GLC Analysis of Thioridazine, Mesoridazine, and Their Metabolites

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Abstract □ A GLC method for measuring thioridazine, mesoridazine, their metabolites, and possibly other phenothiazines was developed. By using this method, seven different phenothiazine derivatives, thioridazine, and six known thioridazine metabolites were extracted and separated. This method was tested by assaying plasma samples from 30 hospitalized psychiatric patients receiving thioridazine or mesoridazine.

Keyphrases □ Thioridazine and metabolites—GLC analysis, plasma □ Mesoridazine and metabolites—GLC analysis, plasma □ Phenothiazines—seven derivatives, GLC analysis, plasma □ GLC—analysis, thioridazine, mesoridazine, metabolites, and seven phenothiazine derivatives, plasma

Studies of thioridazine metabolism have been made difficult by the lack of specific methodologies for measuring thioridazine and its metabolites. The fluorometric method (1, 2), although sensitive, is not specific, because it measures the sum of the fluorescence of thioridazine and its metabolites (1, 3). Although phenothiazines and metabolites can be separated by TLC (3, 4), the components cannot be quantitated.

A GLC method specific for thioridazine was reported (5); but to study thioridazine metabolism and pharmacodynamics, a method capable of separating and quantitating thioridazine and its metabolites is needed. Therefore, the published method (5) was modified to measure thioridazine, mesoridazine [thioridazine (*S*)-sulfoxide], and five other known metabolites as well as other phenothiazines. Data are presented on the recovery, sensitivity, and precision of this method for thioridazine and mesoridazine and on the generality of the method. Plasma samples from 16 subjects receiving mesoridazine and 14 receiving thioridazine were analyzed with the described method (3).

EXPERIMENTAL

Reagents and Chemicals—Spectrophotometric grade heptane[1] was used; all other reagents were analytical reagent grade. Pure thioridazine, mesoridazine, and their metabolites[2] were used in the stock solutions, spiked plasma samples, and standards. Thioridazine, mesoridazine, thioridazine (*R*)-sulfoxide, and northioridazine (*S*)-sulfoxide were all used as the hydrochloride salts. Thioridazine disulfoxide, thioridazine (*S*)-sulfone, and northioridazine were used as the free bases. Chlorpromazine hydrochloride[3] was the internal standard.

The other phenothiazine drugs (perphenazine, trifluoperazine, prochlorperazine, and fluphenazine) were obtained[4] as tablets or ampuls, solubilized in distilled water, and used as such.

Plasma Samples—Spiked plasma samples were prepared by adding measured volumes of concentrated stock solution (0.5–1.0 mg/ml) to known measured quantities of pooled plasma[5]. Plasma dilutions due to addition of drugs were always less than 4%. Spiked plasma samples to be used as standards were prepared in a similar way but in bulk quantities (80–160 ml). Aliquots, 4 ml, were immediately pipetted into separate tubes, covered with parafilm, and kept frozen at −20° until use.

Plasma samples were also obtained from hospitalized psychiatric subjects receiving thioridazine (14 subjects) or mesoridazine (16 subjects). The plasma samples were frozen within 2 hr after blood was drawn and were kept frozen until use.

Extraction Procedure—To a 4-ml sample of plasma was added 2.5 ml of 1 *N* NaOH, and the solution was extracted twice with 5 ml of *n*-heptane–toluene (4:1 v/v) in a 15-ml screw-capped[6] culture tube for 30 min using a wrist-action shaker[7]. All samples for one or more patients and the standards were placed horizontally in a carrier box and shaken together.

The tubes were centrifuged and, if needed, the emulsion at the interface was broken up by stirring or freezing and the tubes were recentrifuged. The recovered heptane–toluene mixtures were pooled and transferred to a clean culture tube and similarly extracted with 2 ml of 0.1 *N* HCl for 30 min. The acid aqueous layer was then split into two aliquots: 0.5 ml for the spectrofluorometric assay and 1.4 ml for the GLC assay.

Fluorometric Assay—The fluorometric assay of Pacha (2) was used. To the 0.5-ml aliquot of the 0.1 *N* HCl layer were added 0.5 ml of 0.2 *N* H_2SO_4 and 100 μl of 0.1% $KMnO_4$. After 5 min, 100 μl of 0.2% H_2O_2 was added, and the fluorescence was read[8] at 355-nm excitation and 440-nm emission.

GLC Assay—To the 1.4-ml acid aliquot was added 0.8 ml of 2 *N* NaOH. The solution was extracted for 30 min with 100 μl of heptane–toluene containing chlorpromazine as the internal standard. The chlorpromazine–heptane–toluene mixture was prepared fresh each day of an experiment by mixing 100 μl of an ethanol mixture containing 8 mg of chlorpromazine hydrochloride/10 ml of absolute ethanol and 1.5 ml of heptane–toluene. For this extraction, glass-stoppered, conical, 3-ml tubes were shaken horizontally with a wrist-action shaker in a specially constructed carrier box.

GLC was performed using a chromatograph[9] with dual flame-ionization detectors. Two- to three-microliter aliquots of samples and standards were injected into 3% OV-17 on Chromosorb Q (100–120-mesh[10]) columns, 0.6 cm (0.25 in.) o.d., 1.8 m (6 ft) long, and 2 mm i.d. The conditions were as follows: column oven temperature, 275°; all other temperatures, 325°; column helium flow, 100 ml/min; hydrogen flow, 43 ml/min; and makeup helium flow, 108 ml/min. It is not essential to know the volume applied to the column since the same amount of chlorpromazine is present in all samples and can be used to normalize the calculations regardless of the actual concentration volumes.

The samples were run as are and, where needed, the heptane–toluene solution was concentrated by reduced pressure aspiration and rerun. The samples were concentrated to maximize the signal-to-noise ratio and, thus, increase the minimum detectable quantity. The concentration was performed in the same 3-ml conical centrifuge tubes after most of the aqueous layer was removed with a disposable pasteur pipet. This procedure adjusted the heptane layer in the narrow, conical section of the tube, facilitating removal of the organic layer with the microliter syringe for the GLC assay.

[1] Mallinckrodt Chemical Works, St. Louis, MO 63147
[2] Sandoz Pharmaceuticals, Hanover, NJ 07936
[3] Smith Kline and French Laboratories, Philadelphia, PA 19101
[4] Pharmacy of the Orange County Medical Center, Orange, Calif.
[5] Clinical Laboratory, Department of Pathology, Orange County Medical Center, Orange, Calif.
[6] Lined with Teflon (du Pont).
[7] Burrell Corp., Pittsburgh, Pa.
[8] Aminco-Bowman spectrophotofluorometer, American Instrument Co., Silver Spring, MD 20910
[9] GC-4 and Cliniscreen, Beckman Instruments, Fullerton, Calif.
[10] Applied Science Laboratories, Inc., State College, PA 16801

Table I—Effect of Concentrating the Heptane–Toluene Layer on the GLC Results

	Chlorpromazine				Mesoridazine			
Concentration	1×	3.8×	1×	3.6×	1×	3.8×	1×	3.6×
Peak height × attenuation × 10^{-3}, mm	60	246	64	212	3.1	9.8	3.5	13.2

Table II—Comparison of Fluorescence and GLC Mesoridazine Assays for Patient LB

	Time after Drug Administration, hr				
Assay Method	1	4	8	24	48
Fluorescent concentration, μg/ml plasma	4.25	5.10	2.98	1.49	0.43
GLC concentration, μg/ml plasma	3.03	4.11	0.98	0.35	—

Calculations—Three stock thioridazine, mesoridazine, and/or metabolite standards, 1, 5, and 10 μg/ml in 4 ml of plasma, kept frozen until use, were run together with the unknown samples. The response of the flame-ionization detector per microgram of drug was averaged over the three standards, and the average was used to calculate the concentration in the subjects' plasma samples. The equation used for the calculation incorporates corrections and normalizing factors for the volume of heptane–toluene recovered and for the concentrate volume differences as measured by the internal standard.

RESULTS AND DISCUSSION

To ascertain the effect of concentration on chlorpromazine and mesoridazine peak heights, several experiments were carried out as described under *Experimental*. Then, with a 100-μl syringe, most of the final heptane–toluene layer containing chlorpromazine and mesoridazine was removed, measured, and decanted into the conical part of a 3-ml conical centrifuge tube. An aliquot was assayed by GLC, the sample was concentrated by reduced pressure aspiration, the volume was measured with the syringe, and aliquots of the concentrate were assayed by GLC. The results in Table I show that the chlorpromazine peak heights reflected the concentration volumes and, therefore, could be used as a measure for the heptane–toluene volume.

Table II compares the results of fluorometric and GLC mesoridazine assays for Patient LB after a single intramuscular dose of 2 mg/kg. The fluorometric results were always greater than the GLC results, especially at the later times when little mesoridazine was measured by GLC while considerable amounts were seemingly still measurable by fluorescence. Fluorometrically, one is seeing a summation of the fluorescence of mesoridazine and its metabolites, while mesoridazine alone is measured by GLC. At the later times, metabolites are predominantly present, resulting in the greater disparity between the specific GLC and the nonspecific fluorometric results at these later times.

A GLC standard curve for mesoridazine was prepared using triplicate 4-ml spiked plasma samples and increasing concentrations of mesoridazine from 0.5 to 5 μg/ml. The results fell in a straight line. The line through the experimental points did not go through the origin, however, indicating a loss of drug during the assay procedure independent of drug concentration, perhaps due to nonspecific drug adhesion to the glassware. The drug lost in this way was less than 0.3 μg/assay. The mean and standard deviation of the mesoridazine peak height normalized to an attenuation of one for the 0.5-, 1.0-, 2.0-, 2.5-, and 5.0-μg/ml samples were 250 ± 115, 740 ± 105, 2100 ± 165, 2600 ± 225, and 5750 ± 135 mm, respectively.

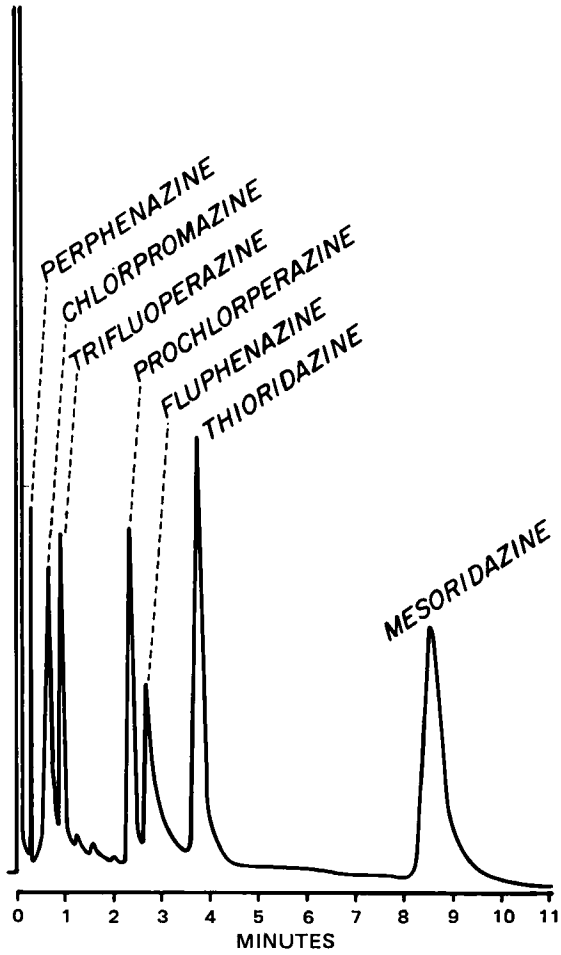

Figure 1—*Gas–liquid chromatogram of a mixture of seven phenothiazine derivatives. The seven phenothiazines were first run singly to find their elution time; then they were pooled and the mixture was injected into the gas chromatograph. The chromatographic conditions were set as indicated under* Experimental.

For a 4-ml plasma sample, the minimum detectable quantity was about 0.3 μg/ml of plasma without concentration. The minimum detectable quantity obtained with the inclusion of the concentration step was 0.05 μg/ml of plasma. The three standards should be included with every assay of unknown samples because of the slight fluctuations in sensitivity. The flame-ionization detector must be carefully optimized for good sensitivity. Relatively minor changes in helium or hydrogen flow can cause disproportionate changes in sensitivity.

The recoveries of parent drug and metabolites from water and plasma as measured by GLC are listed in Table III. The recoveries from water, especially for mesoridazine and thioridazine (*R*)-sulf-

Table III—Recoveries of Thioridazine, Mesoridazine, Thioridazine (*R*)-Sulfoxide, and Thioridazine (*S*)-Sulfone from Water and Plasma as Measured by GLC

	Thioridazine	Mesoridazine	Thioridazine (*S*)-Sulfone	Thioridazine (*R*)-Sulfoxide
Recovery from water, mean ± *SD* (*n* = 3), %	78 ± 0.5	83 ± 0.9	95 ± 0.5	87 ± 1.2
Recovery from plasma, mean ± *SD* (*n* = 8), %	77 ± 6	56 ± 4	82 ± 4	38 ± 3

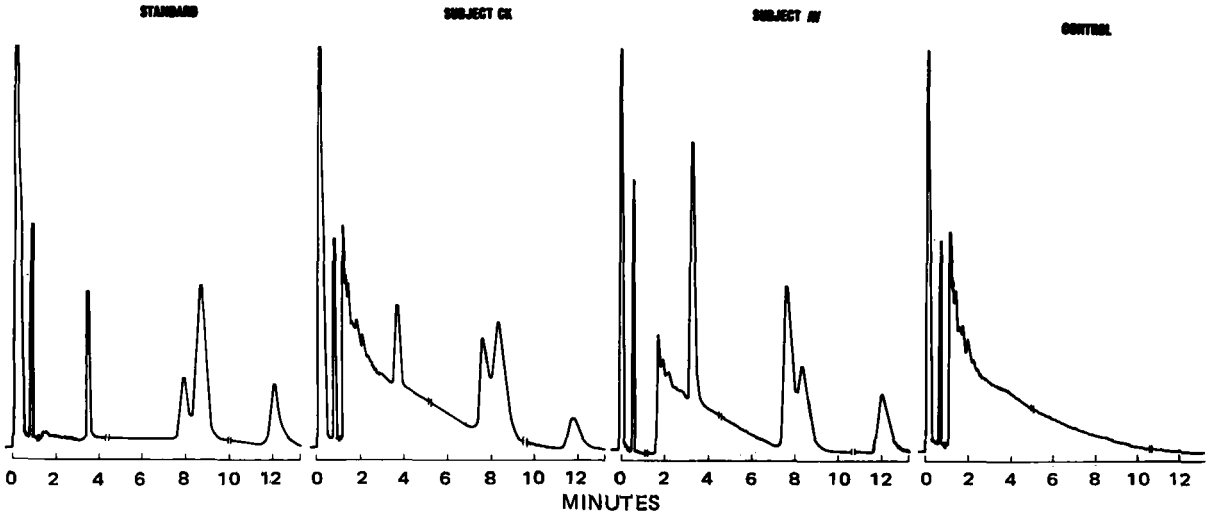

Figure 2—*Gas–liquid chromatograms of a standard, a control, and plasma from two hospitalized psychiatric subjects. The indentation marks (—‖—) signify changes in attenuation from 32,000 to 3200 to 1600 to 160 for the 5-μg/ml plasma standard and from 32,000 to 200 to 80 to 20 for the subject and control plasma samples at about 1.5, 4.5, and 10 min, respectively. Chlorpromazine at 0.6 min was used as the internal standard. Thioridazine, mesoridazine, thioridazine (S)-sulfone, and thioridazine (R)-sulfoxide eluted at about 3.4, 7.9, 8.3, and 12.1 min, respectively.*

oxide, were considerably greater than those from plasma. These data suggest a differential affinity of thioridazine and its metabolites for plasma binding sites. No corrections need to be made for affinity differences because the standards run concurrently with the unknown samples will have the same differential affinities and will thus correct for them.

The possibility that different plasma samples show different affinities for the drug and/or metabolites was investigated by using eight different plasma samples spiked with thioridazine and metabolites. The results (Table III) demonstrate that the differences in observed recoveries were within experimental error. The coefficients of variation for the plasma samples were 7.7, 7.1, 4.8, and 7.9% for thioridazine, mesoridazine, thioridazine (*S*)-sulfone, and thioridiazine (*R*)-sulfoxide, respectively.

The generality of the method is seen in Fig. 1 and Table IV. Figure 1 is a chromatogram of seven different phenothiazines pooled for this experiment. Table IV shows the retention time of several metabolites of thioridazine. The disulfoxide and disulfone metabolites are too polar to be optimally extracted into the heptane–toluene solvent at the pH indicated; however, they are readily extracted by methylene chloride.

A study of the pharmacodynamics of thioridazine and mesoridazine (3) showed a major unknown metabolite in the plasma of 21 human subjects receiving thioridazine or mesoridazine. This metabolite has now been identified (6) as thioridazine (*S*)-sulfone. The plasma concentration of thioridazine for nine subjects given a daily dose of thioridazine at 4 mg/kg po ranged from 1.35 to 6.87 μg/ml of plasma by the fluorometric method, with thioridazine hydrochloride as the standard, and from 0.16 to 2.1 μg/ml of plasma by the specific GLC method. Typical chromatograms of a standard and subject's plasma before and after drug treatment are shown in Fig. 2.

Table IV—Retention Time of Thioridazine and Its Metabolites.

	Minutes[a]
Thioridazine	3.6
Thioridazine (*S*)-sulfoxide	8.3
Thioridazine (*R*)-sulfoxide	12.8
Northioridazine	3.8
Northioridazine (*S*)-sulfoxide	8.7
Thioridazine disulfone	22.8[b]
Thioridazine disulfoxide	20.9[b]
Unknown component	9.1

[a] The elution time was measured from the rising leg of the solvent peak. [b] The extraction solvent was methylene chloride.

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