Gas-Chromatographic Analysis for Chlorpromazine and Some of Its Metabolites in Human Serum, with Use of a Nitrogen Detector

David N. Bailey and John J. Guba

We describe a gas-chromatographic method for measuring chlorpromazine and its metabolites chlorpromazine sulf oxide, mono-N-desmethylechlorpromazine, and di-N-desmethylechlorpromazine at therapeutic concentrations in human serum, with use of a nitrogen detector. The compounds are extracted from serum at pH 10.5 into hexane/isoamyl alcohol, re-extracted into dilute HCl, and then extracted into hexane after alkalization of the HCl. The N-desmethylated metabolites are measured as their respective N-trifluoroacetyl derivatives; the parent drug and its sulfoxide are measured as the unchanged bases. Promazine is the internal standard. As little as 5 μg of chlorpromazine, 20 μg of chlorpromazine sulfoxide, 20 μg of mono-N-desmethylechlorpromazine, and 10 μg of di-N-desmethylechlorpromazine per liter can be measured in 2-mL samples of serum. The within-run coefficients of variation for assays of these drugs at 100 μg/L are 2.7%, 5.6%, 5.1%, and 5.3%, respectively. The procedure was applied to patients receiving therapeutic doses of chlorpromazine and to patients who had ingested an overdose of chlorpromazine.

Chlorpromazine (CPZ); Thorazine, Smith Kline & French Labs. has been widely prescribed for more than 25 years as an effective antipsychotic agent for the treatment of schizophrenia. Despite the long history of the drug, relatively little is known about concentrations of the compound in serum. Because of its extensive catabolism (18 metabolites have been postulated, 1), the parent drug and metabolites attain concentrations of only micrograms per liter in serum, rendering their measurement difficult.

CPZ has been measured by various techniques: colorimetry (2), thin-layer chromatography with densitometry (3), spectrofluorometry (4, 5), and radioassay (6). However, these methods are tedious and usually too insensitive to permit measurement of concentrations in serum after administration of therapeutic doses of the drug. The now-classic electron-capture gas–liquid chromatographic analysis of Curry (7) was the first procedure that was sufficiently sensitive to detect CPZ and some of its relatively nonpolar metabolites in serum. However, that method required multiple extractions, as did subsequent electron-capture methods (8-10), with which only the parent drug could be detected and which were susceptible to contamination with the halogens that are ubiquitous in the clinical laboratory. More recently, radioimmunoassays have been developed for CPZ (11-13); however, the antibodies involved cross react with many CPZ metabolites, greatly diminishing the specificity of the assay. Mass fragmentography has been successfully used for measuring serum CPZ and its metabolites (14, 15) but for technical and economic reasons is not available in most clinical laboratories.

Linnoila and Dorrity (16) recently reported use of gas–liquid chromatography with a nitrogen detector for measurement of CPZ in serum. However, with their method only the mono-N-desmethylated metabolite is measured in addition to the parent drug, and diazepam, a sedative-hypnotic frequently prescribed in the population being considered, interferes.

We report here a procedure for measurement of CPZ and its metabolites chlorpromazine sulf oxide (CPZSO), mono-N-desmethylechlorpromazine (Nor1CPZ), and di-N-desmethylechlorpromazine (Nor2CPZ) at therapeutic concentrations in human serum (Figure 1). We applied the procedure both to patients receiving therapeutic doses of CPZ and to patients who had ingested an overdose of the drug.

Materials and Methods

Apparatus

We used a Model 3920B gas–liquid chromatograph equipped with a nitrogen–phosphorus detector (Perkin-Elmer Corp., Norwalk, CT 06852) and a coated glass column (1.8 m × 2 mm i.d.) containing 3% OV-17 on “Chromosorb W,” 80/100 mesh (Pierce Chemical Co., Rockford, IL 61105).

Reagents and Standards

All reagents were analytical (AR) grade:

- Hexane, distilled in glass (Burdick & Jackson Labs., Inc., Muskegon, MI 49442).
- Isoamyl alcohol. Redistill before use.
- Hexane/isooamy alcohol, 98/2 by vol.
- Methanol, distilled in glass (Burdick & Jackson Labs.).
- Na2CO3 anhydrous.
- Na2CO3, saturated aqueous solution. Wash with hexane before use.
- Na2SO4, anhydrous.
- HCl, 0.1 mol/L. Wash with hexane the distilled water used to prepare this solution.
- Trifluoroacetic anhydride (Pierce Chemical Co.). Redistill before use.
- Stock standards in methanol

Store all standards in the freezer (−20 °C). Prepare at least once a month.

Promazine hydrochloride (internal standard), 1.00 g/L. Dissolve 10 mg of promazine hydrochloride (Wyeth Labora-
Amitriptyline 

I 

Lu 

B 

ID 

Phenacetin 

and 

Fig. 1. Structures of (A), chlorpromazine (R1 = −CH3, R2 = −CH3), mono-N-desmethylchlorpromazine (R1 = −H, R2 = −CH3), and di-N-desmethylchlorpromazine (R1 = −H, R2 = −H); and (B) chlorpromazine sulfoxide.

Fig. 2. Chromatograms of twofold-diluted serum extracts from patient who had ingested 1800 mg of chlorpromazine in overdose.

Table 1. Common Basic Drugs Evaluated for Interference with Assay

<table>
<thead>
<tr>
<th>Drug</th>
<th>Interference with Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amitriptyline</td>
<td>Methamphetamine</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>Methapyriline</td>
</tr>
<tr>
<td>Benzphetamine</td>
<td>Methaqualone</td>
</tr>
<tr>
<td>Caffeine</td>
<td>Methylphenidate</td>
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<tr>
<td>Chlorzepate</td>
<td>Methyprylon</td>
</tr>
<tr>
<td>Chlordiazepoxide</td>
<td>Morphin</td>
</tr>
<tr>
<td>Chlorothiazide</td>
<td>Nortripylone</td>
</tr>
<tr>
<td>Chlorpheniramine</td>
<td>Oxazepam</td>
</tr>
<tr>
<td>Codeine</td>
<td>Perphenazine</td>
</tr>
<tr>
<td>Desipramine</td>
<td>Phencetin</td>
</tr>
<tr>
<td>Diazepam</td>
<td>Prochlorperazine</td>
</tr>
<tr>
<td>Diphenhydramine</td>
<td>Promethazine</td>
</tr>
<tr>
<td>Flurazepam</td>
<td>Protriptylone</td>
</tr>
<tr>
<td>Fluphenazine</td>
<td>Quinine</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>Hydrochlorothiazide</td>
<td>Thoridazine</td>
</tr>
<tr>
<td>Imipramine</td>
<td>Trifluoperazine</td>
</tr>
<tr>
<td>Meperidine</td>
<td>Trihexyphenidyl</td>
</tr>
<tr>
<td>Methadone</td>
<td>Tripelennamine</td>
</tr>
</tbody>
</table>

* Extracts and chromatographs with the internal standard, promazine.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Interference with Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nortriptylane</td>
<td>Methamphetamine</td>
</tr>
<tr>
<td>Prochlorperazine</td>
<td>Methaqualone</td>
</tr>
<tr>
<td>Trihexyphenidyl</td>
<td>Methylphenidate</td>
</tr>
<tr>
<td>Methyprylon</td>
<td>Morphin</td>
</tr>
<tr>
<td>Phencetin</td>
<td>Perphenazine</td>
</tr>
<tr>
<td>Protriptylone</td>
<td>Oxazepam</td>
</tr>
<tr>
<td>Trifluoperazine</td>
<td>Quinine</td>
</tr>
</tbody>
</table>

solution to each 100 mL of hexane/isomyl alcohol used for the initial extraction. This provides 250 mg of internal standard per milliliter of serum extracted. Store in an amber-colored glass bottle. This reagent is stable for one month.

Mixed chlorpromazine standard in methanol, 10 mg of each per liter. Combine 100 mL (100 µg) of each of the 1.00 g/L stock solutions of CPZ, CPZSO, Nor1CPZ, and Nor2CPZ. Evaporate the mixture in a gentle current of nitrogen, and dissolve the residue in 10 mL of methanol. Prepare this standard at the time of analysis.

Working standards in serum

Mixed chlorpromazine standard in serum, 300 µg/L. Evaporate 300 µL (3 µg) of the dilute mixed CPZ standard with a gentle current of nitrogen. Dissolve the residue in 10 mL of drug-free serum. For working standards in serum, appropriately dilute the 300 µg/L mixed serum standard with drug-free serum to produce the desired concentration(s) (usually 5–300 µg/L). Prepare at the time of analysis.

Mixed CPZ control in serum, 200 µg/L. Evaporate 200 µL (2 µg) of the dilute mixed CPZ standard with a gentle current of nitrogen. Dissolve the residue in 10 mL of drug-free serum. The control is stable for four weeks if stored frozen (−20 °C).

Procedure

All glassware was soaked in HCl, 1 mol/L, overnight and then rinsed with water, followed by hexane, before use. Blood samples were drawn into commercial evacuated glass tubes containing no anticoagulant (Vacutainers; Becton-Dickinson, Div. Becton, Dickinson and Co., Rutherford, NJ 7070). The samples were centrifuged and the serum frozen (−20 °C) until analyzed.

“Extract 1” (CPZ, CPZSO). Pipet 2 mL of serum (samples, serum standards, and serum control) into 15-mL glass culture tubes equipped with Teflon-lined screw caps. Adjust the pH to about 10.5 by adding 0.5 mL of saturated Na2CO3. Add 10 mL of hexane/isomyl alcohol containing the internal standard, and extract for 15 min on a mechanical shaker (medium speed). Centrifuge (3000 rpm for 5 min) and transfer the sol-
vent (upper) layer to a second 15-mL screw-capped tube. Add 2 mL of HCl, 0.1 mol/L, extract for 15 min, and, after centrifugation, aspirate and discard the solvent (upper) phase. Wash the acid for 1 min with 2 mL of hexane (no internal standard or isomyl alcohol), centrifuge, and discard the solvent (upper) layer. With a calibrated spatula add about 500 mg of anhydrous Na₂CO₃ to the acid, to adjust the pH to about 10.5. Mix well and extract for 5 min with 2 mL of hexane (no internal standard or isomyl alcohol). After centrifugation, transfer the solvent (upper) layer to a 15-mL glass centrifuge tube and dehydrate it by adding about 500 mg of anhydrous Na₂SO₄ (with a calibrated spatula). Transfer the dried extract to a 5-mL glass centrifuge tube. Evaporate the solvent at room temperature under a gentle current of nitrogen.

Dissolve the extract residue in 10 μL of methanol and chromatograph 4 μL under the following conditions: column temperature program, 270 °C (initial temperature, 4-min hold), 32 °C/min to 290 °C (final temperature, 4-min hold); injector temperature, 250 °C; detector temperature, 300 °C; bead temperature, approximately 350–400 °C; carrier gas (nitrogen) flow rate, 15 mL/min; hydrogen flow rate, 1–2 mL/min; amplifier setting, × 1. Identify CPZ and CPZSO from their respective retention times relative to that of the promazine internal standard: promazine, 1.00; CPZ, 1.35; and CPZSO, 2.95. Calculate the peak-height ratio (ratio of the peak height of each compound to that of the promazine internal standard) for each sample and determine the serum concentration by comparison with the peak-height ratios (relative peak heights) of the extracted serum standards. The N-desmethylated metabolites, Nor₁CPZ and Nor₂CPZ, if present, are not resolved under these conditions and are co-eluted at a relative retention time of 1.65 (Figure 2A). They must be measured in a separate extract of serum (“extract 2”).

“Extract 2” (Nor₁CPZ, Nor₂CPZ). If the N-desmethylated metabolites were noted in “extract 1,” perform the previously outlined extraction procedure on another 2-mL aliquot of serum. Add 100 μL of trifluoroacetic anhydride to the final hexane extract and vortex-mix for 30 s. Evaporate the solvent at room temperature under a gentle current of nitrogen. Be certain that the odor of trifluoroacetic anhydride is no longer detectable after evaporation.

Dissolve the extract residue in 10 μL of methanol and chromatograph 4 μL, using a column temperature of 245 °C (isothermal). The other chromatographic conditions are the same as for “extract 1.” Under these conditions, Nor₁CPZ and Nor₂CPZ are eluted at relative retention times of 2.95 and 2.40, respectively, compared with a relative retention time for promazine of 1.00 (Figure 2B). Because trifluoroacetic anhydride reduces CPZSO to CPZ, yielding “total CPZ,” CPZSO and CPZ cannot be determined separately in “extract 2.” Identify the N-desmethylated metabolites from their relative retention times and calculate their concentrations from the peak-height ratios as for “extract 1.”

Before analyzing samples and standards, it is desirable to adjust the current setting for the nitrogen-detector bead so that the response to 4 μL (40 ng) of the mixed CPZ standard in methanol is kept constant from day to day.

**Results**

A standard curve was prepared by supplementing drug-free serum with the mixed CPZ standard. A plot of concentration against relative peak heights was linear (r = 0.99) for each compound in the concentration range studied (5–300 μg/L). Regression equations by the least-squares method were: CPZ, \( y = 0.0014x + 0.0037 \); CPZSO, \( y = 0.0005x - 0.0042 \); Nor₁CPZ, \( y = 0.0009x - 0.0033 \); and Nor₂CPZ, \( y = 0.010x - 0.0042 \). The analysis of 12 aliquots of serum supplemented to a concentration of 100 μg/L with the mixed CPZ standard gave the following coefficients of variation within-run: CPZ, 2.7%; CPZSO: 5.6%; Nor₁CPZ, 5.1%; and Nor₂CPZ, 5.3%. Ten measurements of a serum pool (300 μg/L) over four weeks gave coefficients of variation between-run as follows: CPZ, 16.7%; CPZSO, 16.8%; Nor₁CPZ, 18.7%; and Nor₂CPZ, 14.1%. The concentrations of the four compounds in serum showed no decrease with time over the four weeks when frozen (−20 °C).

The uncorrected analytical recoveries were calculated by comparing peak heights obtained from supplemented serum with those of nonextracted methanolic standards. The values were: CPZ, 25%; CPZSO, 20%; Nor₁CPZ, 40%; and Nor₂CPZ, 30%. These incomplete recoveries, as well as variations in taking aliquots, are corrected by the use of extracted serum standards and an internal standard (promazine).

Ten different drug-free sera analyzed by the proposed procedure yielded no interfering peaks. Concentrations per liter of as little as 5 μg of CPZ, 20 μg of CPZSO, 20 μg of Nor₁CPZ, and 10 μg of Nor₂CPZ could be measured in 2 mL of serum.

Forty common basic drugs (Table 1) were screened for interference in the analysis.
terference in the proposed assay by comparing retention times of their nonextracted methanolic solutions with those of CPZ, CPZSO, Nor1CPZ, and Nor2CPZ under the conditions of the assay ("extract 1" and "extract 2"). Acidic drugs were not screened because they would not be extracted under the conditions of the assay (pH 10.5). Benztropine (Cogentin; Merck Sharp & Dohme), desipramine (Pertofrane; USV Pharmaceutical), nortriptyline (Aventyl; Lilly), promethazine (Phenergan; Wyeth), and protriptyline (Vivactil; Merck Sharp & Dohme) had the same retention time as the promazine internal standard under the conditions for "extract 1." Under the assay conditions for "extract 2," flurazepam (Dalmane; Roche) had the same retention time as the promazine internal standard, and chlorazepate (Tranxene; Abbott) had the same retention time as Nor2CPZ. Furthermore, these interferences persisted when drug-free serum was supplemented to a concentration of 100 µg/L with the drugs and extracted. Sera from patients receiving any of the six drugs extracting and chromatographed as promazine would thus show falsely depressed concentrations of chlorpromazine and its metabolites because of the enhanced chromatographic response of the internal standard. Sera from patients receiving chlorazepate concurrently with chlorpromazine would show falsely increased concentrations of Nor2CPZ.

We analyzed sera from patients who had received therapeutic doses of chlorpromazine and patients who had ingested nonfatal overdoses of the drug (Table 2). The chromatograms for one analysis are shown in Figure 2.

Discussion

Gas-liquid chromatography with a nitrogen detector is rapidly being integrated into the clinical laboratory because of the much greater sensitivity and selectivity relative to that of the more conventional flame-ionization detector. Its ease of use, inexpensiveness, and ready adaptability to almost all gas chromatographs are additional features. The dramatically enhanced sensitivity afforded by the system has rendered it especially applicable to the analysis of psychoactive drugs, which usually attain only submicrogram per milliliter concentrations in serum (16-19); the sensitivity approaches that of mass fragmentography, which at the present time is not feasible for most clinical laboratories.

Serum concentrations of CPZ after therapeutic doses (Table 2) were in the same general range as that reported by others (7, 10) except for the first sample from patient 4. We suspect, however, that this sample was obtained shortly after ingestion of the drug (before steady-state equilibrium was achieved). The total concentrations (sum of parent drug and metabolites) for the entire series ranged from 14 to 3888 µg/L and generally increased with increasing dosage. Patients 4 and 7, for each of whom two samples were available, showed decreasing concentrations with time.

In the proposed analysis two separate serum extractions are required to resolve CPZ and its metabolites. The N-desmethylated metabolites are measured in a separate extract as their N-trifluoroacetyl derivatives, to achieve peak resolution that is not possible otherwise. "Extract 1" should be analyzed first, in that it serves as a "screening" procedure for the presence of Nor1CPZ and Nor2CPZ. If no peak (Nor1CPZ plus Nor2CPZ) is detected at a relative retention time of 1.65, the second extraction need not be performed. For samples with insufficient volume to permit a second extraction, "extract 1" may be divided into two aliquots and derivatization performed on one aliquot. However, this results in injection of decreased amounts of the compounds into the gas chromatograph.

Generation of the N-trifluoroacetyl derivatives of the N-desmethylated metabolites was confirmed by mass fragmentography of the pure compounds before and after treat-

ment with trifluoroacetic anhydride (Olfax IIA; Vitex Systems, Inc., Div. McDonnell Douglas Corp., Hazelwood, MO 63042). The reduction of CPZSO to CPZ, also reported by others (15), was similarly confirmed.

The recovery of CPZ and its metabolites from serum was improved 50% by increasing the extraction time from 5 min (initially studied) to the now-recommended 15 min. Extraction for 30 min did not enhance the recoveries further. Incomplete recovery was compensated for by the use of extracted serum standards.

In contrast to other nitrogen-detector assays (17-19), we did not encounter interference from the tri(2-butoxyethyl)phosphate contaminant present in Vacutainer Tubes. Analysis of drug-free serum agitated and frozen in Vacutainer Tubes did not show interfering peaks in the chromatogram.

Like Curry (7), we found CPZ to be stable in serum stored frozen in the dark (−20 °C), which is in contrast to the report of Linnoila and Dorrity (16). However, our samples were not permitted to contact the rubber stopper of the Vacutainer for any appreciable length of time. In addition, our samples for the stability study were stored in new glass culture tubes with Teflon-lined screw caps.

Six drugs were extracted and chromatographed with the internal standard, promazine. However, except for benztropine and flurazepam, the six drugs are rarely prescribed concurrently with CPZ. Benztropine is administered in very low doses and flurazepam is extensively metabolized so that interferences would be expected to be minimal. If any of the six drugs is to be present, either from the clinical history or from preliminary screening without the addition of internal standard, another compound can be substituted for promazine. Promazine was selected for the proposed assay because of its similarity in structure and solubility to CPZ. The compounds differ only by the presence of chloride in CPZ.

Chlorazepate was extracted and chromatographed with Nor2CPZ but is extensively metabolized (to nordiazepam) so that interference is expected to be minimal.

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We acknowledge gifts of the following drug samples from pharmaceutical companies: SmithKline French Labs., Division of SmithKline Corp., Philadelphia, PA (chlorpromazine hydrochloride, chlorpromazine sulfoxide hydrochloride, mono-N-desmethylchlorpromazine hydrochloride, di-N-desmethylchlorpromazine hydrochloride, trifluoperazine dihydrochloride, prochlorperazine dimaleate); Wyeth Lab., Inc., Philadelphia, PA (promazine hydrochloride, promethazine hydrochloride); Merck Sharp & Dohme Research Lab., Division of Merck & Co., Inc., Rahway, NJ (amitriptyline hydrochloride, protriptyline hydrochloride, benztropine mesylate); Eli Lily

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4 We have not attempted to use more-polar solvents for the second extraction step to improve recovery, because of the possibility of coextracting polar contaminants if present. We realize that the first extraction step has probably eliminated most of such polar contaminants, but the presence of isomyl alcohol in the initial extraction solvent may result in the recovery of trace polar contaminants, which would be carried through the extraction if polar solvents were used for the second extraction. We have not tried using silanized glassware, again because of our fear of introducing contaminants in the process. Rather, we used new glassware with Teflon-lined caps whenever caps were exchanged. Furthermore, all glassware was acid-washed and solvent-rinsed before use. We felt that the use of extracted serum standards and an internal standard would compensate for most (but perhaps not all) losses.

5 We used Vacutainer Tubes because our samples were gleaned from residual serum submitted to our clinical laboratory. However, in each case the blood was centrifuged immediately upon arrival in the laboratory so that we suspect the contact of blood with the rubber stopper was negligible.

References