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High-Performance Liquid Chromatographic Assay for Nanogram Determination of Chlorpromazine and Its Comparison with a Radioimmunoassay

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Received June 6, 1980, from the **College of Pharmacy, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0, Canada*, the ‡*Drug Research Laboratories, Health Protection Branch, Health and Welfare Canada, Ottawa, Ontario K1A 0L2, Canada*, and the §*Faculty of Pharmacy, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada.* Accepted for publication February 17, 1981.

Abstract □ A specific and sensitive high-performance liquid chromatographic (HPLC) method for the quantitative determination of plasma chlorpromazine concentrations is described. The procedure is capable of determining 1 ng of chlorpromazine/ml and is adequate for following plasma concentration–time profiles after 7-mg single intravenous doses. After a simple organic extraction of the drug and an internal standard (mesoridazine) from plasma, the organic layer was transferred to a vial and evaporated to dryness at 55° under nitrogen. The residue was dissolved in 200 μl of HPLC grade acetonitrile. Aliquots (70–100 μl) were chromatographed, and the drug was quantitated in the range of 1–15 ng/ml of plasma using a fixed-wavelength UV detector. Plasma concentrations determined by the method were compared with those obtained by a previously reported radioimmunoassay specific for chlorpromazine and *N*-desmethylchlorpromazine. The two methods agreed favorably with a correlation coefficient of 0.993 and a slope of 0.994.

Keyphrases □ Chlorpromazine—high-performance liquid chromatographic analysis in plasma and comparison with a radioimmunoassay □ Psychotropic agents—chlorpromazine, high-performance liquid chromatographic analysis □ Antipsychotic phenothiazines—chlorpromazine, high-performance liquid chromatographic analysis and radioimmunoassay comparison

Chlorpromazine is widely used in the treatment of certain psychiatric disorders (1). It undergoes extensive metabolism, and several of its metabolites (2) are considered to be psychoactive. Some of the metabolites can be quantitated in plasma; in some studies, the ratio of plasma concentrations of active to inactive metabolites was correlated with clinical improvement in schizophrenic patients (3–5).

The various chemical methods of quantitation that have been used include GLC with electron-capture detection (6), GLC with mass spectrometric detection (7, 8), fluorescent labeling with dansyl chloride (9), labeled derivative formation (10), and TLC of a quaternary ammonium derivative formed by reaction with 9-bromomethylacridine, followed by UV photolysis and spectrofluorometric determination (11). These methods may have adequate sensitivity to determine plasma concentrations following therapeutic dosage regimens, but they are generally cumbersome and not easily amenable to routine clinical monitoring.

The radioimmunoassay procedures are generally simple, sensitive, and readily applicable to routine analysis. Although sensitive, radioimmunoassays generally are suspect from the aspect of specificity. In the case of chlorpromazine radioimmunoassay (12–14), this specificity concern is augmented by the extremely large number of identified metabolites. To verify the specificity of the radioimmunoassay procedure reported previously (14), which was based on proper designing of the antibody (15), high-performance liquid chromatographic (HPLC) assay, specific and sensitive to 1 ng/ml of plasma, was developed. This HPLC assay is described and compared with the radioimmunoassay reported earlier (14).

EXPERIMENTAL

Materials—Chlorpromazine hydrochloride[1], prochlorperazine[1], mesoridazine besylate[2], 2-chlorophenothiazine[3], chlorpromazine sulfoxide[4], 7-hydroxychlorpromazine sulfoxide[4], *N*-monodesmethylchlorpromazine[4], *N*-monodesmethylchlorpromazine sulfoxide[4], *N*-didesmethylchlorpromazine[4], *N*-didesmethylchlorpromazine sulfoxide[4], and chlorpromazine *N*-oxide[4] were used. All solvents were HPLC grade[5], and all other chemicals were commercial analytical reagent grade.

Apparatus—A liquid chromatographic pump[6] and a valve-loop injector[7] fitted with a 1000-μl loop were connected to a fixed-wavelength detector[8] operated at 254 nm. The detector was attenuated to 0.005 aufs for chlorpromazine and to 0.5 aufs for the internal standard.

Column—A 250 × 3.2-mm i.d. column, packed with 5-μm cyano-bonded column packing[9], was used at ambient temperature with a mobile phase flow rate of 1.6 ml/min.

Mobile Phase—The mobile phase consisted of 10% aqueous 0.015 *M* sodium acetate–acetic acid buffer (pH 6.5) and 90% acetonitrile. It was degassed by refluxing for 5 min and transferred to the solvent reservoir.

Internal Standard—A stock solution of mesoridazine besylate (1000 μg/ml, calculated as free base) was prepared in double-distilled water, stored in the absence of UV light at 4°, and used throughout the experiments. Dilutions of 50 μg/ml were prepared weekly and used for analysis.

Preparation of Standard Curves—An aqueous solution of chlorpromazine hydrochloride (100 μg/ml, calculated as free base) was prepared weekly in double-distilled deionized water and stored in the ab-

[1] Poulenc Ltd., Montreal, Quebec, Canada.
[2] Sandoz Pharmaceuticals, Dorval, Quebec, Canada.
[3] Aldrich Chemical Co., Montreal, Quebec, Canada.
[4] Donated by Dr. A. A. Manian, National Institute of Mental Health, Rockville, Md.
[5] Fisher Scientific Co., Montreal, Quebec, Canada.
[6] Model 110A, Altex, Beckman Instruments, Toronto, Ontario, Canada.
[7] Model 7120, Rheodyne, Technical Marketing Associates, Ottawa, Ontario, Canada.
[8] Model 440, Waters Associates, Mississauga, Ontario, Canada.
[9] Spherisorb CN, Altex, Beckman Instruments, Toronto, Ontario, Canada.

0022-3549/81/0900-1043$01.00/0

Table I—HPLC Retention Times of Chlorpromazine and Its Metabolites

Compound	Retention Time, min
Chlorpromazine	1.90
Chlorpromazine sulfoxide	6.50
7-Hydroxychlorpromazine	2.70
7-Hydroxychlorpromazine sulfoxide	9.14
N-Monodesmethylchlorpromazine	5.86
N-Monodesmethylchlorpromazine sulfoxide	19.16
N-Didesmethylchlorpromazine	3.48
N-Didesmethylchlorpromazine sulfoxide	9.64
Chlorpromazine *N*-oxide	6.10

Table II—HPLC Estimation of Chlorpromazine Added to Plasma [a]

Added, ng	*n*	Mean Peak Height Ratio × 10^2	*SD*	*RSD* [b]
1.0	10	0.101	0.004	3.96
2.5	5	0.250	0.009	3.60
5.0	5	0.498	0.021	4.21
7.5	5	0.758	0.012	1.58
10.0	10	0.995	0.028	2.81
15.0	5	1.418	0.026	1.84

[a] $y = mx$, where $m = 0.0975 \pm 0.0011$; $r^2 = 0.999$. [b] Mean = 3.00.

sence of UV light at 4°. Appropriate final dilutions of 1.0, 2.5, 5.0, 7.5, 10.0, and 15.0 ng/ml were made directly in plasma just prior to analysis.

Extraction of Samples—Plasma samples were extracted using a modification of a method previously used for GLC–mass spectrometric analysis (8).

To a 15-ml centrifuge tube[10] were added 2 ml of plasma, 1 ml of aqueous internal standard (50 μg of mesoridazine), and 0.1 ml of 1 *N* HCl. The sample was mixed[11] for 30 sec, and 4 ml of HPLC grade isopropanol then was added. The sample was mixed[12] for an additional 5 min and then centrifuged[13] at 5000 rpm for 20 min at 0°. The supernate was poured into another tube[10], and the pH was adjusted to 12.5 with 200 μl of 5 *N* NaOH. After mixing[12] for 10 sec, 4 ml of HPLC grade *n*-heptane was added. The sample was mixed[12] for an additional 10 min and centrifuged[14] at 2500 rpm at room temperature.

The upper organic layer then was transferred by pasteur pipet to a 5-ml conical vial[15]. The sample was evaporated[16] to dryness under nitrogen and then cooled to room temperature. Then 200 μl of acetonitrile was added, and the sample was mixed[12] for 2 min. The clear yellow solution was transferred by pasteur pipet to a 1-ml screw-capped disposable vial. For analysis, 75–100 μl was injected into the chromatograph.

Plasma Level Study—Two normal healthy volunteers, 64 and 87 kg, participated in a clinically supervised study. The antecubital veins on both arms of each subject were catheterized by means of infusion sets[17]. The intravenous solutions were prepared by diluting a commercial injectable preparation[18] with 50 ml of normal saline solution. The solutions were infused over a 2-min period *via* the left catheter. The 64-kg subject received 10 mg, and the other subject received 7 mg of chlorpromazine as the hydrochloride. Blood samples were collected in heparinized tubes[19] without touching the rubber stoppers. After centrifugation, the plasma was removed and stored at 4° until just prior to analysis.

Recovery Study—For the determination of chlorpromazine recovery, blank plasma was spiked with 2.5 and 5.0 ng of chlorpromazine/ml as the hydrochloride salt and the samples were analyzed as already described.

The absolute peak heights obtained from the standards were compared with those of fresh standards of chlorpromazine free base in acetonitrile.

Quantitation—Spiked plasma extracts were chromatographed, and standard curves were constructed by plotting the ratios of chlorpromazine to internal standard peak heights *versus* the concentration of the drug (nanograms per milliliter of plasma). Calibration standards were chromatographed each day when the unknown samples were analyzed. The concentrations of the unknown samples were determined by comparison of the peak height ratios to the standard curve obtained that day.

RESULTS AND DISCUSSION

Under the described conditions, chlorpromazine and the internal standard (mesoridazine) gave sharp and symmetrical peaks that eluted in 8 min (Fig. 1B). No interference from endogenous plasma constituents was observed, as can be seen from the chromatogram obtained when the method was applied to blank plasma (Fig. 1A). Figure 1B shows a chromatogram of a spiked plasma sample containing 2.5 ng of chlorpromazine/ml and 50 μg of mesoridazine. Figure 1C shows a chromatogram of a 5-hr postdose plasma sample (2 ml) from the volunteer who received 7 mg of chlorpromazine intravenously, the chlorpromazine concentration being estimated as 6.5 ng/ml. The specificity of the method was established further by chromatographing all available metabolites under the assay conditions. Several of these metabolites are considered to be major metabolic components in humans. All of the metabolites tested eluted at retention times different from those of chlorpromazine and mesoridazine (Table I).

Mass spectral analyses of collected peaks from chromatographed plasma extracts of bulk plasma samples from dosed volunteers and spiked blank plasma were identical, suggesting that there was no interference from chlorpromazine metabolites that were not tested.

Figure 1—*Chromatograms of an extract of 2 ml of plasma. Key: A, blank; B, spiked with chlorpromazine (2.5 ng/ml) and the internal standard mesoridazine (25 μg/ml); and C, sample from a volunteer 5 hr postdose (7 mg iv) estimated to contain 6.5 ng of chlorpromazine/ml. Peak I is chlorpromazine, and peak II is mesoridazine.*

[10] Corex, Fisher Scientific Co., Montreal, Quebec, Canada.
[11] Vortex Genie, Fisher Scientific Co., Montreal, Quebec, Canada.
[12] Evapomix, Fisher Scientific Co., Montreal, Quebec, Canada.
[13] Sorval RC2-B refrigerated centrifuge, Ivan Sorvall Inc., Newton, CT 06470.
[14] IEC model HNS, Fisher Scientific Co., Montreal, Quebec, Canada.
[15] Reacti-vial, Chromatographic Specialties, Brockville, Ontario, Canada.
[16] Thermolyne Dri-Bath, Fisher Scientific Co., Montreal, Quebec, Canada.
[17] Butterfly, Abbott Laboratories Ltd., Montreal, Quebec, Canada.
[18] Largactil, Poulenc Ltd., Montreal, Quebec, Canada.
[19] Vacutainers, Becton Dickinson and Co., Mississauga, Ontario, Canada.

Table III—Recovery of Chlorpromazine from Plasma

Nanograms Added to 1 ml of Plasma	n	Mean Nanograms Recovered	Percent Recovery, mean ± SD
5.00	8	1.73	34.59 ± 0.57
2.5	6	0.86	34.31 ± 0.76

During the development of this assay, one major problem was the loss of chlorpromazine during plasma precipitation, drug extraction, or solvent evaporation. This problem was reported previously (16–18), and a simple method requiring minimum sample manipulation was sought to overcome it. Precipitation of plasma proteins was the process chosen because it would improve recovery of chlorpromazine by facilitating its release from high affinity bound plasma protein sites (19). Of the various reagents tried for plasma protein precipitation, trichloroacetic acid and acetonitrile resulted in complete occlusion of chlorpromazine. Precipitation with methanol was abandoned since evaporation was required before chlorpromazine could be extracted into nonpolar organic solvents. The use of isopropanol not only precipitated plasma proteins without occlusion of the drug but also reduced its loss due to adsorption and/or absorption onto the surfaces of the glass tubes used. Furthermore, the miscibility of isopropanol with a nonpolar solvent like heptane avoided the evaporation step completely. Mixtures of isopropanol and heptane were successfully used for extracting chlorpromazine and its metabolites from plasma (8).

The problem of the loss of chlorpromazine at the lower nanogram levels (≤10 ng) still existed and was visibly evident from the negative intercepts of the standard curves compiled using 50 ng of prochlorperazine/ml as the internal standard. To avoid the loss of chlorpromazine due to adsorption and/or absorption onto glass surfaces, the concentration of the internal standard was raised to 600 ng/ml in the hope that it would saturate the adsorption and/or absorption sites. This amount did not rectify the problem completely since the standard curves still showed some negative intercepts. A further increase in the prochlorperazine concentration resulted in a peak that interfered with that of chlorpromazine. This peak was thought to be due to a small impurity present in the prochlorperazine used.

A solution of 10 μg of 2-chlorophenothiazine used as an antioxidant (11) along with 50 ng of prochlorperazine was tried to solve the described problem. However, on chromatographic analysis, the peak due to 2-chlorophenothiazine tailed into that of chlorpromazine. Ultimately, the use of mesoridazine as an internal standard at a concentration of 50 μg appeared to prevent adsorption and/or absorption as indicated by the lack of negative intercept observed with other internal standards tried. It also provided an acceptable retention time with no interference from plasma constitutents.

Table II shows a composite standard curve for the quantitation of chlorpromazine from plasma. The curve is linear with a negligible intercept over the concentration range of 1–15 ng/ml. The ratio of chlorpromazine to mesoridazine plotted against chlorpromazine concentration gave a straight line passing through the origin (r^2 = 0.999). A mean slope

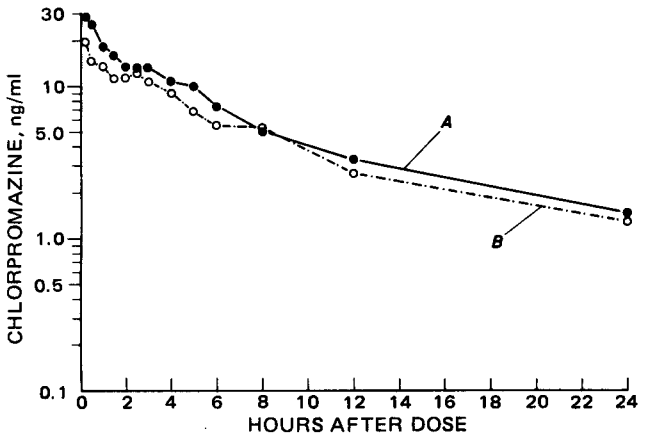

Figure 2—*Chlorpromazine concentrations in the plasma of two normal healthy volunteers. Key: A, volunteer (64 kg) who received 10 mg of an injectable chlorpromazine; and B, volunteer (87 kg) who received 7 mg of the same preparation.*

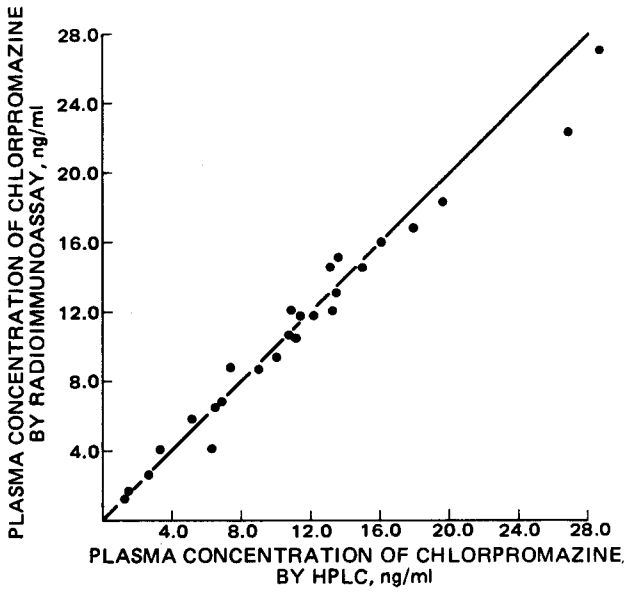

Figure 3—*Plot of plasma chlorpromazine concentration in humans by radioimmunoassay (14)* versus *plasma concentration of chlorpromazine measured by the described HPLC method;* n = 26, r^2 = 0.993, *and slope* = 0.994.

value of 0.0975 ± 0.0011 was obtained. This linearity of the curve was observed to 30 ng/ml, the highest concentration tested. For plasma samples containing concentrations of >30 ng/ml, aliquots smaller than 2 ml should be extracted.

The sensitivity of the described assay 1 ng/ml of plasma was adequate for following plasma concentration–time profiles of subjects receiving low single intravenous doses of the drug (7–10 mg). However, it was not adequate for following plasma profiles in volunteers who were orally administered single low doses (up to 25 mg) of the drug.

The overall recovery of chlorpromazine is given in Table III. Results were from at least six determinations at the 2.5- and 5.0-ng/ml plasma levels. A mean percent recovery of 34.45 ± 0.67 was obtained. Although the recoveries of chlorpromazine in the present assay are low, they are reproducible. Attempts to improve the recovery of the drug led to either added complexities or irreproducibility.

Application of the described HPLC procedure to plasma concentration determinations in subjects who received single intravenous doses of chlorpromazine[17] (7 or 10 mg) is shown in Fig. 2. As can be seen, the method is of sufficient sensitivity to analyze specimens obtained as late as 24 hr after this low single intravenous dose.

The described HPLC procedure was compared to the earlier radioimmunoassay procedure in which the antibody used cross-reacted only with chlorpromazine and *N*-desmethylchlorpromazine (14). There was excellent agreement between the values obtained by the two methods (Fig. 3). The correlation coefficient (r^2) of 0.993 and the slope value of 0.994 were obtained for 26 samples from the two volunteers, analyzed by the two methods. This excellent correlation suggests that *N*-desmethylchlorpromazine is not a major metabolite following single low intravenous doses.

The described HPLC procedure is precise, accurate, and specific for the drug. Furthermore, it is of sufficient sensitivity to determine plasma levels following single intravenous doses (7 or 10 mg) of the drug and should be suitable for single- or multiple-dose pharmacokinetic or bioavailability studies. It compared favorably with a correlation coefficient of 0.99 with the radioimmunoassay procedure reported earlier, thereby suggesting that both procedures are suitable for therapeutic monitoring of patients undergoing treatment with chlorpromazine. However, it may not have the sensitivity for following plasma profiles in volunteers who are orally administered single doses of up to 25 mg of the drug.

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ACKNOWLEDGMENTS

Presented in part at the American Society of Pharmacology and Experimental Therapeutics meeting, Portland, Oreg., August 1979.

Analysis of Pilocarpine and Isopilocarpine in Ophthalmic Solutions by UV Spectrophotometry–Polarimetry

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Received September 12, 1980, from *New Product Research/Analytical Chemistry, Alcon Laboratories, Inc., Fort Worth, TX 76101.* Accepted for publication March 4, 1981.

Abstract □ An improved analytical method was developed that simultaneously quantitates pilocarpine and isopilocarpine in the presence of each other and pilocarpic acid. Pilocarpine and isopilocarpine are first separated from any pilocarpic acid present in the sample by eluting with water-washed chloroform through a column packed with acid-washed diatomaceous earth. The concentrations of pilocarpine and isopilocarpine then are determined by a combination of UV spectrophotometric and polarimetric measurements. UV absorbance is measured at the absorption maximum (215 nm), and optical rotation is measured at the 254-nm line of mercury. Standard curve and standard recovery data are presented. The method is applicable to several commercially available ophathalmic solutions of pilocarpine and is compared to both the USP colorimetric method and a high-performance liquid chromatographic method.

Keyphrases □ Pilocarpine—analysis in the presence of isopilocarpine and pilocarpic acid, ophthalmic solutions □ Isopilocarpine—analysis in the presence of pilocarpine and pilocarpic acid, ophthalmic solutions □ UV spectrophotometry—analysis of pilocarpine and isopilocarpine, ophthalmic solutions □ Polarimetry—analysis of pilocarpine and isopilocarpine, ophthalmic solutions

Pilocarpine is an alkaloid used in the treatment of glaucoma to lower intraocular pressure. It has been reported to isomerize into isopilocarpine or to form pilocarpic acid reversibly in basic solution (1, 2). Both processes result in a loss of pharmacological activity (3, 4).

BACKGROUND

Several satisfactory nonspecific methods have been developed for the analysis of pilocarpine (5–11), but only recently have numerous specific analytical methods been reported (1, 2, 12–17). Two procedures combine colorimetry and polarimetry (1, 12); the first measures a chromium complex of pilocarpine (5), and the second measures an iron complex of the hydroxamic acid of pilocarpine (8). While both methods are theoretically sound in that the colorimetric methods should quantitate total pilocarpine and isopilocarpine and the polarimetric measurements should determine the amount of pilocarpine, these methods are not widely used for routine analysis.

One specific NMR spectrometry method, requiring a 100-MHz instrument, distinguishes between pilocarpine, isopilocarpine, pilocarpic acid, isopilocarpic acid, and pilocarpate and isopilocarpate ions (2). However, this procedure is not designed for multiple analyses and requires expensive instrumentation and reagents. A GLC method that separates pilocarpine and isopilocarpine was also described, but it involves a tedious derivatization of pilocarpine prior to analysis (13). Several specific high-performance liquid chromatographic (HPLC) methods for the quantitation of pilocarpine and isopilocarpine were reported (14–17) and were discussed previously (18).

The present report describes a specific procedure involving a combination of column chromatography, UV spectrophotometry, and polarimetry to analyze for pilocarpine and isopilocarpine in the presence of each other and pilocarpic acid. Validation data for the procedure and data resulting from the analysis of commercially available ophthalmic solutions by this and two other analytical methods are presented.

EXPERIMENTAL

Reagents—USP reference standard pilocarpine nitrate, USP grade pilocarpine hydrochloride[1], and ACS reagent grade isopilocarpine nitrate[2], hydrochloric acid, dibasic potassium phosphate, monobasic potassium phosphate, water-washed chloroform, and acid-washed diatomaceous earth[3] were used.

Solutions—The following were used: 0.1 *M* HCl and pH 5.8 potassium phosphate buffer (prepared by mixing one volume of 1 *M* dibasic potassium phosphate with nine volumes of 1 *M* monobasic potassium phosphate and adjusting to pH 5.8 with the appropriate potassium phosphate solution).

Equipment—A UV spectrophotometer[4], polarimeter[5], and 60-MHz NMR instrument[6] were used.

Optical Rotation Measurements—Stock solutions of pilocarpine hydrochloride and isopilocarpine nitrate were prepared in 0.1 *M* HCl. From these stock solutions, a series of solutions was prepared in concentrations ranging from 0.17 to 1.70 mg/ml of pilocarpine or isopilo-

[1] Quimitra S. A., Merck.
[2] Aldrich Chemical Co.
[3] Celite 545, Johns-Manville Product Corp.
[4] Beckman model ACTA CV with matched 1-cm quartz cells.
[5] Perkin-Elmer model 241 MC with a 100 × 4-mm i.d. cell.
[6] Varian model EM360A.

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