

creased amounts injected, and the use of larger extraction columns for trace enrichment of the samples may provide sensitivity comparable to that of the more conventional assays. The separation of cyclic AMP from other biological substances using a simple isocratic HPLC system may be a difficult task. However, the use of gradient elution with a reverse-phase column (10) or the use of ion-exchange chromatography may separate V from the coeluting compound(s) (16).

It should also be pointed out that the chromatogram in Fig. 2 is a good example of the extra dimension offered to the chromatographic process by ion pairing. All compounds analyzed with mobile phase A eluted in the reverse order of their polarity as expected in conventional reverse-phase chromatography. The only exceptions were V and IV. Although V is slightly less hydrophobic than IV, it has a greater retention under the conditions used due to the presence of tetramethylammonium hydroxide. The interaction of the tetramethylammonium cation with the negatively charged V increases its retention. On the other hand, the positively charged IV is not affected by this cation (7). The result is that while IV and V coelute without this cation, its presence causes V to elute after IV so that separation of these two compounds can be achieved.

By the use of the methods described here, it has been shown that the N^6 -butyryl derivative is the major product formed following the perfusion of rat lung with dibutyryl cyclic AMP. It is estimated that ~24% of the dibutyryl derivative taken up by the lung during the perfusion period can be recovered as the N^6 -monobutyryl analogue from the perfusate and lung compartments. The remaining portion has most likely been further metabolized to the straight-chain monophosphate by the action of phosphodiesterase, since it is not recovered in the lung as the unmetabolized dibutyryl cyclic AMP. We have also shown that 35-min perfusion with 100 μM dibutyryl derivative results in a sevenfold increase in the cyclic AMP content of the lung measured by the protein binding assay of Gilman (13) (data not shown). HPLC analysis reveals that 90% of this increased protein binding can be attributed to the formation of the N^6 -butyryl derivative in the lung tissue.

In light of recent findings that different cyclic nucleotide analogues can selectively activate different isozymes of protein kinase (17, 18), it has become important to know the intracellular distribution of these analogues following treatment with cyclic nucleotide derivatives. This analytical procedure provides a specific, sensitive, and simple method for measuring dibutyryl cyclic AMP and its metabolites in biological systems. This technique can potentially be applied for analysis of many of the other cyclic nucleotide analogues. Also, not only can it be used for

perfusion studies, but could also be adapted to cell culture and whole animal experiments.

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Subnanogram Quantitation of Chlorpromazine in Plasma by High-Performance Liquid Chromatography with Electrochemical Detection

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Abstract □ A specific and sensitive high-performance liquid chromatographic (HPLC) method for the quantitative determination of subnanogram levels of chlorpromazine in plasma is described. Following extraction of chlorpromazine and the internal standard, prochlorperazine, HPLC analysis is carried out on a cyano column with a mobile phase consisting of 0.1 M ammonium acetate in acetonitrile (10:90 v/v). The use of oxidative thin-layer amperometric detection allowed the quantitation of 0.25 ng of chlorpromazine/ml of plasma with a coefficient of variation of 5.1%. The HPLC method has adequate sensitivity to follow

plasma concentration-time profiles up to 24 hr following low single oral doses of chlorpromazine in healthy volunteers.

Keyphrases □ Chlorpromazine—human plasma, high-performance liquid chromatographic determination of subnanogram levels, electrochemical detection □ High-performance liquid chromatography—electrochemical detection, subnanogram levels, chlorpromazine, human plasma

Chlorpromazine is the most widely used phenothiazine antipsychotic agent. It is extensively metabolized (1–6) both systemically and presystemically (7, 8) to numerous

metabolites, some of which are psychoactive. The quantitative analysis of chlorpromazine in plasma or serum has been achieved by several methods which include GLC–

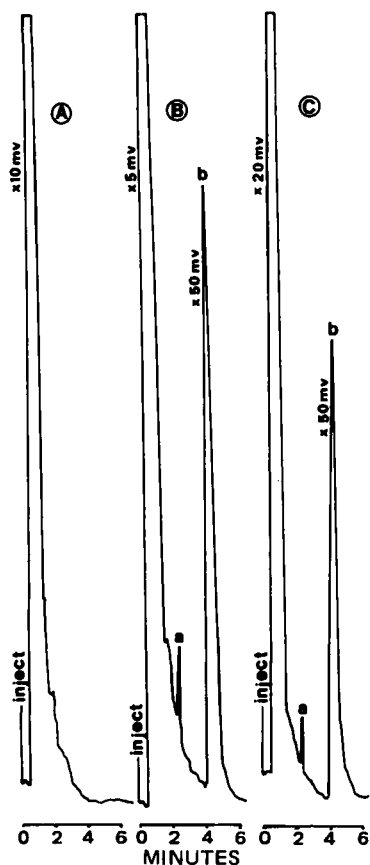


Figure 1—Chromatograms of plasma extracted as outlined in Experimental with recorded sensitivity changes. Key: (A) blank plasma; (B) spiked plasma containing chlorpromazine (peak a, 0.5 ng/ml) and prochlorperazine (peak b, 50 ng/ml); (C) plasma sample 10 hr postdose from a volunteer (73.0 kg) who received 2 × 25-mg tablets orally of chlorpromazine hydrochloride. Chlorpromazine (peak a, 1.70 ng/ml) and prochlorperazine (peak b, 50 ng/ml).

ECD (9), radioassay (10), radioimmunoassay (11, 12), fluorometry (13), GLC-NPD (14, 15), GLC-MS (1, 16, 17), and high-performance liquid chromatography (HPLC) (18–21).

HPLC methods, in general, are facile, easy to adopt, and are well suited for the therapeutic monitoring of drugs. Two of the HPLC procedures for chlorpromazine quantitation employ UV detection (18, 19), and the more sensitive (1 ng/ml) of these methods (18) was applicable to plasma level determinations in volunteers receiving chlorpromazine intravenously. Administration of chlorpromazine orally in low doses (25–50 mg), however, results in plasma levels that are generally in the subnanogram range at the end of the plasma concentration–time curve (7). Therefore, more sensitive analytical methods are needed to study the pharmacokinetics of this drug following low single oral doses. The development of such a method was achieved using electrochemical detection coupled with an improved extraction procedure that gave a greater recovery of the drug from plasma. Oxidative thin-layer amperometric detection (electrochemical detection) of chlorpromazine was investigated by Curry and Brown (20) and showed promise, but details of the assay were lacking. The procedure of Murakami *et al.* (21) was based on a reverse-phase HPLC system in which the extraction procedure was complex and plasma concentrations <1 ng/ml were not determined. The normal-phase

Table I—HPLC Estimation of Chlorpromazine Added to Plasma ^a

Amount Added, ng	n	Mean Peak Height Ratio × 5	SD	CV ^b
0.25	5	0.0348	0.0018	5.10
0.50	5	0.0667	0.0031	4.60
1.0	5	0.1330	0.0036	2.71
2.5	5	0.3317	0.0071	2.14
5.0	5	0.6853	0.0292	4.26
10.0	5	1.3843	0.0276	1.99

^a $y = mx + b$, where $m = 0.1368$ and $b = 0.0027$. ^b Mean = 3.47, $r = 0.999$.

HPLC method described here is simple, sensitive, and requires no specially treated glassware or apparatus. The improvement in the recovery of chlorpromazine from plasma was achieved by modification of an extraction procedure used for trifluoperazine (22).

EXPERIMENTAL

Materials—All solvents used both for extraction and in the HPLC mobile phase were HPLC grade¹. Chlorpromazine hydrochloride², prochlorperazine mesylate³, and 25-mg chlorpromazine tablets⁴ were used as received. All other chemicals were commercial analytical grade.

HPLC—A liquid chromatographic pump⁵ and valve loop injector⁶ with a 500- μ l sample loop were connected to an electrochemical detector⁷. The detector was fitted with a glassy carbon electrode and set at +0.9 V in the oxidation mode with a fixed 10-nA feed connected to a recorder⁸. All changes in attenuation were made only with the recorder to avoid baseline stabilization problems. The column (250 mm × 4.6-mm i.d.) was packed with 10- μ m Spherisorb CN⁹. The mobile phase consisted of 0.1 M ammonium acetate–acetonitrile (1:9) and was deaerated before use by filtration¹⁰. The column was maintained at ambient temperature with a flow rate of 4 ml/min.

Preparation of Stock Solutions and Standard Curves—A stock solution of the internal standard, prochlorperazine mesylate (100 μ g/ml, calculated as free base), was prepared monthly in double-distilled deionized water. The solution was stored in the dark at 4°. Daily dilutions were made in double-distilled deionized water to give a 100-ng/ml working solution. An aqueous solution of chlorpromazine hydrochloride (100 μ g/ml, calculated as free base) was prepared monthly in double-distilled deionized water and stored as described for the internal standard. Daily dilutions of 100 ng/ml were made in 1:1 water–plasma and microliter amounts were added to give chlorpromazine concentrations of 0.25, 0.5, 1.0, 2.5, and 10 ng/ml in plasma.

Extraction of Samples—Plasma samples were extracted by a modification of a GLC-MS method developed for trifluoperazine (22). To a test tube¹¹ were added 2 ml of plasma and 1 ml of aqueous internal standard (prochlorperazine, 100 ng/ml). The sample was mixed¹² for 10 sec, and 0.5 ml of saturated sodium carbonate was then added. The sample was again mixed, and 5 ml of 3% isopropyl alcohol in pentane was added. The sample was capped and mixed for 20 min¹³ and then centrifuged¹⁴ at 1725×g for 5 min. The upper organic layer was transferred by Pasteur pipet to a clean 10-ml tube, and the extraction was repeated with another 5 ml of 3% isopropyl alcohol in pentane. The organic extracts were combined and a few antibumping granules¹⁵ were added. The sample was evaporated to dryness at 65°¹⁶. To the cooled tube was added 200 μ l of

¹ Caledon Laboratories, Georgetown, Ontario, Canada.

² Poulenc Ltd., Montreal, Quebec, Canada.

³ Sandoz Pharmaceutical, Dorval, Quebec, Canada.

⁴ Smith Kline & French, Philadelphia, Pa.

⁵ Waters model M45; Waters Associates, Mississauga, Ontario, Canada.

⁶ Rheodyne model 7125; Technical Marketing Associates, Calgary, Alberta, Canada.

⁷ Model LC4A; Bioanalytical Systems through Technical Marketing Associates, Calgary, Alberta, Canada.

⁸ Perkin-Elmer model 56; Perkin-Elmer, Montreal, Quebec, Canada.

⁹ Beckman Instruments, Toronto, Ontario, Canada.

¹⁰ Millipore Corp., Bedford, Mass.

¹¹ Polytef-lined screw-capped (13 × 100-mm) tubes; Corning Glass, Corning, N.Y.

¹² Vortex-Genie; Fisher Scientific Co., Edmonton, Alberta, Canada.

¹³ Büchler Evapomix; Fisher Scientific Co., Edmonton, Alberta, Canada.

¹⁴ TJ6 Centrifuge; Beckman Instruments, Toronto, Ontario, Canada.

¹⁵ BDH Chemicals, Toronto, Ontario, Canada.

¹⁶ Thermolyne Dri-Bath; Fisher Scientific Co., Edmonton, Alberta, Canada.

Table II—Recovery of Chlorpromazine and Prochlorperazine from Plasma

Drug	Amount Added to 1 ml of Plasma, ng	n	Mean Amount Recovered, ng	Recovery, % ^a
Chlorpromazine	5.00	10	4.31	86.12 ± 2.26
	1.00	10	0.86	85.76 ± 2.90
Prochlorperazine	50.00	7	43.16	86.31 ± 1.33

^a Mean ± SD.

HPLC-grade acetonitrile, the sample was mixed for 20 sec, and aliquots of 100 μ l were injected into the chromatograph.

Plasma Level Study—Three healthy male volunteers weighing 73.0, 77.8, and 87.8 kg participated in the study, which was performed under medical supervision. Each volunteer was administered orally two 25-mg tablets of a commercial formulation of chlorpromazine with 250 ml of water. No xanthine-containing beverage was allowed during the study and no alcoholic beverages were allowed 24 hr before and 24 hr after the last blood sample. A lemon-lime beverage (200 ml) was provided 2 hr after dosing, and a standard lunch at 4.5 hr. Blood samples (10 ml each) were obtained at 0, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 15, and 24 hr following ingestion of the drug. The samples were collected by means of venipuncture into heparinized evacuated tubes¹⁷ taking care to not allow the blood to come in contact with the rubber stopper¹⁸. After centrifugation the plasma was removed and stored at -20° until analysis; subsequently, thawed samples were stored at 4°.

Recovery Study—For the determination of the recovery of chlorpromazine and the internal standard, blank plasma was spiked at 1 and 5 ng/ml for chlorpromazine and 50 ng/ml for prochlorperazine. The samples were extracted as described and peak heights were compared with those obtained for absolute injection of the same amounts of chlorpromazine and prochlorperazine in acetonitrile.

Quantitation—Standard curves were constructed by chromatographing spiked plasma standards and plotting the peak height ratio of the drug to the internal standard *versus* the concentration of the drug. Unknown samples were analyzed along with calibration standards, and concentrations were determined by comparison of the peak height ratios to the standard curve obtained for that day. Patient samples above the stated linear range of 10 ng/ml were diluted as required with fresh blank control plasma and reanalyzed.

RESULTS AND DISCUSSION

Figure 1A shows a typical chromatogram of an extract of fresh blank plasma, and Fig. 1B shows a chromatogram obtained when the method was applied to spiked plasma containing 0.5 ng/ml of chlorpromazine and 50 ng/ml of the internal standard. Chlorpromazine and the internal standard gave well-separated sharp symmetrical peaks with retention times of 2.4 and 4.4 min, respectively. There were no extraneous peaks in chromatograms obtained for blank control plasma. Figure 1C shows a 10-hr postdose plasma sample (2 ml) of a volunteer (73.0 kg) who received 50 mg of chlorpromazine hydrochloride orally; the chlorpromazine concentration was estimated to be 1.7 ng/ml in this sample.

Mass spectral analysis of collected peaks from extracts of pooled plasma from volunteers and spiked samples were found to be identical and gave no evidence of possible interferences. The known metabolites of chlorpromazine either eluted in the same order and relative retention times as in the previous HPLC method using UV detection (18) or did not give any response with electrochemical detection. The use of a more concentrated ammonium acetate buffer increased the retention time of chlorpromazine by 2 min over that observed in the earlier study. This change in retention time resolved the peak due to chlorpromazine from components that eluted in the void volume. When the concentration of acetonitrile was decreased to 70%, no significant change in the retention time of chlorpromazine was observed; however, there was a significant decrease in the signal-to-noise ratio. Also, separation of chlorpromazine metabolites is best carried out using a 90% acetonitrile-based mobile phase. Linear extraction of chlorpromazine at low concentrations was observed to be a problem during the development of the HPLC-UV procedure reported from this laboratory (18). To correct this problem an extraction scheme was developed that provided linearity, but resulted

¹⁷ Vacutainers; Becton, Dickinson & Co., Mississauga, Ontario, Canada.

¹⁸ Necessary to avoid distortions in plasma chlorpromazine concentrations (23).

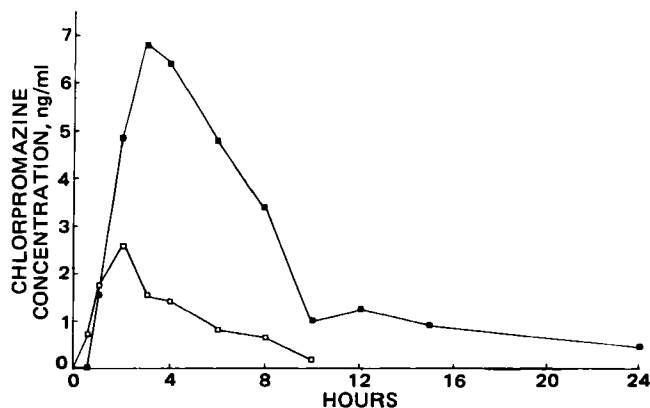


Figure 2—Plasma concentration-time profiles for two volunteers (A = 77.8 kg, B = 87.8 kg) after each received a single oral dose of chlorpromazine (2 × 25-mg) tablets. Key: (■) volunteer A; (□) volunteer B.

in low recoveries: 34.5%. In the present method using 3% isopropyl alcohol in pentane increased the recovery to 86%, and produced reproducible results over the concentration range of 0.25–10.0 ng/ml. Furthermore, the use of the alternate detection system, electrochemical detection, allowed the quantitation of subnanogram amounts of chlorpromazine in plasma. In addition, no problems of occlusion, as observed in the HPLC-UV procedure (18), were encountered since deproteinization was unnecessary, and loss of drug due to adsorption to glass surfaces was not evident.

The electrochemical detector provides at least a 10-fold increase in sensitivity; this finding is in agreement with Curry and Brown (20). An apparent limitation of electrochemical detection is the presence of wide solvent fronts, which may be due to the response of endogenous materials coextracted from plasma. This limitation prevents operation of the detector at its lowest limits of detection. Despite this problem it is evident from Fig. 1B, where no appreciable interferences are observed with the recorder operated at 5 mV, that 0.5-ng amounts of drug can be measured.

Table I shows a composite standard curve obtained from the quantitation of chlorpromazine in plasma. The curve is linear in the range of 0.25–10 ng/ml. The ratio of chlorpromazine to prochlorperazine plotted against chlorpromazine concentration gave a straight line, which passed through the origin with a correlation coefficient of 0.999, a mean slope value of 0.1368, and an overall mean coefficient of variation of 3.47%. The limit of detection of this assay is ~0.1 ng/ml in plasma, and the limit of accurate quantitation is 0.25 ng/ml when 2-ml plasma samples are used. It is adequate for following plasma concentration-time profiles in patients receiving low oral doses of chlorpromazine.

The overall recoveries of chlorpromazine and the internal standard are shown in Table II. Results are based on at least 10 determinations at the 5- and 1-ng/ml levels for chlorpromazine and 7 determinations at the 50-ng/ml level for the internal standard. Mean recoveries of 85.94 ± 2.58% and 86.31 ± 1.33% were obtained for chlorpromazine and the internal standard (prochlorperazine), respectively. Application of this method is shown in Fig. 2, where plasma concentration *versus* time profiles are shown for two healthy male volunteers each receiving 2 × 25-mg tablets of chlorpromazine hydrochloride. Intersubject variability in plasma levels, as shown in Fig. 2, makes it clear that analysis of chlorpromazine should be carried out with the most sensitive procedure available especially when low oral doses are administered.

The described HPLC method is simple, requiring minimum sample handling and time. It is sensitive enough for single- as well as multiple-dose pharmacokinetic or bioavailability studies of low oral doses of chlorpromazine. Furthermore, multiple-dose studies and therapeutic monitoring could be carried out with smaller plasma sample volumes than previously required.

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Maintenance-Dose Prediction Based on a Single Determination of Concentration: General Applicability to Two-Compartment Drugs with Reference to Lithium

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Abstract □ A general approach to the selection of the maintenance dose (D_m) required to give a desired steady-state concentration of drug based on a single determination of concentration after a test dose (C^*) is extended to drugs with two-compartment pharmacokinetic characteristics. Using the equation developed, the value of the proportionality factor relating $1/D_m$ to C^* was found to be within 3.2% of the value calculated from a published nomogram for lithium. The inherent error is shown to be a function of the value of the hybrid rate constants α and β , as well as the value of an intercompartmental transfer rate constant, k_{21} , in an individual.

Keyphrases □ Dose, maintenance—steady-state concentration, prediction by single determination of concentration, two-compartment pharmacokinetics, lithium □ Concentration, steady-state—maintenance dose, prediction by single determination of concentration, two-compartment pharmacokinetics, lithium □ Pharmacokinetics—two-compartment, maintenance dose for steady-state concentration, prediction by single determination of concentration, lithium

In 1973, Cooper *et al.* observed a correlation ($r = 0.972$) between the serum lithium concentration obtained 24 hr after the administration of a 600-mg dose of lithium carbonate and the eventual steady-state concentration if that dose were continued three times a day (1). From that observation, they constructed a nomogram that predicted the maintenance dose required to achieve a therapeutic steady-state concentration of lithium in plasma (0.6–1.2 meq/liter) based on the concentration determined 24 hr after administration of a test dose of the drug. The same

group published a report 2 years later confirming the success of the method (2).

Similar techniques have since been proposed for drugs with widely differing pharmacokinetic characteristics (3–9). Montgomery *et al.* (5) proposed that blood samples taken 24 or 48 hr after an oral test dose of nortriptyline could adequately predict steady-state concentrations of that drug using a justification similar to that used by Cooper *et al.* Koup *et al.* suggested that a strong correlation between steady-state levels and drug concentrations 6 hr after administering a single dose of chloramphenicol or theophylline would exist based on a series of pharmacokinetic simulations (7), and later provided clinical data to support the method (8). The appropriate sampling times for those drugs seemed to correspond to their average half-life in the population. It thus became apparent that this approach to maintenance-dose prediction could be applied to many drugs, and that its successful use depended on implicit knowledge of the individual pharmacokinetic characteristics of a drug within the population.

A theoretical framework was provided to explain and evaluate the empirical clinical observations. The theory was founded on the essential clinical observation that there existed an optimal time at which a blood sample could be obtained from an individual, in which the concentration