Simultaneous Assay of Thioridazine and Its Major Metabolites in Plasma at Single Dosage Levels with a Novel Report of Two Ring Sulfoxides of Thioridazine

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Abstract □ A highly sensitive and selective method of analysis of plasma for thioridazine and its major metabolites, including two isomers of the ring sulfoxide, is presented. It is suitable for following the metabolism of thioridazine for 24 hr after a single dose. The method involves extraction of the materials from plasma, high-performance liquid chromatographic separation, and postcolumn oxidation and fluorometric detection. The sensitivity of the method to thioridazine and its metabolites is 2 ng/ml. Recoveries ranged from 87.8 to 100.6% at levels between 20 and 400 ng/ml.

Keyphrases □ Thioridazine—isomeric ring sulfoxides, metabolism, high-performance liquid chromatography with fluorometric detection, determination in plasma □ High-performance liquid chromatography—thioridazine metabolism, formation of two isomeric ring sulfoxides, determination in plasma

In preparation for a clinical study designed to compare the bioavailability of various brands of thioridazine tablets, it was necessary to develop a sensitive, specific method for the determination of thioridazine and its major metabolites in plasma. The metabolites of interest were sulforidazine (thioridazine 2-sulfone), mesoridazine (thioridazine 2-sulfoxide), and the two isomers of thioridazine ring sulfoxide (thioridazine 5-sulfoxide) for which analyses have not been reported in the literature.

The concentrations of thioridazine and its metabolites in biological fluids have been measured by fluorometric (1, 2), TLC (2–5), GLC (2, 6–14), and high-performance liquid chromatographic (HPLC) (15–17) methods. Most of these methods were developed to measure thioridazine and its metabolites in the blood of patients who were placed on a therapeutic regimen of thioridazine. For the study of the metabolism of thioridazine arising from a single dose, a method capable of detecting these compounds in the range of 10–500 ng/ml of plasma was needed. None of the methods reported in the literature provide both the sensitivity and specificity necessary.

Although the fluorometric methods provide the required sensitivity, they are not specific and do not differentiate among the metabolites. The GLC methods are not suitable at levels <50 ng/ml because of the instability of the metabolites on the chromatographic columns. The TLC procedures lack the necessary sensitivity.

The method of Muusze and Huber (15), which employs an HPLC separation of thioridazine and its metabolites followed by postcolumn oxidation and fluorescence, showed the most promise. However, at a sensitivity that would allow the detection of the eluted compounds at levels <50 ng/ml, the baseline signal from the fluorometer became excessively noisy. To eliminate the noisy baseline and allow the detection of lower levels of the metabolites, the detection system was modified to extract the eluted products into a phosphoric acid solution of potassium permanganate for oxidation.

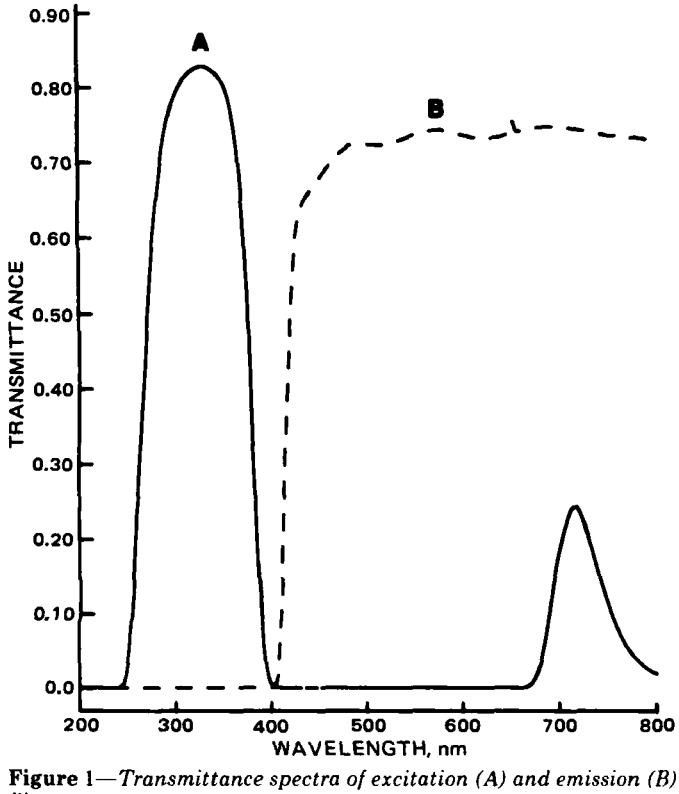

Figure 1—*Transmittance spectra of excitation (A) and emission (B) filters.*

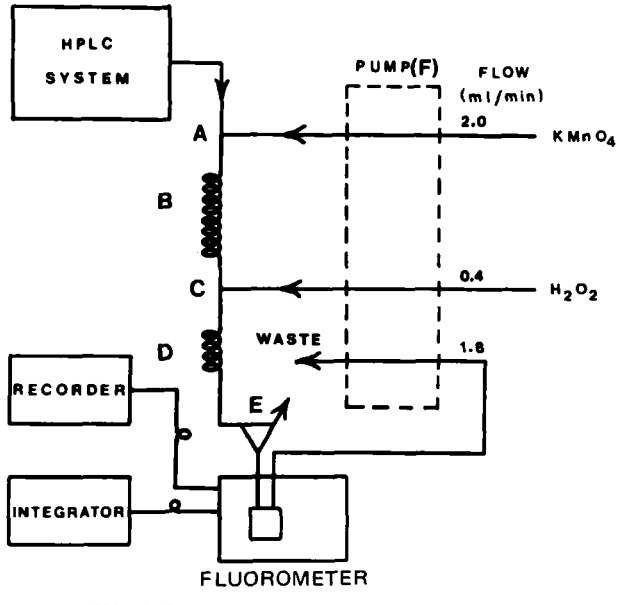

Figure 2—*HPLC detection system. Key: A, PT18; B, 14-turn phasing coil, 2.4-mm i.d.; C, A6; D, 1X mixing coil, 1-mm i.d.; E, B0; and F, Pump I*[6].

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Table I—Linearity Data for Thioridazine and Metabolites (Spiked Plasma)

Concentration[b]	Ratio[a] I C[c]	I I[d]	II C[c]	II I[d]	III C[c]	III I[d]	IV C[c]	IV I[d]
Blank[e]	0.089	0.090	—	—	—	—	—	—
10	0.346	0.347	0.131	—[f]	0.079	0.068	0.050	—[f]
20	0.755	0.741	0.287	0.278	0.170	0.163	0.117	0.107
50	1.45	1.42	0.572	0.569	0.343	0.332	0.241	0.230
100	—[g]	2.76	1.09	1.10	0.673	0.672	0.480	0.486
250	—[g]	6.42	—[g]	2.70	—[g]	1.58	—[g]	1.16
400	—[g]	10.49	—[g]	4.30	—[g]	2.45	—[g]	1.78
500	—[g]	12.62	—[g]	5.48	—[g]	3.15	—[g]	2.26
Slope	36.2	39.6	95.7	92.6	154.2	161.7	213.2	224.2
Intercept	−3.9	−6.3	−4.8	−2.7	−3.8	−4.4	−2.3	−5.1
Correlation Coefficient	0.9942	0.9997	0.9987	0.9998	0.9991	0.9997	0.9989	0.9998

[a] Standard peak height to internal standard peak height. I = thioridazine; II = thioridazine ring sulfoxide (fast eluter); III = thioridazine ring sulfoxide (slow eluter); IV = mesoridazine. [b] ng/ml of plasma. [c] Peak height taken from recorder chart. Recorder set at 20 mV full scale. [d] Peak height reported by integrator. [e] No II, III, or IV detected in blank. [f] Integrator failed to detect peak. [g] Peaks were off-scale on the recorder.

Although that study (15) reported only a single thioridazine ring sulfoxide, another study (18) reported the presence of two isomers. The mobile phase used in the HPLC system was modifed to permit the separation of all the metabolites of interest, including the two ring sulfoxide isomers, and to allow the use of an internal standard.

EXPERIMENTAL

Apparatus—The HPLC system consisted of a pump[1], a syringe injector[1], a recorder[2], an integrator[3], a 3.2- × 250-mm column packed with 10-μm spherical particle silica[4], a 2- × 50-mm precolumn of solid-core silica[5], a peristaltic pump[6], mixing coils and connecting tees[6], and a fluorometer[7] equipped with excitation[8] and emission[9] filters. The transmittance spectra of these filters are shown in Fig. 1. Samples were stored in a freezer[10] at −40°. A vortex mixer[11] and a centrifuge[12] were used in the extraction of the plasma samples. The detection system was assembled as shown in Fig. 2.

Reagents—Potassium permanganate, sodium nitrite, diethylamine, hydrogen peroxide, and phosphoric acid were reagent grade. Ethyl acetate, heptane, 1-chlorobutane, methylene chloride, and 2-propanol were HPLC grade. The water was deionized. Thioridazine hydrochloride[13], mesoridazine besylate[13], thioridazine ring sulfoxide nitric acid salts[14] (18), sulforidazine[14], and triflupromazine hydrochloride[15] were used as standards. Plasma was obtained from the Red Cross blood bank.

The potassium permanganate solution was prepared by dissolving 100 mg of potassium permanganate in 1 liter of 5% aqueous phosphoric acid. The hydrogen peroxide solution was prepared by diluting 1 ml of 30% hydrogen peroxide to 250 ml with water.

HPLC Operating Conditions—The mobile phase was 1-chlorobutane–2-propanol–water–diethylamine (92:7.9:0.08:0.016). The flow rate was 1.5 ml/min at 25°. The potassium permanganate solution flow rate was 2.0 ml/min, and the hydrogen peroxide solution flow rate was 0.4 ml/min. The flow rate through the fluorometer flowcell was 1.8 ml/min. The recorder sensitivity was set at 20 mV full scale (the output of the fluorometer was 100 mV full scale). The fluorometer sensitivity was adjusted so that the internal standard peak height was ~ 80% of full scale on the recorder when 100 μl of sample extract was injected. The integrator was set to measure peak heights. Instrument operating conditions were maintained for 0.5 hr before injection of the first sample or standard solution.

Preparation of the Internal Standard Solution—An aqueous solution of triflupromazine hydrochloride (10 mg/25 ml) was transferred to a separatory funnel. A 1-ml portion of 0.1 *N* hydrochloric acid and a 1-ml portion of sodium nitrite solution (10 g/100 ml) were added, and the funnel was shaken for 2 min. The solution was made alkaline with concentrated ammonium hydroxide, the oxidized triflupromazine was extracted with one 50-ml portion of methylene chloride, and the extract was diluted to 100.0 ml with methylene chloride. A 3.0-ml aliquot of the

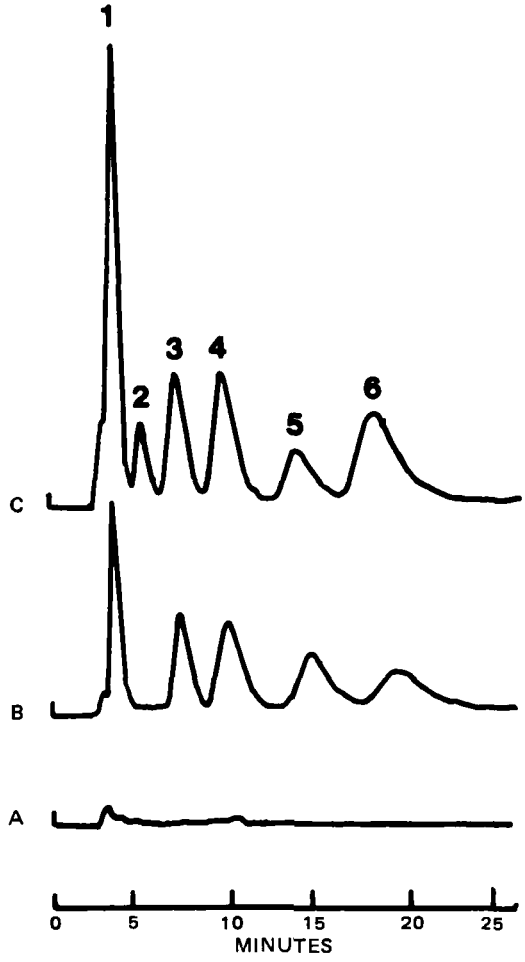

Figure 3—*HPLC chromatograms. Key: A, extract of blank plasma; B, standards added to plasma at a level of ~100 ng/ml each; C, extract from whole blood of patient on thioridazine therapy; (1) thioridazine; (2) sulforidazine; (3) internal standard; (4 and 5) thioridazine ring sulfoxides; and (6) mesoridazine.*

[1] Model 6000A pump and model U6K injector, Waters Associates, Milford, MA 01757.
[2] Model PS01W6A, Texas Instruments, Inc., Houston, TX 77001.
[3] Model 3390A, Hewlett-Packard, Palo Alto, CA 94304.
[4] 10-μm LiChrosphere Si-500, Alltech Associates, Inc., Deerfield, IL 60015.
[5] Corasil, 30–50 μm, Waters Associates.
[6] Technicon Instruments Corp., Tarrytown, NY 10591. See Fig. 2 for part numbers.
[7] Ratio Fluorometer 2, Farrand Optical Co., Inc., Valhalla, NY 10595.
[8] Schott UG-11.
[9] Wratten 2B.
[10] Model ULT 1535-A-J-6, Rheem Manufacturing Co., West Columbia, SC 29169.
[11] Model M-16715, Thermolyne Corp., Dubuque, IA 52001.
[12] Spinette, International Equipment Co., Damon Corp., Needham Heights, MA 02194.
[13] Sandoz Pharmaceuticals, Hanover, NJ 07936.
[14] Prepared in our laboratory.
[15] USP reference standard.

Table II—Recovery Data for Thioridazine (ng/ml of Plasma) from Four Spiked Samples of Plasma

Sample	Sample[a] 1	2	3	4
1	377.1	147.3	75.3	17.7
2	373.1	149.7	75.2	19.2
3	376.9	150.8	73.1	18.4
4	393.7	141.6	71.5	18.3
Mean	380.2	147.3	73.8	18.4
SD	9.19	4.10	1.82	0.62
CV, %	2.42	2.78	2.47	3.35
Added	407.8	163.0	81.5	20.4
Percent recovery	93.2	90.4	90.5	90.2

[a] For concentrations of other components in these samples, see Tables III–V.

methylene chloride solution was diluted to 250 ml with heptane–ethyl acetate–diethylamine (100:100:1). The internal standard solution gave an HPLC peak with a retention time identical to that of triflupromazine sulfoxide prepared from a previous procedure (19). The latter material was crystallized from ethyl acetate and dried (55°, 3 mm, 3 hr) to give a product, mp 94–96°, which was submitted for carbon and hydrogen elemental analysis.

Anal.—Calc. for $C_{18}H_{19}F_3N_2OS$: C, 58.68; H, 5.20. Found: C, 58.43; H, 5.33.

Preparation of Sample Solutions—A 2.0-ml aliquot of plasma was transferred to a 16- × 100-cm screw-capped test tube containing 2.0 ml of the internal standard solution and 3 ml of heptane–ethyl acetate (1:1). The tube was capped, mixed on a vortex mixer for 2 min, centrifuged for 5 min, and placed in the freezer until the plasma was frozen. The organic layer was decanted into a second tube. The frozen plasma was allowed to thaw and was re-extracted with 3 ml of heptane–ethyl acetate (1:1). The organic layers were combined and evaporated to dryness (steam bath, nitrogen stream). The residue was dissolved in 0.5 ml of the HPLC mobile phase.

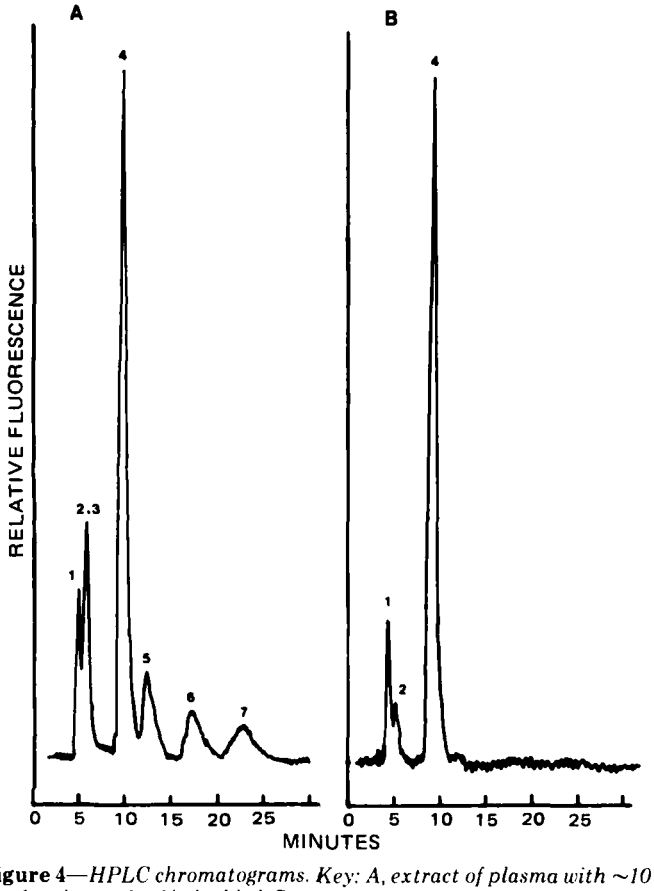

Figure 4—*HPLC chromatograms. Key: A, extract of plasma with ~10 ng of each standard/ml added; B, extract of blank plasma with internal standard added; (1 and 2) plasma blanks; (3) thioridazine; (4) internal standard; (5 and 6) thioridazine ring sulfoxides; and (7) mesoridazine.*

Table III—Recovery Data for Thioridazine Ring Sulfoxide (Fast Eluter) (ng/ml of Plasma) from Four Spiked Samples of Plasma

Sample	Sample[a] 1	2	3	4
1	42.9[b]	38.9	207.7	393.2
2	18.7	35.9	201.2	388.7
3	16.6	35.5	188.1	394.6
4	16.6	40.8	193.7	390.9
Mean	17.3	37.8	197.7	391.8
SD	1.21	2.52	8.57	2.60
CV, %	7.00	6.68	4.34	0.66
Added	19.7	39.3	196.8	393.6
Percent recovery	87.8	96.2	100.4	99.5

[a] For concentrations of other components in these samples, see Tables II, IV, and V. [b] Sample contaminated; not used in calculation of mean, *SD*, and *CV*.

Preparation of Standard Solutions—Portions of the salts of thioridazine, mesoridazine, and the ring sulfoxides equivalent to 20 mg of the corresponding free bases were each dissolved in 95% ethanol and diluted to 100.0 ml. These solutions were diluted with 95% ethanol to give mixed standard solutions containing 0.8, 1.6, 4, 8, 20, 32, and 40 μg of each standard/ml. A 25-μl aliquot of each solution was added to 2 ml of plasma. At this low level, the ethanol neither denaturated the plasma nor influenced the partition coefficients in the extractions. The sample-preparation procedure was used for the extraction of the standard solutions.

Recovery of Thioridazine and Metabolites from Plasma—Four plasma samples containing thioridazine and each of its metabolites at levels of 20 to 400 ng/ml were prepared. Aqueous solutions of the ethanolic standard solutions were added to the plasma to avoid denaturing of the plasma and to avoid changes in the partition coefficients in the extraction steps. Four aliquots of each prepared plasma sample and four aliquots of blank plasma were extracted.

Stability of Thioridazine and Its Metabolites in Plasma—Aliquots of one of the plasma samples used in the recovery experiment were transferred to test tubes and stored in the freezer. At intervals over a 3-week period, the samples were removed from the freezer and extracted. The dried extracts were stored in the freezer until the end of the 3-week period; all extracts were then injected into the HPLC system on the same day. In a second experiment, samples were removed from the freezer and stored at room temperature for 0, 1, 2, 5, and 24 hr before extraction; all five of these extracts were injected on the same day.

HPLC Separation and Analysis—A 100-μl aliquot of the extract of each standard and sample was injected into the liquid chromatograph. The ratios of each peak height to the peak height of the internal standard were calculated from the integrator output and from the recorder chart for those peaks that remained on scale. Linear regression calculations were performed on the standards, and the slope and intercept so obtained were used to calculate the concentration of the sample solutions.

RESULTS AND DISCUSSION

Preliminary tests showed that, at levels < 50 ng of metabolite per ml of plasma, the peak height measurement by the integrator was more reliable than the area measurement. Integration would sometimes stop before the baseline was reached at the end of the peak, causing relatively large errors in the calculations based on peak area.

The peak height ratios of the plasma standards were subjected to a

Table IV—Recovery Data for Thioridazine Ring Sulfoxide (Slow Eluter) (ng/ml of Plasma) from Four Spiked Samples of Plasma

Sample	Sample[a] 1	2	3	4
1	28.8[b]	39.2	206.3	378.2
2	20.2	37.2	206.1	396.6
3	18.0	40.0	199.7	410.0
4	19.0	36.6	197.9	400.2
Mean	19.1	38.2	202.5	396.2
SD	1.10	1.61	4.33	13.33
CV, %	5.77	4.21	2.14	3.36
Added	20.7	41.4	206.9	413.8
Percent recovery	92.2	92.3	97.9	95.7

[a] For concentrations of other components in these samples, see Tables II, III, and V. [b] Sample contaminated; not used in calculation of mean, *SD*, and *CV*.

Table V—Recovery Data for Mesoridazine (ng/ml of Plasma) from Spiked Samples of Plasma

Sample	Sample[a] 1	2	3	4[b]
1	81.0	379.6	198.5	—
2	81.4	355.7	197.3	—
3	79.2	384.9	195.7	—
4	78.1	362.0	197.6	—
Mean	79.9	370.6	197.3	—
SD	1.55	13.9	1.17	—
CV, %	1.94	3.76	0.59	—
Added	79.5	397.4	198.7	20.0
Percent recovery	100.5	93.3	99.3	—

[a] For concentrations of other components in these samples, see Tables II–IV. [b] Level of mesoridazine in sample 4 not detectable in the presence of 400 ng of the ring sulfoxide/ml of plasma.

linear regression analysis (Table I). Each of the four compounds tested showed a linear response in the 10- to 500-ng/ml range. The peak height ratios calculated from the recorder chart could be used interchangeably with those calculated by the integrator. Occasionally the integrator failed to detect peaks at levels of 10–20 ng/ml. The recorder chart then provided a convenient backup for measurement of the peak height ratio.

Recoveries (Tables II–V) ranged from 87.8 to 100.5%, and coefficients of variation ranged from 0.6 to 7.0%. The largest variations were found for the ring sulfoxides in the 20- to 40-ng/ml range. The recovery of sulforidazine was not studied due to lack of sufficient material.

The results of the stability tests (Tables VI and VII) show that thioridazine and its metabolites are stable in frozen plasma for at least 3 weeks and are stable in plasma at room temperature for at least 24 hr.

Figure 3 shows the integrator curves for blank plasma, plasma with each standard added at the 100-ng/ml level, and a whole blood sample from a patient on thioridazine therapy. Figure 4 shows the recorder tracing of a plasma sample with each standard added at the 10-ng/ml level and that of a plasma blank with the internal standard added. The estimated detection limit for each standard in plasma is 2 ng/ml.

The HPLC system gives good resolution of thioridazine and its major metabolites. When used with the new sample extraction procedure and the new procedures for postcolumn extraction, oxidation, and fluorometric detection reported here, the system provides a highly sensitive and specific method of analysis with minimum interferences.

A modification of the procedure has been used to measure thioridazine and its metabolites in postmortem whole blood samples. Thioridazine,

Table VI—Stability of Compounds[a] in Frozen Plasma Samples (Sample 3)

Days in Freezer	Peak Height Ratios[b] I	II	III	IV
1	1.761	1.839	1.200	0.912
2	2.062	2.132	1.362	1.035
2	1.957	1.969	1.282	0.955
5	2.096	2.032	1.339	1.012
14	1.870	2.034	1.274	0.955
14	2.067	1.993	1.304	0.963
21	1.997	2.162	1.372	1.031
21	2.048	2.005	1.289	0.958
Mean	1.982	2.021	1.303	0.978
SD	0.1154	0.1000	0.0556	0.0435
CV, %	5.82	4.92	4.27	4.45

[a] I = thioridazine; II = thioridazine ring sulfoxide (fast eluter); III = thioridazine ring sulfoxide (slow eluter); IV = mesoridazine. [b] Standard peak height–internal standard peak height.

Table VII—Stability of Compounds[a] in Plasma Samples at Room Temperature

Hours	Peak Height Ratios[b] I	II	III	IV
0	1.951	1.805	1.223	0.911
1	1.870	1.744	1.142	0.862
2	1.971	1.818	1.216	0.922
5	2.073	1.844	1.228	0.919
24	1.963	1.842	1.195	0.920
Mean	1.966	1.811	1.201	0.907
SD	0.0723	0.0411	0.0350	0.0253
CV, %	3.66	2.25	2.95	2.80

[a] I = thioridazine; II = thioridazine ring sulfoxide (fast eluter); III = thioridazine ring sulfoxide (slow eluter); IV = mesoridazine. [b] Standard peak height–internal standard peak height.

sulforidazine, the two ring sulfoxides, and mesoridazine were detected at levels that ranged from 20 ng to 20 μg/ml[16].

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