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# Determination of the enantiomers of thioridazine, thioridazine 2-sulfone, and of the isomeric pairs of thioridazine 2-sulfoxide and thioridazine 5-sulfoxide in human plasma

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**Abstract**

Thioridazine is a commonly prescribed phenothiazine drug administered as a racemate and it is believed that its antipsychotic effect is mainly associated with (*R*)-thioridazine. A method based on high-performance liquid chromatography has been developed for the determination of the enantiomers of thioridazine and thioridazine 2-sulfone (THD 2-$SO_2$ or sulforidazine) and of the enantiomers of the diastereoisomeric pairs of thioridazine 2-sulfoxide (THD 2-SO or mesoridazine) and thioridazine 5-sulfoxide (THD 5-SO) in the plasma of thioridazine-treated patients. The method involves sequential achiral and chiral HPLC. The limits of quantitation for total (*R*) + (*S*) concentrations were found to be 15 ng/ml for thioridazine and 5 ng/ml for its metabolites. The limits for the determination of the (*R*)/(*S*) ratios were found to be 60 ng/ml for racemic THD and 10 ng/ml for racemic THD 2-SO, THD 2-$SO_2$, THD 5-SO (FE) and THD 5-SO (SE). The method has been used to determine the concentrations of the enantiomers of thioridazine and of its metabolites in the plasma of a patient treated with 100 mg of racemic thioridazine hydrochloride per os per day for 14 days. The results show a high enantioselectivity in the metabolism of this drug: the (*R*)/(*S*) ratios for THD, THD 2-SO (FE), THD 2-SO (SE), THD 2-$SO_2$, THD 5-SO (FE) and THD 5-SO (SE) were found to be 3.90, 1.22, 6.10, 4.10, 0.09 and 28.0, respectively.

## 1. Introduction

Thioridazine (THD) is a commonly prescribed phenothiazine neuroleptic drug for the treatment of schizophrenia and other psychiatric disorders. It is extensively biotransformed in the organism (see Fig. 1), and its main metabolites are thioridazine 2-sulfoxide (THD 2-SO or mesoridazine), thioridazine 2-sulfone (THD 2-$SO_2$ or sulforidazine) and thioridazine 5-sulfoxide (THD 5-SO). THD, THD 2-SO and THD 2-$SO_2$ are believed to exert their antipsychotic and side effects mainly via their antidopaminergic functions in the CNS [1,2], while THD 5-S0 seems to have no antipsychotic effect but contributes more potently than the parent compound [3,4] to the cardiotoxicity of the drug.

THD possesses an asymmetrical carbon atom at position 2 in the piperidyl ring and is currently administered as a racemate. Because the oxidation of the ring sulphur atom creates an additional chiral centre, THD 5-SO exists in the form of

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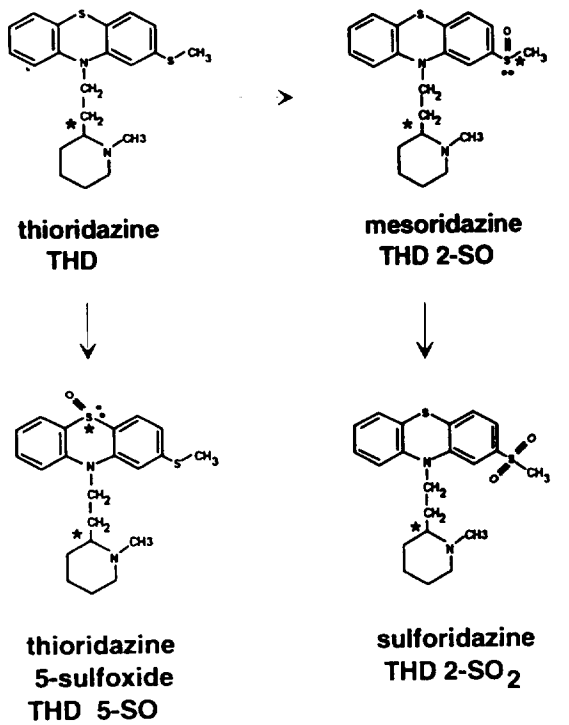


Fig. 1. Chemical formulae of thioridazine and its main metabolites (the chiral centres are indicated by an asterisk).

two diastereoisomeric pairs of enantiomers called "THD 5-SO fast eluting" (FE) and "THD 5-SO slow eluting" (SE) owing to their separation properties by different chromatographic methods [5]. Oxidation of the side chain also creates another chiral centre, and mesoridazine (but not sulforidazine) is constituted of two diastereoisomeric pairs of enantiomers. To our knowledge, the separation of the two diastereoisomeric pairs of mesoridazine with an achiral column, which is theoretically possible, has as yet never been shown.

The stereochemistry of thioridazine is important because (*R*)-thioridazine has a 2.7 times higher affinity than (*S*)-thioridazine for $D_2$-receptors in isolated rat brain preparations, while (*S*)-thioridazine has a 10 times higher affinity for the $D_1$-receptor [6]. As activation of $D_2$-receptors in the CNS leads to an inhibition of adenylate cyclase activity, while activation of $D_1$-receptors stimulates this enzyme, it is believed that the antipsychotic effect of administered racemic thioridazine is mainly associated with (*R*)-thioridazine [7]. Nevertheless, it has been suggested by neurochemical studies that antagonism of $D_1$ receptors may be more pertinent to atypical neuroleptic responses [8].

As a consequence, it would be useful to measure the enantiomers of thioridazine and of its metabolites in the blood of patients treated with this neuroleptic, and to determine whether a possible stereoselectivity in its metabolism exists. A study has been published using (*R*)-N-α-phenethyl-N-propylurea covalently bonded to Spherisorb silica for the separation of thioridazine and its metabolites [9]. However, as with many chiral liquid chromatography columns, the low number of theoretical plates does often not allow the direct and simultaneous separation of the enantiomers of the parent drug and of its metabolites without preliminary achiral analysis steps. Recently, a sequential achiral and chiral HPLC system has been used to measure the enantiomers of thioridazine in human serum [7] by which has been shown a stereoselectivity as well as an interindividual variability in the metabolism of this drug. In this paper, a method is described for the determination of thioridazine and its four metabolites in human plasma using, in a first step, a previously published method [10] with an achiral column and, in a second step, three types of chiral columns.

## 2. Experimental

### 2.1. Reagents

THD base, (−)-THD fumarate, (+)-THD fumarate, THD 2-SO benzosulfonate (mixture of diastereoisomers), THD 2-$SO_2$ base, THD 5-SO HCl (SE) (mixture of diastereoisomers) and nor-THD 2-SO benzosulfonate were supplied by Sandoz (Basle, Switzerland). According to a recent publication [8], (−)- and (+)-THD are the (*S*)- and (*R*)-enantiomers, respectively [the (*R*)- and (*S*)-forms relate to the chiral carbon at position 2 in the piperidyl ring]. THD 5-SO

HNO3 (FE) (mixture of diastereoisomers) was synthesized and purified as previously described [5]. Stock solutions of thioridazine and its metabolites were prepared in methanol at a concentration of 1 mg base per ml and stored at −20°C. All other reagents used were of analytical or HPLC grade. As THD and its metabolites have been shown to be light-sensitive [11], special care was taken to protect all solutions from light sources, and all procedures during the extraction steps were performed either in subdued or complete absence of light.

### 2.2. Instrumentation

An HPLC pump, Beckman Model 116 (Beckman Instruments, Nyon, Switzerland) was used together with a Perkin Elmer fluorescence detector LC 240 (Perkin Elmer, Le Mont-sur-Lausanne, Switzerland). Excitation was set at 262 nm and emission at 458 nm for THD, THD 2-SO, THD 2-$SO_2$ and pipothiazine (the internal standard), and at 274 nm and at 380 nm for THD 5-SO.

### 2.3. Extraction

Extraction was performed according to previously published methods [10]. To summarize, 500 ng of pipothiazine (internal standard), 400 μl of 2 *M* sodium hydroxide, and 4 ml of diethyl ether–hexane (3:1, v/v) were added to a 1-ml aliquot of heparinized plasma in a tube and the extraction performed on a shaker for 20 min. After centrifugation (5 min, 2800 *g*), the organic layer was transferred to another tube containing 1.2 ml of 0.1 *M* hydrochloric acid. After 15 min of shaking and centrifugation, the organic phase was discarded, and the acid phase transferred to another tube and extracted twice with 200 μl of 2 *M* sodium hydroxide and 2 ml of diethyl ether–hexane. The organic phase was then evaporated under nitrogen at 40°C, the residue was dissolved in 120 μl of the mobile phase consisting of hexane–methylene chloride–methanol (8.3:1:0.7), and 100 μl were injected into the HPLC.

### 2.4. Achiral separations

Chromatographic separations were performed as previously described [10]. To summarize, a Waters Z-module radial compression separation system (Waters, Kloten, Switzerland) was used with a cartridge (8 mm internal diameter) packed with 5 μm diameter microparticulate silica gel and a guard column filled with the same material. The mobile phase was 83% 2,2,4-trimethylpentane–10% methylene chloride–7% methanol and the flow-rate was set to 2.25 ml/min. Peaks corresponding to THD, THD 2-SO, THD 2-$SO_2$, THD 5-SO (FE) and THD 5-SO (SE) were manually collected and dried under $N_2$ at 40°C. The residues were then dissolved with 125 μl of the HPLC solvent used for the chiral separation and 100 μl injected into the HPLC.

### 2.5. Chiral separations

For the enantiomeric separations of THD (see Figs. 3A and 4A), a β-acetylated cyclodextrin column (Cyclobond I 2000 ac, 250 × 4.6 mm, Astec, Whippany, USA) was used. The flow-rate was set to 1.2 ml/min, and the HPLC solvent was 16% acetonitrile–84% TEA buffer (1% triethylamine titrated to pH 3.0 with ortho-phosphoric acid). At the end of each day, the column was washed with 90% acetonitrile–10% water and stored overnight in this solution. For longer periods of storage, 100% methanol was used, as recommended by the manufacturer.

For the enantiomeric separations of THD 2-SO (see Figs. 3B and 4B), an amylose derivative containing column (Daicel Chiralpak AS, 250 × 4.6 mm, J.T. Baker, Deventer, Netherlands) was used. The flow-rate was set to 1 ml/min, and the HPLC solvent was 81% hexane–19% ethanol–0.2% diethylamine. The column was washed at the end of each day with a washing and storing solution (90% hexane–10% isopropanol).

For the enantiomeric separations of THD 2-$SO_2$, THD 5-SO (SE) and THD 5-SO (FE) (see Figs. 3C–E and 4C–E), a cellulose carbamate derivative containing column (Daicel Chiracel OD, 250 × 4.6 mm, J.T. Baker, Deventer,

Netherlands) was used. The flow-rate was set to 1 ml/min, and the HPLC solvents were 85% hexane–15% ethanol–0.05% diethylamine for THD 5-SO (FE) and (SE) and 90% hexane–10% isopropanol–0.1% diethylamine for THD 2-$SO_2$. The column was washed at the end of each day with a washing and storing solution (90% hexane–10% isopropanol).

The reproducibility of the chiral step was measured by two experiments. Firstly, multiple ($n = 6$) direct injections into the chiral columns of appropriate HPLC solvents containing various relative concentrations of the (*R*)- and (*S*)-enantiomers of THD and metabolites (final concentration for each compound: 2 ng/$\mu$l, 100 $\mu$l injected) were performed. Secondly, a blank plasma was spiked with THD and metabolites (final concentration of THD 600 ng/ml, THD 2-SO 600 ng/ml, THD 2- $SO_2$ 200 ng/ml, THD 5-SO (FE) 600 ng/ml, and THD 5-SO (SE) 600 ng/ml) at various relative concentrations of the (*R*)- and (*S*)-enantiomers, extracted ($n = 8$) and injected into the achiral column. Samples were collected at the appearance of the peaks corresponding to each compound, recovered and injected into the appropriate chiral columns.

### *2.6. Identification of the enantiomers of thioridazine and metabolites on the chromatogram*

As pure enantiomers were only available for THD [(*S*)-THD and (*R*)-THD], the (*S*)- and (*R*)-forms of the metabolites of THD were obtained as previously described [12]. To summarize, (*S*)- and (*R*)-THD were incubated separately with rat liver microsomes (identical results were obtained with mouse liver microsomes, data not shown), extracted, injected into the achiral column, and the peaks of the metabolites manually collected. The (*R*)- and (*S*)-forms of each metabolite were then injected into the chiral columns to determine their order of elution.

### *2.7. Subject*

The blood samples were collected from a patient participating in a double-blind study on the efficacy of moclobemide and placebo versus moclobemide and thioridazine for the treatment of therapy-resistant severely depressed patients. The patient received per os daily for 28 days 450 mg moclobemide (150 mg after breakfast, lunch and dinner), and 100 mg thioridazine hydrochloride (after dinner). Determinations of the concentrations of the enantiomers of THD and metabolites were performed on blood drawn in the morning of day 14, that is at least 12 h after the last medication.

## 3. Results and discussion

### *3.1. Evaluation of the methods*

Concentrations of THD and metabolites were first measured using the achiral column (see Fig. 2). The achiral method used is that previously described [10] except that the UV detector was replaced by a fluorimeter. The result was a lower limit of quantitation [defined as the minimal plasma concentration which, after extraction and injection, gave a signal-to-noise ratio of at least 5, a coefficient of variation (C.V.) for replicate determination ($n = 6$) of 15% or less, and a deviation from the theoretical value of 20% or less], which was found to be 15 ng/ml for THD and 5 ng/ml for its metabolites. Another advantage of the fluorimeter over the UV detector is the higher specificity, but it has to be mentioned that connection of a UV detector at the outlet of the fluorimeter yielded, as expected, comparable results (data not shown).

To determine the enantiomeric ratios, peaks corresponding to each compound were manually collected at the detector outlet, dried, injected into the chiral column, and the relative area of each enantiomer measured. In Fig. 3 are shown the enantiomeric separations of pure standards of THD, THD 2-SO, THD 2-$SO_2$, THD 5-SO (FE) and THD 5-SO (SE) (for the type of columns used and the conditions, see Section 2). Due to the high number of metabolites of THD, it is not surprising that it is rather difficult to find one column which can successfully separate the enantiomers of all compounds. We have found that the $\alpha_1$-acid glycoprotein column was one of

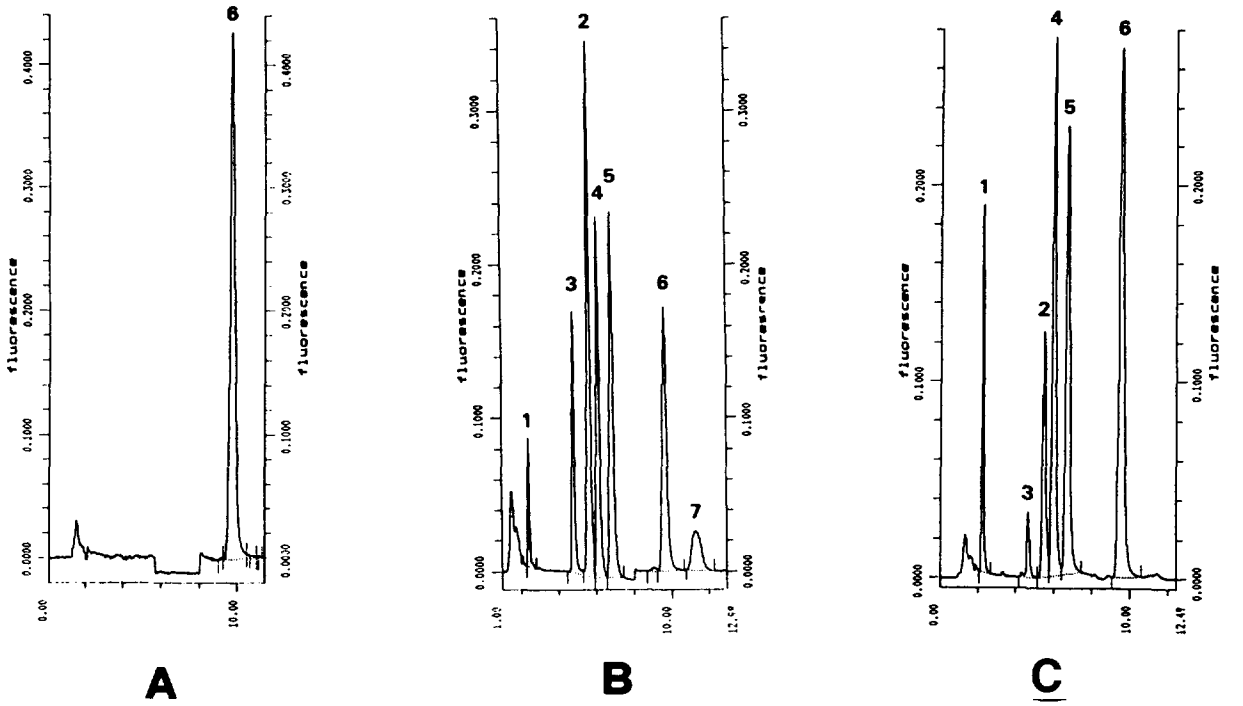


Fig. 2. HPLC chromatograms obtained on an achiral column (see Section 2) after extraction of (A) a blank plasma; (B) a standard plasma containing 250 ng/ml of thioridazine (peak 1, 2.32 min), 500 ng/ml of a mixture of diastereoisomers of thioridazine 2-sulfoxide (peak 2, 5.35 min), 150 ng/ml of thioridazine 2-sulfone (peak 3, 4.62 min), 400 ng/ml of thioridazine 5-sulfoxide fast eluting (FE) peak (peak 4, 5.87 min), 400 ng/ml of thioridazine 5-sulfoxide slow eluting (SE) peak (peak 5, 6.53 min), 150 ng/ml of northioridazine 2-sulfoxide (peak 7, 11.16 min), and 500 ng/ml of pipothiazine (internal standard, peak 6, 9.42 min); (C) a plasma of a patient treated with 100 mg/day of racemic thioridazine hydrochloride for 14 days.

them [13], but we were neither satisfied with the stability of the column (even of the second generation type) nor with the very long retention times (more than one hour) necessary for the separation of the enantiomers of some compounds (in particular THD) which, in turn, resulted in broad peaks (data not shown), thus impairing the limit of detection. Furthermore, an achiral step was also necessary before the chiral separation because the different metabolites would otherwise coelute from the $\alpha_1$-acid glycoprotein column (data not shown). The problem of long retention times can also be observed with a (*R*)-N-α-phenetyl-N-propylurea column [9]. In the method described here, three types of columns were used for five compounds, which allowed to obtain reasonable retention times ($< 25$ min) while still providing good separation.

Limits of quantitation, given in ng/ml for the initial plasma, after extraction, injection into the achiral column, recovery and injection into the chiral column and defined as giving a signal-to-noise ratio of at least 3, were found to be 60 ng/ml for racemic THD and 10 ng/ml for racemic THD 2-SO, THD 2-$SO_2$, THD 5-SO (FE) and THD 5-SO (SE). Higher values than those obtained with the achiral columns were probably due to losses during the recovery steps, but this result for THD is similar to another published method [7]. In Table 1 are shown some statistical data concerning the reproducibility of the chiral method. The relative concentrations of the (*R*)- and (*S*)-enantiomers of THD and metabolites with regard to the sum of all isomers were determined either after direct injection into the chiral columns or after extraction from a spiked plasma, injection into the chiral column, recovery and injection into the chiral

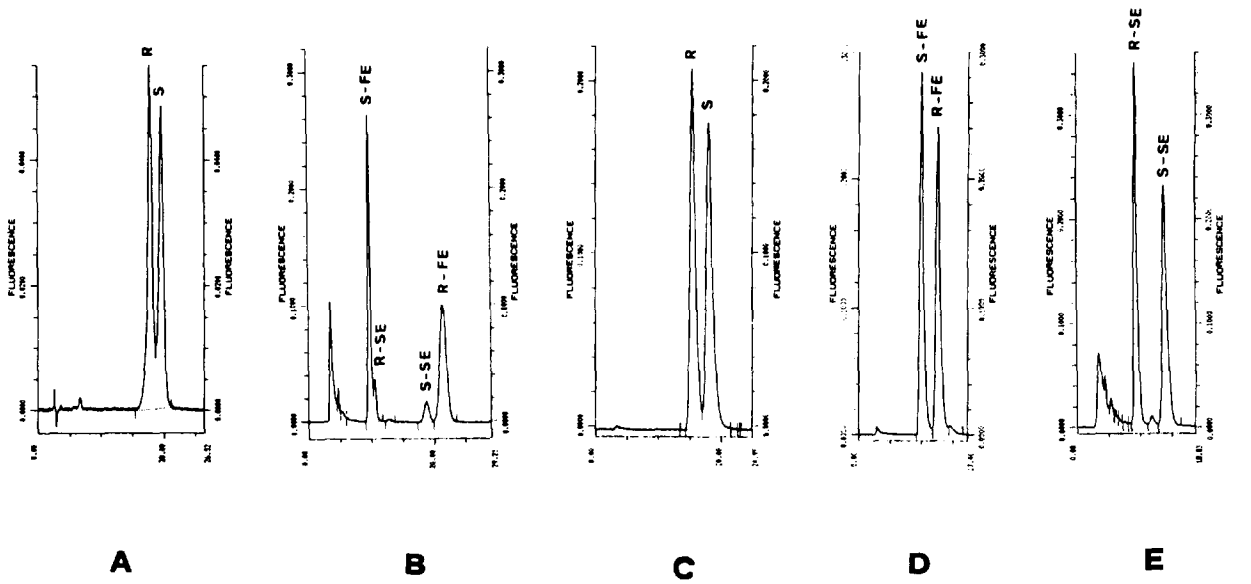


Fig. 3. HPLC chromatograms obtained on chiral columns for pure standards of (A) thioridazine on Cyclobond I 2000 Ac; typical retention times: 17.9 min and 19.7 min; (B) thioridazine 2-sulfoxide on Chiralpak AS; (FE): 9.9 min, 21.6 min and (SE): 10.7 min, 19.0 min; (C) thioridazine 2-sulfone on Chiracel OD; 15.4 min and 18.0 min; (D) thioridazine 5-sulfoxide (FE) on Chiracel OD; 9.9 min and 12.6 min and (E) for thioridazine 5-sulfoxide (SE) on Chiracel OD; 9.3 min and 13.9 min.

columns. A good concordance was found between the theoretical relative concentrations and the measured relative concentrations, and small standard deviations were calculated. Furthermore, the results show that the relative concentrations of one enantiomer of THD can still be measured with an acceptable precision in the presence of differing amounts of its antipode. Due to the lack of pure standards of the (*R*)- and (*S*)-enantiomers of THD metabolites, the experiments for these compounds were performed using racemic mixtures only.

### *3.2. Identification of the enantiomers of thioridazine and metabolites on the chromatogram*

Pure substances of (*R*)- and (*S*)-THD were available and (*R*)- and (*S*)-THD 2-SO, THD 2-$SO_2$ and THD 5-SO were obtained by separate incubations of (*R*)- and (*S*)-THD with rat liver microsomes, as previously described [12]. Subsequently, identification of the peaks corresponding to (*R*)-THD, (*S*)-THD, (*R*)-THD 2-$SO_2$ and (*S*)-THD 2-$SO_2$ on the chromatograms obtained with the chiral columns was straightforward. For THD 5-SO, two peaks corresponding to the pairs of diastereoisomers were first isolated by means of the achiral column and called FE (for fast eluting) and SE (for slow eluting), as previously described [5]. On the chiral column, the two pairs of enantiomers were separated and the (*R*)- and (*S*)-forms relating to the chiral carbon at position 2 in the piperidyl ring were identified as mentioned above. The four isomers were then tentatively called (*R*)-THD 5-SO (FE), (*S*)-THD 5-SO (FE), (*R*)-THD 5-SO (SE) and (*S*)-THD 5-SO (SE). It must be mentioned that, in order to obtain a complete separation of the four stereoisomers, the pair of diastereoisomers THD 5-SO (FE) and THD 5-SO (SE) must first be separated with the achiral column. Indeed, if a mixture of THD 5-SO (FE) and THD 5-SO (SE) is injected into the chiral column, three and not four peaks are seen on the chromatogram because there is coelution of (*R*)-THD 5-SO (SE) and (*S*)-THD 5-SO (FE).

For THD 2-SO, it was not possible to separate the two isomeric pairs of enantiomers on the

Table 1
Relative concentrations of the isomers of thioridazine and metabolites with regard to the sum of all isomers after direct injection into the chiral column or after extraction from a spiked plasma, injection into the achiral column, recovery and injection into the chiral column

| | Direct injection[a] | | Injection after extraction[b] | |
|---|---|---|---|---|
| | Theoretical relative concentration (%) | Measured relative concentration (%, mean ± S.D., C.V.) | Theoretical relative concentration (%) | Measured relative concentration (%, mean ± S.D., C.V.) |
| (*R*)-THD | 50 | 50.0 ± 0.4 (0.8) | 50 | 50.3 ± 0.5 (0.9) |
| | | | 66.7 | 66.3 ± 0.8 (1.3) |
| | | | 33.3 | 33.7 ± 0.4 (1) |
| | | | 80 | 78.3 ± 0.5 (0.7) |
| | | | 20 | 20.7 ± 0.5 (2.6) |
| (*S*)-THD | 50 | 50.0 ± 0.4 (0.8) | 50 | 49.7 ± 0.5 (0.9) |
| | | | 33.3 | 33.7 ± 0.9 (2.6) |
| | | | 66.7 | 66.3 ± 0.3 (0.5) |
| | | | 20 | 21.7 ± 0.5 (2.5) |
| | | | 80 | 79.3 ± 0.5 (0.7) |
| (*R*)-THD 2-SO (FE) | unknown | 44.1 ± 1.1 (2.6) | unknown | 43.4 ± 0.4 (1) |
| (*S*)-THD 2-SO (FE) | unknown | 42.6 ± 1.0 (2.4) | unknown | 42.2 ± 0.5 (1.2) |
| (*R*)-THD 2-SO (SE) | unknown | 6.6 ± 0.2 (2.3) | unknown | 7.2 ± 0.4 (6) |
| (*S*)-THD 2-SO (SE) | unknown | 6.7 ± 0.3 (4.5) | unknown | 7.3 ± 0.6 (8) |
| (*R*)-THD 2-$SO_2$ | 50 | 50.2 ± 1.9 (3.7) | 50 | 49.4 ± 0.6 (1) |
| (*S*)-THD 2-$SO_2$ | 50 | 49.8 ± 1.8 (3.7) | 50 | 50.6 ± 0.6 (1) |
| (*R*)-THD 5-SO (FE) | 50 | 52.3 ± 0.8 (1.4) | 50 | 51.3 ± 1.8 (2) |
| (*S*)-THD 5-SO (FE) | 50 | 47.7 ± 0.8 (1.7) | 50 | 48.7 ± 1.8 (4) |
| (*R*)-THD 5-SO (SE) | 50 | 48.2 ± 1.1 (2.4) | 50 | 49.4 ± 1.4 (2.8) |
| (*S*)-THD 5-SO (SE) | 50 | 51.8 ± 1.1 (2.2) | 50 | 50.6 ± 1.4 (2.7) |

[a] Direct injection into the chiral column ($n = 6$).
[b] Injection after extraction, injection and recovery from the achiral column ($n = 8$).

achiral column with the HPLC conditions as mentioned in Section 2 (Fig. 2). On the other hand, four peaks can be seen on the chiral column (Figs. 3 and 4). By lowering the flow-rate and the methanol content of the HPLC eluent, a partial separation was obtained with the achiral column (chromatogram not shown). The two peaks were also called fast eluting (FE) and slow eluting (SE) and collected. Although the two fractions were contaminated by each other, it was possible to see that the (FE) fraction corresponded to the first and fourth peak and the (SE) fraction to the second and third peak on the chromatogram obtained with the chiral column. The (*R*)- and (*S*)-forms corresponding to the chiral carbon at position 2 in the piperidyl ring were again identified, and the four peaks on the chiral column were tentatively called (*R*)-THD 2-SO (FE), (*S*)-THD 2-SO (FE), (*R*)-THD 2-SO (SE) and (*S*)-THD 2-SO (SE). It is interesting to mention that our sample of THD 2-SO, which was supplied by Sandoz, is mainly constituted of the (FE) pair of enantiomers (about 87%, see Table 1 and Fig. 3B).

### 3.3. Analysis of a human sample

Figs. 2 and 4 show the HPLC chromatograms obtained on achiral and chiral columns for the analysis of THD and its metabolites in the plasma of a patient treated with 100 mg of racemic thioridazine hydrochloride per os per day for 14 days. The concentrations of THD and metabolites as measured with the achiral column (total concentrations, analysis in duplicate) and the (*R*)/(*S*) ratios as measured with the chiral

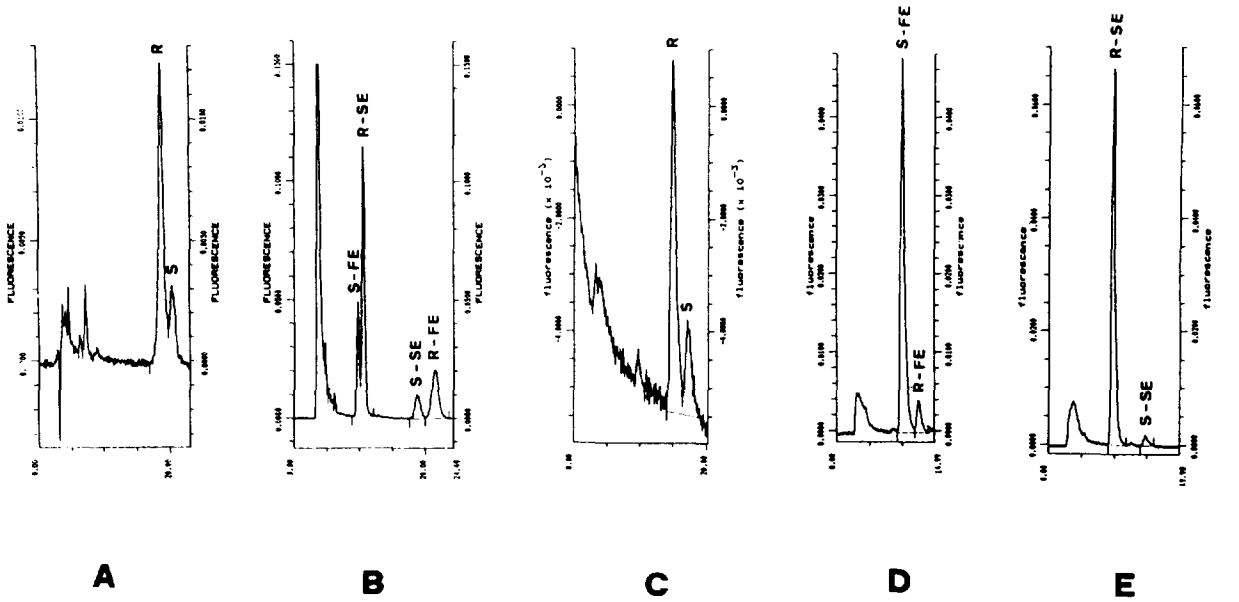


Fig. 4. HPLC chromatograms obtained on chiral columns (see Fig. 3) for thioridazine (A), thioridazine 2-sulfoxide (B), thioridazine 2-sulfone (C), thioridazine 5-sulfoxide (FE) (D), and thioridazine 5-sulfoxide (SE) (E), isolated from the plasma of a patient treated with 100 mg of racemic thioridazine hydrochloride per day for 14 days.

column (analysis in duplicate) are listed in Table 2. These results show a high enantioselectivity in the metabolism of THD; they confirm those of a recent study which measured the concentrations of THD only [7]. It must be said, however, that a reverse enantiomeric ratio for THD [i.e. higher concentration of (−)-(*S*)-THD than (+)-(*R*)-THD] was found by other authors [7]. The reasons of this discrepancy are unknown, but it should be mentioned that it was not possible for the authors of the study [7] to obtain pure standards of (−)- and (+)-THD. In our case and as described in Section 2, these pure standards were supplied to us by Sandoz (Basel), and we have also checked and confirmed the optical rotations (in methanol) of the enantiomers (data not shown).

Table 2
Concentrations of total [(*R*) + (*S*)] and of the enantiomers [(*R*) and (*S*)] of thioridazine and metabolites in the plasma of a patient receiving 100 mg/day of thioridazine hydrochloride per os for 14 days

| | Concentration (ng/ml) | | | (*R*)/(*S*) ratio |
|---|---|---|---|---|
| | (*R*) + (*S*) | (*R*) | (*S*) | |
| Thioridazine | 556 | 443 | 113 | 3.9 |
| THD 2-SO (FE) | 68 | 39 | 33 | 1.22 |
| THD 2-SO (SE) | 112 | 93 | 15 | 6.1 |
| THD 2-$SO_2$ | 36 | 29 | 7 | 4.1 |
| THD 5-SO (FE) | 499 | 40 | 459 | 0.09 |
| THD 5-SO (SE) | 448 | 433 | 15 | 28 |

Considering the differences in the pharmacological profiles of (*R*)-THD and (*S*)-THD and being reminded that (*R*)-THD is considered to be the active neuroleptic drug (cf. Section 1), these results are most interesting. We have now completed a clinical study, during which patients phenotyped as being extensive or poor metabolizers of debrisoquine (for a review, see Refs. [14–16]) were medicated with thioridazine: the concentrations of thioridazine and its metabolites, including the enantiomers, were measured, and the results will be published elsewhere [17].

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