland). Stock standard solutions were prepared in methanol at concentrations of 2.00, 4.00, 10.0, 20.0, 40.0, 80.0, 200 and 400 μg mL^{-1} and were stored at −20 °C in the absence of light. It is important to mention that the drugs used in the present work consist of the FE pair of enantiomers for THD-2-SO (racemic mixture of (R)-THD-2-SO (FE) and (S)-THD-2-SO (FE)) and of the SE pair of enantiomers for THD-5-SO (racemic mixture of (R)-THD-5-SO (SE) and (S)-THD-5-SO (SE)). HPLC-grade hexane, methanol, diethyl ether were purchased from Mallinckrodt Baker Inc. (Paris, France) and ethanol from Merck (Darmstadt, Germany). All other chemicals were of analytical-grade in the highest purity available. Water was distilled and purified using a Millipore Milli Q Plus system (Bedford, USA).

2.2. Instrumentation and analytical conditions

The analyses were carried out using a Shimadzu (Kyoto, Japan) HPLC system, consisting of a LC 10 AS model solvent pump, a Rheodyne model 7125 injector with a 50 μL loop, a SPD-10 A UV–vis detector operating at 262 nm and a CR6-A model integrator. The simultaneous resolution of THD-2-SO and THD-5-SO stereoisomers was performed on a CHIRALPAK® AS 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, Darmstadt, Germany), using a mobile phase consisting of hexane:ethanol:methanol (92:6:2, v/v/v) and 0.5% diethylamine at a flow rate of 1.4 mL min^{-1}.

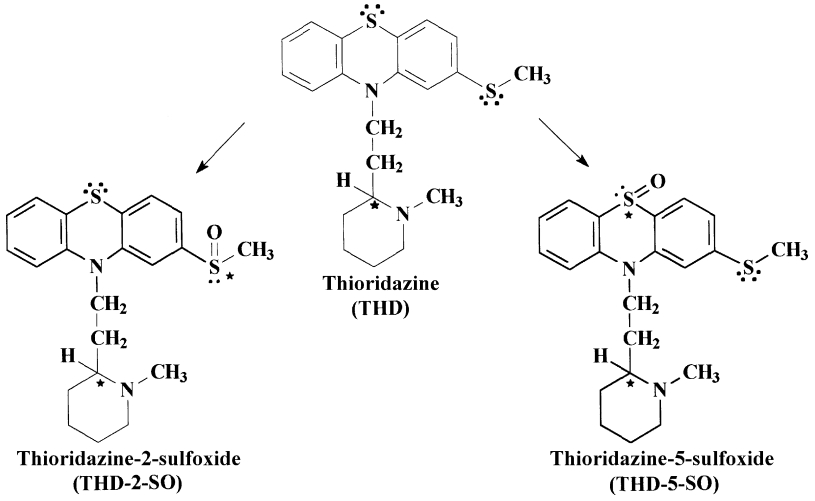

Fig. 1. Molecular structure of thioridazine, thioridazine-2-sulfoxide and thioridazine-5-sulfoxide (the chiral centers are indicated by an asterisk).

The analyses were carried out in an acclimatized room with the temperature set at 23 (±2) °C.

2.3. Extraction procedure

During the extraction procedure, some special care was taken: the solutions were protected from light sources by maintaining windows blinds closed to prevent photo degradation [20,21,22] and the room temperature was set at 20 °C in order to prevent solvent evaporation during sample preparation. Aliquots of 1 mL of medium (see 2.6 item) spiked with 25 μL of standard solutions of THD-2-SO and THD-5-SO (or samples obtained in the biotransformation process) were alkalinized with 200 μL 4 mol L^{-1} sodium hydroxide solution and extracted with 3 mL diethyl ether. The tubes were capped and submitted to vortex mixing for 2 min and then centrifuged at 1800 × *g* for 5 min. The upper organic phases (2 mL) were transferred to conical tubes and evaporated under nitrogen. The residues were dissolved in 100 μL of the mobile phase and 50 μL were chromatographed.

2.4. Method validation

Calibrations curves were obtained by spiking aliquots of 1 mL medium with standard solutions of *rac*-THD-2-SO (FE) and *rac*-THD-5-SO (SE), prepared in methanol, in the range of 2.00–400 μg mL^{-1}, resulting in concentrations of 25.0–5000 ng mL^{-1} for each enantiomer. No internal standard was used in this method.

To determine the extraction recovery, medium samples (1 mL) were spiked with THD-2-SO (FE) and THD-5-SO (SE) enantiomers in the concentrations of 125, 500 and 2500 ng mL^{-1} for each enantiomer ($n = 3$) and submitted to the extractor procedure. Another set of samples were prepared extracting 1 mL aliquots of medium and them spiking the extract with the same amount of THD-2-SO (FE) and THD-5-SO (SE) enantiomers. The recovery was determined by comparing the areas obtained before and after extraction and was expressed as percentage of the amount extracted.

The detectability of the method was evaluated by determining the quantification limit (LOQ). The LOQ was defined as the lowest stereoisomer concentration that could be determined with accuracy and precision below 20% [23] over five analytical run and it was obtained using medium samples (1 mL, $n = 5$) spiked with concentrations of 25.0 ng mL^{-1} of each enantiomer.

The precision and accuracy of the method were evaluated by within-day ($n = 5$) and between-day ($n = 3$) assays using medium samples spiked with THD-2-SO (FE) and THD-5-SO (SE) at the concentrations of 125, 500 and 2500 ng mL^{-1} of each stereoisomer. The results obtained were expressed as coefficient of variation (CV, %) and relative error (E, %).

The selectivity of the method was evaluated by analyzing sterile medium, sterile medium added of *N*,*N*-dimethylformamide and endophytic fungal mycelium under the conditions previously established. We have already studied the stability of thioridazine and its metabolites in previous studies [20,21,22]. Among the parameters studied (pH, temperature and light), the exposition to light was the only one that resulted in epimerization of THD-5-SO and THD-2-SO. So in the present study, all experiments were carried out in the light absence.

2.5. Elution order for THD-2-SO and THD-5-SO enantiomers

The elution order for THD-2-SO (FE) and THD-5-SO (SE) stereoisomers was established based on the method reported by Eap et al. [18,24]. Twenty-five microliters of standard methanol solutions of THD-2-SO (FE) and THD-5-SO (SE) were transferred to tubes, the solvent was evaporated under nitrogen and the residues were dissolved in the mobile phase and analyzed by HPLC. To obtain the other stereoisomers (THD-2-SO (SE) and THD-5-SO (FE)), the tubes with standard methanol solutions of THD-2-SO (FE) and THD-5-SO (SE) were exposed to UV light (254 nm) during 4 h [20,21,22]. A CHIRALPAK® AS column and a mobile phase consisting of hexane:ethanol (81:19, v/v) containing 0.2% diethylamine, at flow rate of 1.0 mL min^{-1} [18,24] were used for the resolution of THD-2-SO (FE) stereoisomers. For THD-5-SO (SE) stereoisomers, a Chiralcel OD column and a mobile phase consisting of hexane:ethanol (85:15, v/v) and 0.05% diethylamine, at a flow rate of 1.0 mL min^{-1} were used [18]. The separated stereoisomers of THD-2-SO and THD-5-SO were collected at the end of the columns, the solvent was evaporated and the residues were analyzed according to the conditions described in the present paper.

2.6. Endophytic fungi isolation and THD biotransformation

The fungi utilized in these experiments were previously isolated as endophytes from the plants *Tithonia diversifolia*, *Viguiera robusta* and *Viguiera arenaria* (Asteraceae). Cultures of endophytes were maintained on potato dextrose agar slants and store at 4 °C. The strains have been deposited in the "Laboratório de Enzimologia Industrial—FCFRP/USP". An initial screening of twelve strains of endophytic fungi was performed to evaluate if they were able to produce mammalian metabolites of THD, allowing us to select four of them were selected for this study. The selected strains were *Phomopsis* sp. (TD2), isolated from *T. diversifolia*, *Glomerella cingulata* (VA1), isolated from *V. arenaria*, *Diaporthe phaseolorum* (VR4) and *Aspergillus fumigatus* (VR12) both isolated from *V. robusta*. All fungi were identified utilizing molecular biology techniques (data not shown).

The fungi were cultured using a two-step process. First, suspension mycelium was aseptically inoculated into 125 mL Erlenmeyers flasks containing 25 mL of seed medium [25]. The flasks were incubated for 2 days (48 h) at 30 °C on a rotary shaker (New Brunswick Scientific Co., Inc., model INNOVA™ 4300, New Jersey, USA) operating at 120 rpm (preculture). The resulting mycelia were harvested, rinsed and transferred into 125 mL Erlenmeyer flasks containing 50 mL of Czapek medium [26] adjusted to pH 5.0 and then 1 mg of THD dissolved in 100 μL of *N*,*N*-dimethylformamide was added to each flask. Control flasks consisted of culture broth without THD and fungi, only sterile medium with *N*,*N*-dimethylformamide, sterile medium with *N*,*N*-dimethylformamide and THD, sterile

medium with *N*,*N*-dimethylformamide and endophytic fungal mycelium (each endophytic fungus studied). Biotransformation experiments were carried out at 30 °C, with shaking at 120 rpm for additional 48 h in light absence. Aliquots of the filtrates (1 mL) were submitted to extraction procedure and analyzed by chiral HPLC.

3. Results and discussion

3.1. Method validation

The chiral resolution of THD-2-SO and THD-5-SO has already been studied by our group. De Gaitani et al. [21] reported a method for the stereoselective analysis of THD-5-SO in human plasma by capillary electrophoresis using hydroxypropyl-β-cyclodextrin and sulfated-β-cyclodextrin as chiral selectors. Further, the degradation and epimerization THD-2-SO in human plasma, buffer and methanolic solutions were studied using an enantioselective HPLC method with a Chiralpak AD [22]. Unfortunately, these methods are suitable only for the resolution of the isolated metabolites. The analysis of THD and/or its metabolites has also been discussed by other groups, using sequential achiral and chiral separations, with the peaks manually collected [18,27,28]. We have also observed that among the several chiral stationary phases based on polysaccharide derivatives, the amylose derived phases resulted in better resolution for THD-2-SO and THD-5-SO stereoisomers [18,24,29]. So, a CHIRALPAK® AS column, an amylose-based chiral stationary phase (tris ((S)-α-methylbenzylcarbamate derivative of amylose)), was evaluated for the simultaneous resolution of THD-2-SO and THD-5-SO stereoisomers. The optimized chromatographic conditions are described on Table 1 as well as the obtained chromatographic parameters. Under these chromatographic conditions THD elutes at 2.0 min and THD-2-SO_2, another THD metabolite, elutes at 13.4 min and do not interfere with the analysis of THD-2-SO and THD-5-SO stereoisomers. Fig. 2A shows the chromatograms referring to the separation of THD-2-SO (FE) and THD-5-SO (SE). Fig. 2B shows the chromatograms referring to the separation of THD-2-SO and THD-5-SO exposed to UV light. In addition, the chromatogram of THD-5-SO stereoisomers in the chiral column showed only three peaks due to coelution of (R)-THD-5-SO (SE) and (S)-THD-5-SO (FE).

The validation of the method was performed only for THD-2-SO (FE) and THD-5-SO (SE) enantiomers because the other pairs of enantiomers (THD-2-SO (SE) and THD-5-SO (FE)) were not available. The calibration curves were linear over the concentration range of 25.0–5000 ng mL^{-1} for (R)-THD-2-SO (FE), (S)-THD-2-SO (FE), (R)-THD-5-SO (SE) and (S)-THD-5-SO (SE), with correlation coefficients (r) ≥0.9998 (Table 2). The lowest concentration quantified by the validated method was 25.0 ng mL^{-1} with coefficient of variation and relative errors lower than 10% and 15%, respectively (Table 2). Recoveries were around 100% for THD-2-SO and THD-5-SO stereoisomers with coefficients of variation lower than 15% (Table 3). Within-day ($n = 5$) and between-day ($n = 3$) precision and accuracy presented coefficients of variation and relative errors lower than 10%, respectively (Table 4). In spite of not using an internal standard, the precision results were acceptable. All fungi evaluated did not produce any secondary metabolites that presented retention times close to those of THD-2-SO and THD-5-SO stereoisomers (Fig. 3).

3.2. Biotransformation of THD in mammalian metabolites by endophytic fungi

The percentage of metabolites production was calculated considering that 1 mg of THD was added in 50 mL of culture medium (20,000 ng mL^{-1}), resulting in 10,000 ng mL^{-1} of each enantiomer, (R)-THD and (S)-THD. As mentioned previously, the validation of the method was carried out only for the THD-2-SO (FE) and THD-5-SO (SE) enantiomers pairs. The concentrations for the forms THD-2-SO (SE) and THD-5-SO (FE) were based on the linear equation of the adjacent peaks. So, the concentration of (R)-THD-2-SO (SE) (peak 2) was determined using the calibration curve of (S)-THD-2-SO (FE) (peak 1) and for the quantification of (S)-THD-2-SO (SE) (peak 3), the calibration curve of (R)-THD-2-SO (FE) (peak 4) was used. For THD-5-SO (SE), the calibration curve of (R)-THD-5-SO (SE) (peak 5) was used to quantify (R)-THD-5-SO

Table 1
Chromatographic parameters for the chiral separation of THD-2-SO and THD-5-SO stereoisomers on CHIRALPAK® AS column; mobile phase: hexane:ethanol:methanol (92:6:2, v/v/v) + 0.5% DEA; flow rate of 1.4 mL min^{-1}; detection at 262 nm

Peaks	1	2	3	4	5	6	7
Rs		1.00	4.67	0.75	1.40	1.27	1.27
k	4.00	4.50	8.00	8.75	10.50	12.25	14.00
α		1.13	1.78	1.09	1.20	1.17	1.14

Rs, resolution; k, retention factor for the first eluted stereoisomer ($t_M = 1.94$ min, defined as the first significant baseline disturbance, corresponding to the retention time of a non-retained solute); α, separation factor. (1) (S)-THD-2-SO (FE), (2) (R)-THD-2-SO (SE), (3) (S)-THD-2-SO (SE), (4) (R)-THD-2-SO (FE), (5) (R)-THD-5-SO (SE) + (S)-THD-5-SO (FE), (6) (S)-THD-5-SO (SE) and (7) (R)-THD-5-SO (FE).

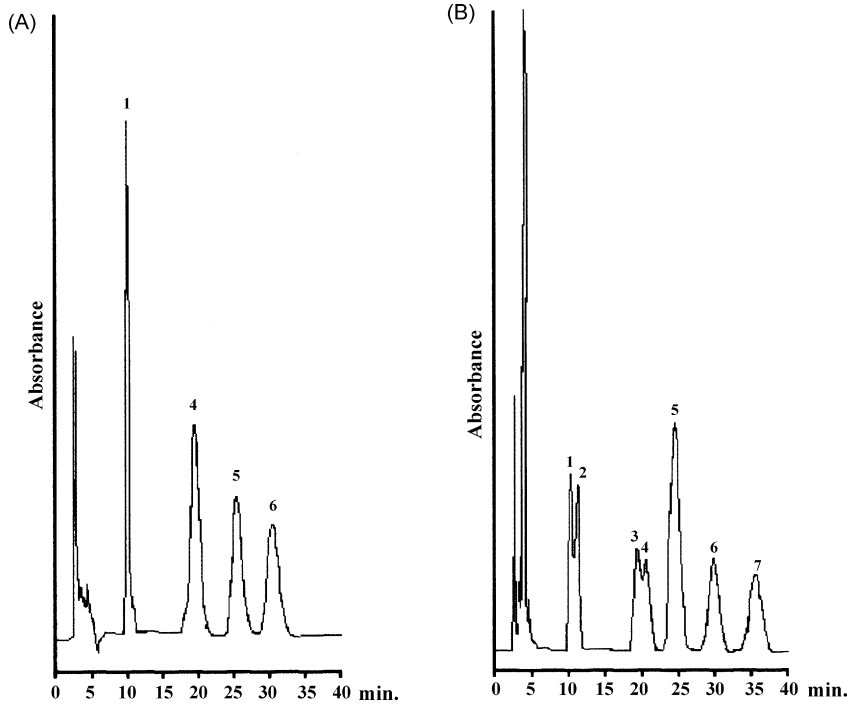

Fig. 2. (A) Chromatograms referring to the separation of THD-2-SO (FE) and THD-5-SO (SE): (1) (*S*)-THD-2-SO (*FE*), (4) (*R*)-THD-2-SO (*FE*), (5) (*R*)-THD-5-SO (*SE*) e (6) (*S*)-THD-5-SO (*SE*); (B) Chromatograms referring to separation of THD-2-SO and THD-5-SO exposed to UV light: (1) (*S*)-THD-2-SO (*FE*), (2) (*R*)-THD-2-SO (*SE*), (3) (*S*)-THD-2-SO (*SE*), (4) (*R*)-THD-2-SO (*FE*), (5) (*R*)-THD-5-SO (*SE*) + (*S*)-THD-5-SO (*FE*), (6) (*S*)-THD-5-SO (*SE*) and (7) (*R*)-THD-5-SO (*FE*). Chromatographic conditions: CHIRALPAK® AS column; mobile phase: hexane:ethanol:methanol (92:6:2, v/v/v) + 0.5% diethylamine at a flow rate 1.4 mL min^{-1}; detection at 262 nm.

Table 2
Linearity and quantification limit of the method

Stereoisomers	Linear equation	Correlation coefficient	CV (%)[a]	Analyzed concentration (ng mL^{-1})[b]	E (%)[c]	CV (%)[d]
(R)-THD-2-SO (FE)	$y = 821.39x + 15287$	0.9998	7.7	27.6	10.4	7.0
(S)-THD-2-SO (FE)	$y = 845.79x + 39484$	0.9999	8.1	26.7	6.8	5.8
(R)-THD-5-SO (SE)	$y = 712.86x - 6460.5$	0.9999	7.8	28.0	12.8	2.3
(S)-THD-5-SO (SE)	$y = 688.3x + 10186$	0.9998	8.6	25.3	1.2	9.1

Calibration curves with the following concentrations: 25.0, 50.0, 125, 250, 500, 1000, 2500, 5000 ng mL^{-1} for each stereoisomers, $n = 2$ for each concentration.
[a] CV, coefficient of variation on the slope of the calibration curve.
[b] Quantification limit, $n = 5$.
[c] E, relative error of the quantification limit.
[d] CV, coefficient of variation of the quantification limit.

Table 3
Recoveries of THD-2-SO and THD-5-SO

Concentration (ng mL^{-1}, $n = 3$)[a]	(S)-THD-2-SO (FE)		(R)-THD-2-SO (FE)		(S)-THD-5-SO (SE)		(R)-THD-5-SO (SE)	
	Recovery (%)	CV (%)[b]	Recovery (%)	CV (%)[b]	Recovery (%)	CV (%)[b]	Recovery (%)	CV (%)[b]
125	100.7	8.9	101.9	6.6	110.4	0.9	114.9	1.7
500	99.1	9.1	100.6	10.6	101.4	12.6	101.9	14.0
2500	96.8	2.9	97.9	3.0	99.4	2.1	97.4	1.4
Mean	98.9	8.1	100.2	7.1	103.7	7.5	104.7	8.7

[a] Number of determinations.
[b] CV, coefficient of variation.

Table 4
Precision and accuracy of the method for analysis of THD-2-SO and THD-5-SO stereoisomers in medium

	(S)-THD-2-SO (FE)			(R)-THD-2-SO (FE)			(S)-THD-5-SO (SE)			(R)-THD-5-SO (SE)		
Nominal concentration (ng mL^{-1})	125	500	2500	125	500	2500	125	500	2500	125	500	2500
Within-day ($n=5$)												
Concentration (ng mL^{-1})	126.4	462.7	2572	123.0	503.8	2666	133.1	465.0	2550	134.8	480.9	2476
Precision (CV, %)[a]	3.3	1.3	2.7	5.7	7.1	2.7	2.4	1.1	1.9	3.5	6.4	2.2
Accuracy (E, %)[b]	1.1	−7.5	2.9	−1.6	0.8	6.2	6.5	−7.0	2.0	7.9	−3.8	−1.0
Between-day ($n=3$)												
Concentration (ng mL^{-1})	128.4	480.7	2586	126.4	477.9	2567	137.5	471.8	2500	125.9	465.1	2434
Precision (CV, %)[a]	7.4	9.4	7.6	6.1	6.4	7.0	3.7	5.3	5.4	6.3	5.8	6.3
Accuracy (E, %)[b]	2.7	−3.9	3.5	1.1	−4.4	2.7	10.0	−5.6	0	0.7	−7.0	−2.6

n, number of determinations: five for within-day assay and three for between-day assay.
[a] Expressed as coefficient of variation, CV.
[b] Expressed as relative error, E.

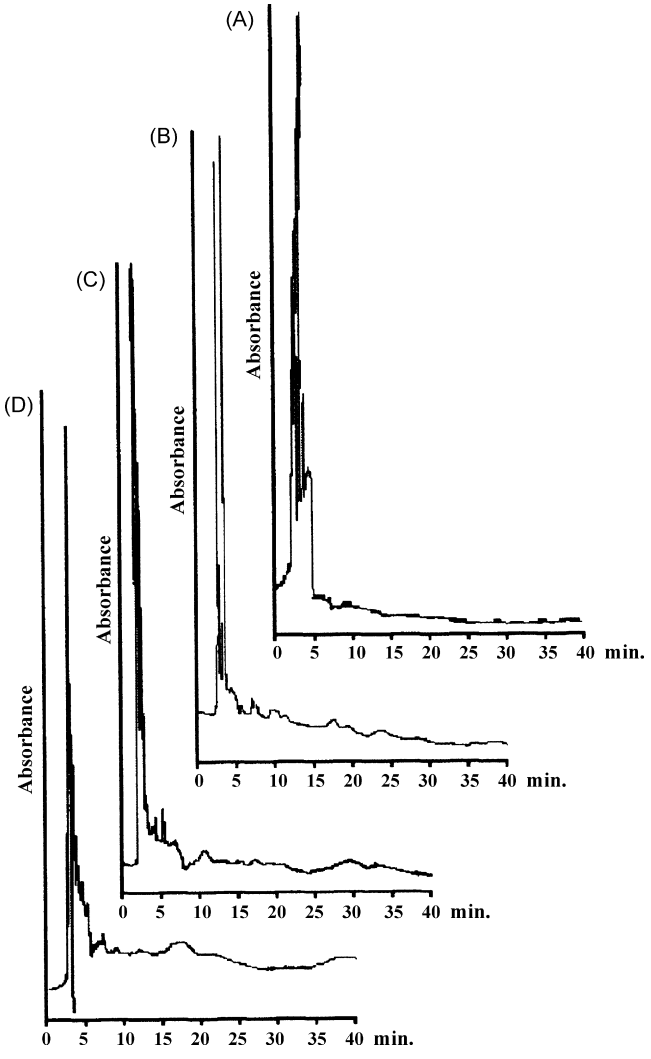

Fig. 3. (A) Chiral HPLC profile of the extract of two days incubation without THD with a *Phomopsis* sp. (TD2) culture; (B) Chiral HPLC profile of the extract of two days incubation without THD with a *Glomerella cingulata* (VA1) culture; (C) Chiral HPLC profile of the extract of two days incubation without THD with a *Diaporthe phaseolorum* (VR4); (D) Chiral HPLC profile of the extract of two days incubation without THD with a *Aspergillus fumigatus* (VR12). Chromatographic conditions as reported in Fig. 2.

(SE) + (S)-THD-5-SO (SE) (peak 5). Peak 5 can indicate the presence of (R)-THD-5-SO (SE), (S)-THD-5-SO (FE) or the mixture of these species. As the method does not discriminate these forms, we are denoting them as (R)-THD-5-SO (SE) + (S)-THD-5-SO (FE). Finally, the calibration curve of (S)-THD-5-SO (SE) (peak 6) was used to quantify (R)-THD-5-SO (FE) (peak 7).

The endophytes *Phomopsis* sp. (TD2), *G. cingulata* (VA1), *D. phaseolorum* (VR4) and *A. fumigatus* (VR12) were able to biotransform THD into the major human metabolites THD-2-SO and THD-5-SO (Fig. 4) by oxidating the sulphur atom in the lateral chain (position 2) or in the phenothiazine ring (position 5). However, the mono-2-sulfoxidation occurred with higher frequency. In addition, (R)-(+)- and (S)-(−)-THD were metabolized in different patterns. *Phomopsis* sp. (TD2) presented greater mono-2-sulfoxidation to the forms (S)-(SE) (12.1%); *G. cingulata* (VA1) presented greater mono-5-sulfoxidation to the forms (S)-(SE) + (R)-(FE) (10.5%); *D. phaseolorum* (VR4) presented greater mono-2-sulfoxidation to the forms (S)-(SE) and (R)-(FE) (84.4% and 82.5%, respectively) and *A. fumigatus* (VR12) presented greater mono-2-sulfoxidation to the forms (S)-(SE) and (R)-(SE) (31.5% and 34.4%, respectively). The results of the analyses of medium samples are shown on Table 5.

Aspergillus sp., *Glomerella* sp. and *Diaporthe* sp. have already been used for biotransformation of some other compounds showing high potential for stereoselective oxidations. *Aspergillus niger* converted (−)-menthol to 1-, 2-, 6-, 7- and 9-hydroxymenthol and the mosquito repellent-active 8-hydroxymenthol, whereas (+)-menthol was converted to 7-hydroxymenthol. *Aspergillus cellulose* biotransformed (−)-menthol specifically to 4-hydroxymenthol [30]. Miyazawa and Hashimoto [31] reported the resolution of (±)-2-*endo*-acetoxy-1,8-cineole by *G. cingulata* resulting (+)-2-*endo*-acetoxy-1,8-cineole and (−)-2-*endo*-hydroxy-1,8-cineole in enantiomerically pure form (yield 50%; e.e. 100%). Agusta et al. [12] reported the use of the endophytic fungus *Diaporthe* sp., isolated from a tea plant *C. sinensis*, for the stereoselective oxidation of (+)-catechin and (−)-epicatechin. Therefore, the production of a specific

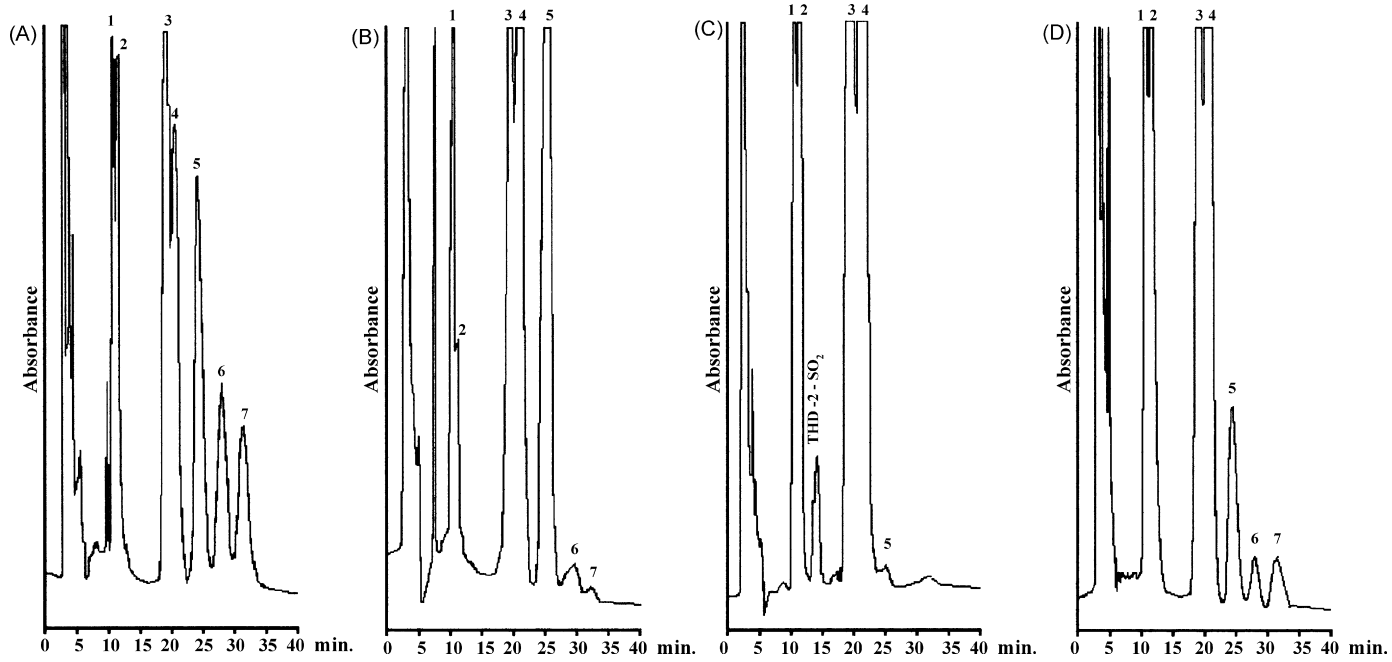

Fig. 4. (A) Chiral HPLC profile of the extract of 2 days incubation of THD (1 mg–100 μL DMF) with a *Phomopsis* sp. (TD2) culture; (B) Chiral HPLC profile of the extract of 2 days incubation of THD (1 mg–100 μL DMF) with a *Glomerella cingulata* (VA1) culture; C) Chiral HPLC profile of the extract of 2 days incubation of THD (1 mg–100 μL DMF) with a *Diaporthe phaseolorum* (VR4); D) Chiral HPLC profile of the extract of 2 days incubation of THD (1 mg–100 μL DMF) with a *Aspergillus fumigatus* (VR12). Chromatographic conditions as in Fig. 2. The peaks are: (1) (*S*)-THD-2-SO (*FE*), (2) (*R*)-THD-2-SO (*SE*), (3) (*S*)-THD-2-SO (*SE*), (4) (*R*)-THD-2-SO (*FE*), (5) (*R*)-THD-5-SO (*SE*) + (*S*)-THD-5-SO (*FE*), (6) (*S*)-THD-5-SO (*SE*) and (7) (*R*)-THD-5-SO (*FE*).

Table 5
Formation of the THD metabolites (expressed in % of drug added)

Strains[a]	Time (hours)	% (of drug added)						
		THD-2-SO				THD-5-SO		
		(S)-(FE)	(S)-(SE)	(R)-(SE)	(R)-(FE)	(R)-(SE) + (S)-(FE)	(S)-(SE)	(R)-(FE)
Phomopsis sp. (TD2)	48	3.1	12.1	2.9	6.9	11.4	6.1	5.8
Glomerella cingulata (VA1)	48	2.4	6.9	0.9	9.0	10.5	0.5	0.3
Diaporthe phaseolorum (VR4)	48	18.1	84.4	13.2	82.5	1.1	0.8	0.9
Aspergillus fumigatus (VR12)	48	20.7	31.5	34.4	29.0	6.9	0.8	0.9

[a] The control flasks do not present formation of the THD metabolites.

metabolite could be improved by selecting the appropriate fungus.

4. Conclusions

A suitable high-performance liquid chromatography method was developed and validated for the stereoselective determination of thioridazine-2-sulfoxide and thioridazine-5-sulfoxide simultaneously in a fermentative medium.

This method was used to study the biotransformation of thioridazine by endophytic fungi, showing that different metabolites could be obtained by using different types of fungi and that metabolite production was stereoselective. The results reported here could be further used to obtain specific metabolites for pharmacodynamic, toxicological or other kind of studies. For example, the fungus *D. phaseolorum* (VR4) can be used to obtain the forms (S)-(SE) and (R)-(FE) (84.4% and 82.5%, respectively) of THD-2-SO.

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References

[1] A.L. Demain, Appl. Microbiol. Biotechnol. 52 (1999) 455–463.
[2] C.P. Kurtzman, Mycologia 75 (1983) 374–382.
[3] S. Korol, P. Natale, J. Moretton, P. Santini, M. D'Aquino, Rev. Microbiol. 22 (1991) 313–318.
[4] R. Azerad, Adv. Biochem. Eng. Biot. 63 (1999) 169–218.
[5] R.V. Smith, J.P. Rosazza, Arch. Biochem. Biophys. 161 (1974) 551–558.
[6] R.V. Smith, J.P. Rosazza, J. Pharm. Sci. 64 (1975) 1737–1759.

[7] A.M. Clark, C.D. Hufford, Med. Res. Rev. 11 (1991) 473–501.
[8] D.G. Freitag, R.T. Foster, R.T. Coutts, M.A. Pickard, F.M. Pasutto, Drug Metab. Dispos. 25 (1997) 685–692.
[9] I.A. Parshikov, J.P. Freeman, A.J. Williams, J.D. Moody, J.B. Sutherland, Appl. Microbiol. Biotechnol. 52 (1999) 553–557.
[10] J.D. Moody, J.P. Freeman, P.P. Fu, C.E. Cerniglia, Drug Metab. Dispos. 30 (2002) 1274–1279.
[11] B. Schulz, C. Boyle, Mycol. Res. 109 (2005) 661–686.
[12] A. Agusta, S. Maehara, K. Ohashi, P. Simanjuntak, H. Shibuya, Chem. Pharm. Bull. 53 (2005) 1565–1569.
[13] R. Axelsson, Curr. Ther. Res. Clin. Exp. 21 (1977) 587–605.
[14] D.B. Bylund, J. Pharmacol. Exp. Ther. 217 (1981) 81–86.
[15] E. Richelson, A. Nelson, Eur. J. Pharmacol. 103 (1984) 197–204.
[16] L.A. Gottschalk, E. Dinovo, R. Biener, B.R. Nandi, J. Pharm. Sci. 67 (1978) 155–157.
[17] P.W. Hale Jr, Poklis, Toxicol. Appl. Pharmacol. 86 (1986) 44–55.
[18] C.B. Eap, L. Koeb, K. Powell, P. Baumann, J. Chromatogr. B 669 (1995) 271–279.
[19] C.B. Eap, T.W. Guentert, M. Schaublin-Loid, M. Stabl, L. Koeb, K. Powell, P. Baumann, Clin. Pharmacol. Ther. 59 (1996) 322–331.
[20] C.M. De Gaitani, A.S. Martinez, P.S. Bonato, Chirality 15 (2003) 479–485.
[21] C.M. De Gaitani, A.S. Martinez, P.S. Bonato, Electrophoresis 24 (2003) 2723–2730.
[22] C.M. De Gaitani, A.S. Martinez, P.S. Bonato, J. Pharm. Biomed. Anal. 36 (2004) 601–607.
[23] Guidance for industry, Bioanalytical Method Validation, Center for Drug Evaluation and Research, United states Food and Drug Administration, http://www.fda.gov/eder/guidance/index.htm.
[24] C.B. Eap, A. Souche, L. Koeb, P. Baumann, Ther. Drug Monit. 13 (1991) 356–362.
[25] M. Jackson, J.P. Karwowski, P.E. Humphrey, W.L. Kohl, G.J. Barlow, S.K. Tanaka, J. Antibiot. 46 (1993) 34–38.
[26] C.S. Alviano, S.R. Farbiarz, L.R. Travassos, J. Angluster, W. Souza, Mycopathologia 119 (1992) 17–23.
[27] S.A. Jortani, A. Poklis, J. Anal. Toxicol. 17 (1993) 374–377.
[28] S.A. Jortani, J.C. Valentour, A. Poklis, Forensic Sci. Int. 64 (1994) 165–170.
[29] P.S. Bonato, R. Bortocan, C.M. de Gaitani, F.O. Paias, M.H. Iha, R.P. Lima, J. Braz. Chem. Soc. 13 (2002) 190–199.
[30] Y. Asakawa, H. Takahashi, M. Toyota, Y. Noma, Phytochemistry 30 (1991) 3981–3987.
[31] M. Miyazawa, Y. Hashimoto, Tetrahedron: Asymmetry 12 (2001) 3185–3187.