Combining Eqs. A2 and A3 and integrating with these boundary conditions give:

$$C_f = \frac{D_m}{D_f} C_m + \left(C_f{}^0 + \frac{D_m C_m{}^0}{D_f}\right)\left(1 - \frac{x}{h}\right) \quad \text{(Eq. A4)}$$

for $(0 \leq x \leq h)$.

Substituting Eq. A4 into Eq. A3 and rearranging give:

$$\frac{d^2 C_m}{dx^2} - \frac{k_f}{D_m}\left(\frac{D_m}{D_f} + \frac{1}{k}\right) C_m = -\frac{k_f}{D_m}\left(C_f{}^0 + \frac{D_m C_m{}^0}{D_f}\right)\left(1 - \frac{x}{h}\right) \quad \text{(Eq. A5)}$$

Equation A5 can be solved analytically for C_m to give:

$$C_m = \left[C_m{}^0 - \left(\frac{D_f C_f{}^0 + D_m C_m{}^0}{D_m + D_f/K}\right)\left(\frac{e^{x\sqrt{A}} - e^{-x\sqrt{A}}}{1 - e^{2h\sqrt{A}}}\right)\right] - \left(\frac{D_f C_f{}^0 + D_m C_m{}^0}{D_m + D_f/K}\right)\left(1 - \frac{x}{h}\right) \quad \text{(Eq. A6)}$$

where:

$$A = \left(\frac{k_f}{D_f} + \frac{k_f}{D_m K}\right) \quad \text{(Eq. A7)}$$

By differentiating Eq. A6 with $(dC_m/dx)_{x=0} = 0$, $C_m{}^0$ can be obtained algebraically:

$$C_m{}^0 = \frac{\frac{D_f C_f{}^0}{D_m + D_f/K}\left[1 + \frac{(1 - e^{2h\sqrt{A}})}{h\sqrt{A}(1 + e^{2h\sqrt{A}})}\right]}{1 - \left(\frac{D_m}{D_m + D_f/K}\right)\left(1 + \frac{(1 - e^{2h\sqrt{A}})}{h\sqrt{A}(1 + e^{2h\sqrt{A}})}\right)} \quad \text{(Eq. A8)}$$

The total diffusional flux of cholesterol is:

$$J = \frac{D_m C_m{}^0}{h} + \frac{D_f C_f{}^0}{h} \quad \text{(Eq. A9)}$$

and the flux from free cholesterol $(D_f C_f{}^0/h)$ can be neglected in this case. Substituting Eq. A8 into Eq. A9 and rearranging give:

$$J = \frac{D_m C_f{}^0}{h}\left[\frac{(1 - e^{2h\sqrt{A}}) + h\sqrt{A}(1 + e^{2h\sqrt{A}})}{\frac{h\sqrt{A}}{K}(1 + e^{2h\sqrt{A}}) - \frac{D_m}{D_f}(1 - e^{2h\sqrt{A}})}\right] \quad \text{(Eq. A10)}$$

For $h\sqrt{A} \gg 1$, Eq. A10 can be reduced to:

$$J = \frac{K C_f{}^0}{\frac{h}{D_m} + \frac{K}{\sqrt{k_f D_f}}} \quad \text{(Eq. A11)}$$

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Chlorpromazine Metabolism VII: New Quantitative Fluorometric Determination of Chlorpromazine and Its Sulfoxide

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Abstract ☐ A new, sensitive assay is described for chlorpromazine and/or its sulfoxide. The method is based on reacting the tertiary amine base with 9-bromomethylacridine to form a quaternary compound which, on photolysis, yields highly fluorescent products that are determinable fluorometrically. The procedural steps were standardized, and an optimum assay procedure was developed. The method shows a less than 3% coefficient of variation when applied directly to chlorpromazine samples and is capable of determining 15–20 ng of the drug. The method is readily adaptable to clinical and bioavailability studies.

Keyphrases ☐ Chlorpromazine and its sulfoxide—fluorometric analysis after quaternization with 9-bromomethylacridine, pharmaceutical formulations and blood levels ☐ 9-Bromomethylacridine—quaternization reagent for fluorometric analysis of chlorpromazine and its sulfoxide, pharmaceutical formulations and blood levels ☐ Fluorometry—analysis, chlorpromazine and its sulfoxide, pharmaceutical formulations and blood levels ☐ Tranquilizers—chlorpromazine, fluorometric analysis, pharmaceutical formulations and blood levels

Of the many assay methods (1–12) developed for chlorpromazine, those possessing adequate sensitivity to determine its levels in blood include a GC method and its modifications (2, 3, 5), *in vitro* radioassay (6), radioimmunoassay (9, 10), and GC–mass spectrometry (4). The lack of precision in GC methods has been adequately reiterated (11–13). The *in vitro* radioquantitation method has not been applied successfully, perhaps because of its complicated nature and poor precision. A reported radioimmunoassay (9)

is unable to differentiate between chlorpromazine and its 7-hydroxy metabolite, whereas a more recent method (10) remains to be assessed for its precision and clinical applicability. The GC–mass spectrometric method requires not only relatively expensive equipment but also specially trained personnel for day-to-day operation.

A new approach to assaying subnanomole quantities of chlorpromazine and its sulfoxide, adaptable to routine use in a clinical laboratory, was described previously (12, 14, 15). In essence, the method is based on reacting the appropriate tertiary amine with 9-bromomethylacridine to form a nonfluorescent quaternary compound. This product can be isolated by TLC and photolyzed to generate measurable fluorescence. The major component of the fluorescent photolytic products was shown to be 9-methylacridine (14, 15). Both the quaternization reaction and the photolysis are stoichiometric and reproducible.

This paper details the development and standardization of the evolved fluorometric method of assay for chlorpromazine and/or chlorpromazine 5-oxide (the sulfoxide), which invariably occurs as one metabolite of the drug.

EXPERIMENTAL

Materials and Equipment—The following were used: 9-bromomethylacridine, synthesized as described previously (14); chlorpromazine and chlorpromazine 5-oxide[1]; cadmium carbonate, cadmium bromide, lead oxide, mercuric cyanide, nickel carbonate, palladium carbonate, and zinc carbonate, all as catalysts; hydroquinone, cadmium sulfate, stannous chloride, and 2-chlorophenothiazine, all as antioxidants; acetone[2]; acetonitrile[2]; ammonium hydroxide[2]; benzene[2]; chloroform[2]; dichloromethane[2]; 1,2-dichloroethane[2]; n-hexane[2]; tetrahydrofuran[2]; 200-μm glass beads[3]; 100-μm thick silica gel TLC plates without fluorescent indicator and with terephthalate backing[4]; TLC saturation chambers[5]; a UV lamp emitting in the range of 200–300 nm (λ_{max} 254 nm)[6]; a microbalance; a pH meter; a sonicator; and vortex shakers.

General Assay Procedure—Chlorpromazine hydrochloride and/or its sulfoxide were converted to base by dissolving in 0.1 N NaOH. The base was extracted with n-hexane, and the solvent was evaporated. The appropriate base residue was dissolved in 100 μl of acetonitrile and reacted with excess 9-bromomethylacridine in the presence of glass beads as the catalyst. The reaction mixture was incubated at various temperatures for various times.

After the reaction period, the reaction mixture was used as such or evaporated to dryness and reconstituted to its original 100-μl volume. An aliquot was subjected to a TLC procedure to separate the quaternary salts. The isolated quaternary salts on the plates were photolyzed under UV light, and the fluorescent material was eluted with methanolic sulfuric acid for fluorometric determination[7].

Standardization of Reaction Conditions—The criteria for the evaluation of optimum reaction conditions were the magnitude and the precision of relative fluorescence produced in the final step of the general assay procedure. In the final application of the evolved assay procedure to blood, the drug in the biological fluid is generally extracted into the solvent. In this process, blood extractables are also extracted into the solvent. These extractables usually can interfere in the chemical reactions employed for assay purposes. Therefore, most experiments on standardization of the quaternization reaction were carried out in the presence of a blank blood extract.

Stoichiometry—To be useful as an analytical technique, the quaternization reaction as well as the photolyic reaction must be stoichiometric relative to the concentrations of the drugs to be assayed. Therefore, chlorpromazine and its sulfoxide, in amounts varying from 0.16 to 3.13 nmoles, were reacted with an excess of the acridine derivative and carried through the photolysis and fluorometric determination to establish the stoichiometry of the reaction.

9-Bromomethylacridine Concentration—Varying amounts of the acridine derivative, ranging from 50 to 313 nmoles, were added to a fixed quantity of chlorpromazine and reacted in the presence of 50 mg of glass beads for 18 hr at 50° to determine the amount of the reagent necessary to produce the most quaternary product.

Reaction Medium—The reaction of 3.13 nmoles of chlorpromazine with 94 nmoles of 9-bromomethylacridine in the presence of 50 mg of glass beads was carried out in organic solvents of varying polarity for 14 hr at 30° to determine the effect of solvent on the quaternization reaction.

Time–Temperature Dependence—Three sets of reaction tubes, with each tube containing 3.13 nmoles of the drug, 94 nmoles of the acridine reagent in 100 μl of acetonitrile, and 50 mg of glass beads, were allowed to react at 25, 50, and 70°. At definite times ranging from 1 to 18 hr, replicates of three tubes were removed from each temperature set and processed through the remainder of the general assay procedure to assess the progress of the reaction.

Catalysis—To accomplish the quaternization reaction in a relatively short time, various substances were evaluated as catalysts. Initial experiments revealed that inclusion of 200-μm glass beads increased the rate of formation of the reaction product significantly ($p < 0.05$) compared to the reaction not containing beads. Varying, but predetermined, weights of beads corresponding to known surface areas were added to the reaction mixtures, and their effect on the reaction was determined. The increase in surface area was accomplished either by increasing the quantity of beads or by crushing a fixed weight of beads to decrease the particle size.

The other agents tested as catalysts included cadmium carbonate (16), cadmium bromide, mercuric cyanide, nickel carbonate, palladium carbonate, zinc carbonate, and lead oxide.

Effect of Antioxidants—In the quaternization reaction of chlorpromazine, a minor product was invariably formed with an R_f value identical to that of the chlorpromazine sulfoxide quaternary product. This product might have been derived by quaternization of the sulfoxide present originally as a contaminant in the chlorpromazine stock, but TLC analysis of the chlorpromazine stock solution revealed that there was no detectable sulfoxide. Therefore, the secondary product might have resulted from oxidation of the drug to its sulfoxide during the quaternization reaction. If so, addition of a suitable antioxidant to the reaction mixture would inhibit this conversion.

On this basis, hydroquinone, cadmium sulfate, stannous chloride, and 2-chlorophenothiazine, all possessing a redox potential greater than that of the drug, were evaluated for their effectiveness in preventing the supposed oxidation. To the reaction mixture containing 3.13 nmoles of chlorpromazine in the presence of an eightfold molar quantity of the acridine derivative and 50 mg of glass beads were added twofold molar quantities of each antioxidant.

In other experiments, three different concentrations of chlorpromazine, with and without 12.6 nmoles of 2-chlorophenothiazine, were reacted with 94 nmoles of the acridine derivative in the presence of glass beads for 18 hr at 50°. The relative fluorescence values of the photolyzed quaternary product of the drug and its minor product each were separately analyzed by a two-factor analysis of variance, *i.e.*, 2-chlorophenothiazine (Factor A) and chlorpromazine concentration (Factor B).

Fractionation of Reaction Products—Various solvent systems and adsorbents for TLC were evaluated for their ability to separate the quaternary salts of chlorpromazine and its sulfoxide from the reactants, from the reaction by-products, and from each other. Fifty-two solvent systems were tested with alumina, different variants of cellulose, and polyamide plates. Another set of 81 solvent systems and some of their ratio variations were evaluated as the developing solvents with the silica gel plates. The presence

[1] Obtained from the Psychopharmacology Research Branch, National Institute of Mental Health.
[2] Nanograde or analytical reagent grade, Mallinckrodt Chemical Works, St. Louis, Mo.
[3] Arthur H. Thomas Co., Philadelphia, Pa.
[4] Eastman Kodak, Rochester, N.Y.
[5] Kensco, Oakland, Calif.
[6] Ultraviolet Products Ltd., San Gabriel, Calif.
[7] Aminco Bowman spectrophotofluorometer.

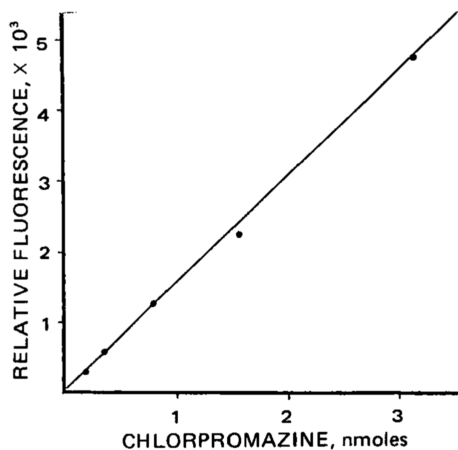

Figure 1—*Correlation between the quantity of chlorpromazine reacted with 9-bromomethylacridine and the relative fluorescence obtained following completion of the quaternization assay. Each point represents the mean of three values.*

of the phenothiazine nucleus at the spots that generated fluorescence following photolysis of the quaternary salts was confirmed by spraying the TLC plates with Forrest reagent (5% ferric chloride in 50% sulfuric acid) and noting the development of a pink color for chlorpromazine and a pinkish-purple color for the sulfoxide at their respective spots.

Photolysis—The quaternary ammonium product formed by the reaction of chlorpromazine with the acridine derivative had no fluorescence of its own. However, UV irradiation of the quaternary salt on a 100-μm silica gel TLC plate produced fluorescence. The stoichiometry of photolysis was determined by applying varying amounts of the isolated quaternary product to the silica gel plates, subjecting the plates to photolysis, eluting the fluorescent products thus generated, and measuring the fluorescence of the eluates. The relative rate of photolysis was determined with the UV lamp at different heights from the TLC plate. The products of photolysis were prepared on a large scale by preparative TLC for structure elucidation.

Determination of Fluorescence—For final quantitation in the assay procedure, the fluorescent products generated by photolysis on a spot were not fractionated to measure individual photolytic products. Instead, the spot was cut out and eluted by vortexing in 10 ml of 20% methanolic sulfuric acid (0.01 *N*), and the eluate was determined at excitation and emission wavelengths of 350 and 474 nm, respectively.

RESULTS AND DISCUSSION

The nature of the reaction between chlorpromazine and 9-bromomethylacridine to form a 1:1 quaternary adduct, the possible mechanism of photolysis of the quaternary product, and the characterization of the fluorescent products of photolysis were described previously (14). When various concentrations of the phenothiazine were reacted with an excess of the acridine reagent and the relative fluorescence of the corresponding photolyzed quaternary product was determined, a linear correlation was seen between the drug concentration and the fluorescence (Fig. 1).

Whenever chlorpromazine was reacted with the reagent in acetonitrile, a minor product with an R_f value equivalent to that of the quaternary product of the sulfoxide invariably developed.

Table I—Relative Yield of Quaternary Product Formed by Reacting Chlorpromazine with 9-Bromomethylacridine in Various Organic Solvents

Solvent	Maximum Yield ± *SD*, %
Acetonitrile	100 ± 1.2
Methanol	96 ± 1.5
Acetone	75 ± 1.6
Chloroform	62 ± 2.2
Tetrahydrofuran	16 ± 4.0

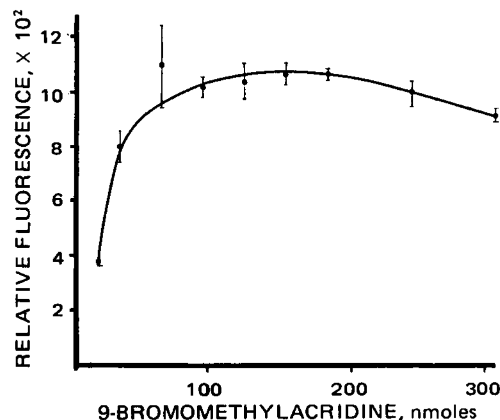

Figure 2—*Effect of varying concentrations of 9-bromomethylacridine on the formation of the quaternary product of chlorpromazine. Each point represents the mean of three values* ± SD.

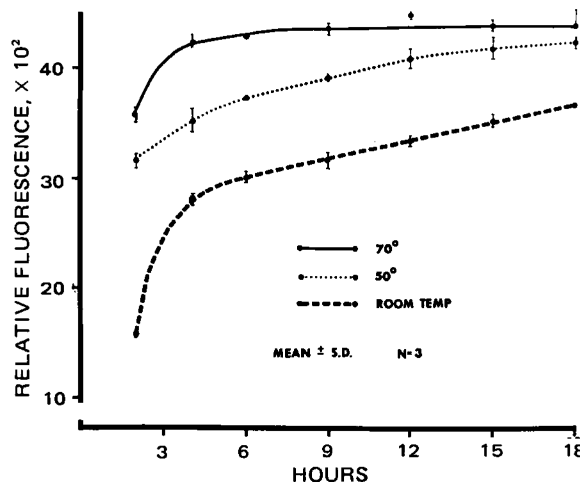

Figure 3—*Temperature dependence of the reaction of 3.13 nmoles of chlorpromazine in the presence of 94 nmoles of 9-bromomethylacridine reacted at 25, 50, and 70°. The points represent the mean of three values* ± SD.

However, in all cases during the standardization phase where 2-chlorophenothiazine was included as an antioxidant in the reaction mixture, the fluorescence value of this minor secondary spot was less than 5% of the value due to the major product of chlorpromazine. For this reason, the contribution of the minor spot was ignored in all analyses.

Reaction Conditions—The nucleophilic quaternization reaction was enhanced in relatively polar solvents (Table I). Although both acetonitrile (bp 81.6°) and methanol (bp 65.5°) appeared to facilitate the reaction, acetonitrile was selected for routine use due to its lower vapor pressure, resulting in relative ease and accuracy in the quantitative transfers involved in the assay procedure.

There was no significant difference ($p > 0.01$) in the fluorescence values resulting from the photolysis of the quaternary product of chlorpromazine formed when 94–250 nmoles of the acridine derivative were used relative to 3.13 nmoles of chlorpromazine (Fig. 2). Since higher concentrations of the acridine yielded significantly higher ($p < 0.05$) blank values, 94 nmoles was used for routine reactions.

The data in Fig. 3 indicate that the quaternization reaction was enhanced by increasing temperature. At 25°, the reaction proceeded to only 87% of the maximum. However, after an 18-hr reaction, there was no significant difference ($p > 0.05$) between the amounts of the quaternary salts formed at 50 and 70°. Occasionally, a few tubes of the higher temperature group showed additional spots, which were not observed in the tubes kept at 50°. For this reason, 50° was chosen as the optimum temperature for subsequent reactions. On completion of the reaction, the contents of all tubes were evaporated at room temperature, reconstituted in 100 μl of acetonitrile, and processed for the rest of the assay procedure.

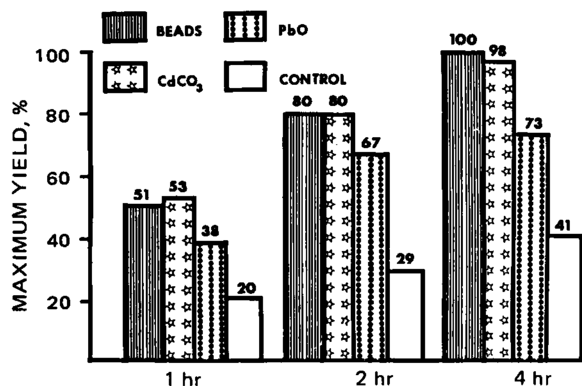

Figure 4—*Effect of 200-μm glass beads, cadmium carbonate ($CdCO_3$), and lead oxide (PbO) on the reaction of 3.13 nmoles of chlorpromazine with an excess of 9-bromomethylacridine at 50°*

The results of catalysis experiments indicated that glass beads and cadmium carbonate were equally effective as catalysts for the quaternization reaction (Fig. 4), whereas cadmium bromide, zinc carbonate, and other agents were of no value. A bead surface area of approximately 1 m^2, equivalent to 50 mg of glass beads/100-μl reaction volume, was optimum. Greater surface areas of glass beads had no further effect.

Of the various antioxidants used to prevent formation of the minor secondary product during the quaternization reaction, 2-chlorophenothiazine significantly ($p < 0.05$) reduced the formation. The other compounds had either no effect or actually caused a significant ($p < 0.05$) reduction in the formation of the primary reaction product, *i.e.*, the chlorpromazine quaternary compound. In the presence of the antioxidant, the relative values of the minor spot decreased as compared to those in its absence, with a corresponding increase in the primary product (Table II).

The statistical analysis on the data relative to the minor product is summarized in Table III. The data indicate that the presence of 2-chlorophenothiazine had a significant effect ($p < 0.05$) on the formation of the minor product. Also, the dependence of the minor spot on the concentration of chlorpromazine was significant ($p < 0.01$).

Separation of Reaction Products—On alumina, cellulose, and polyamide plates, the quaternary products moved best in relatively polar solvents. However, separation of the two quaternary salts of chlorpromazine and its sulfoxide from the excess reagent and other reaction by-products was difficult. *N,N*-Dimethylformamide–chloroform (7:30) was effective in separating the reaction by-products from the quaternary salts on the alumina plates but not on the cellulose and polyamide plates. Since the quaternary salts did not move from the origin in this system, benzene–dioxane–acetic acid (40:10:3) was required to move and separate the two salts. The fractionation, however, was far from satisfactory for accurate quantitative elution of the spots following photolysis. Therefore, the three adsorbents, *i.e.*, alumina, cellulose, and polyamide, were abandoned.

With the silica gel plates, only three of the 81 solvent systems tested were suitable. One of these utilized development of the plate in methanol–methylal–ammonium hydroxide (50:10:0.6) to move the reaction by-products, followed by development in butanol–acetic acid–water (50:10:40), which moved and separated the quaternary products of chlorpromazine (R_f 0.44) and its sulfoxide (R_f 0.39). However, 8–10 hr was required for development. Furthermore, the separations were not consistently reproducible.

Table III—Summary of the Two-Factor Analysis of Variance of Data Relative to Chlorpromazine Secondary Product

Variation	*SS*	*df*	*MS*	*F*
2-Chlorophenothiazine	1613.33	1	1613.33	7.69[a]
Chlorpromazine concentration	30621.66	2	15310.83	73.05[b]
2-Chlorophenothiazine × chlorpromazine concentration	771.60	2	385.83	1.84[c]
Error	5030.00	24	209.58	

[a] $p < 0.05$. [b] $p < 0.01$. [c] Not significant.

In the second system, the plates were predeveloped in methanol–methylal–benzene (2:2:1) to move the reaction by-products to the solvent front, followed by development in methanol–chloroform–benzene–ammonium hydroxide (35:16:10:5), which moved and separated the quaternary products of chlorpromazine (R_f 0.20) and its sulfoxide (R_f 0.26) from the blood blank. However, the development time was relatively long, the separations were inconsistent, and the fluorescence values following photolysis of the quaternary products lacked precision.

The third solvent system was acetonitrile–water (9:1). To date, this is the only system capable of simultaneously moving the reaction by-products to the solvent front and separating the two quaternary products from each other as well as from the blood blank. However, to increase the separation rather reproducibly, two sequential runs were found to be necessary. In this solvent system and with the TLC plates used in these studies, the quaternary salts of chlorpromazine and its sulfoxide have R_f values of 0.26 and 0.22, respectively.

Photolysis—Figure 5 shows that the correlation between the fluorescence yielded by photolysis and the amount of the quaternary compound in the spot was linear. Over 90% of the fluorescence was developed in 2–3 min with a lamp height of 9, 12, or 15 cm from the TLC plate (Fig. 6). As the time of photolysis increased, the fluorescence decreased, possibly due to the conversion of the fluorescent product(s) to less fluorescent congener(s). The loss of fluorescence was significant ($p < 0.05$) when photolysis times of 4 min or longer were used with either the 9- or 12-cm lamp height. With a 15-cm lamp height, however, no significant ($p > 0.05$) loss of fluorescence occurred until 7 min had elapsed. Therefore, a 15-cm lamp height was optimum for the assay procedure, although any height between 12 and 15 cm would be satisfactory for a 3-min photolysis period.

The fluorescent spot produced by photolysis of the quaternary salt on the silica gel TLC plate was developed in benzene–methanol (9:1) and found to resolve into three components. Of the total fluorescence, the primary component (R_f 0.70) represented about 85% while the other two minor spots represented about 7% (R_f 0.29) and 5% (R_f 0.79). A residual fluorescence remained at the point of origin and contributed about 1–3% of the total fluorescence; it may have been due to an unphotolyzed trace of the original quaternary salt. However, the overall photolytic yield of fluorescence, which is measured in the assay, was invariably constant for a particular concentration of the quaternary compound of chlorpromazine.

On comparing the physicochemical properties of the photolytic

Table II—Formation of the Major and Minor Quaternary Products of Chlorpromazine in the Presence and Absence of 2-Chlorophenothiazine

Chlorpromazine, ng/Sample	Without 2-Chlorophenothiazine		With 2-Chlorophenothiazine (12.6 nmoles)	
	Major Product	Minor Product	Major Product	Minor Product
100	508 ± 13.0[a]	14 ± 4.2	524 ± 29.7	7 ± 2.7
400	1720 ± 83.7	48 ± 4.5	1855 ± 97.5	40 ± 9.4
800	3520 ± 192	103 ± 30.9	3650 ± 150	74 ± 12.9

[a] Relative values. Mean ± *SD*; $n = 6$.

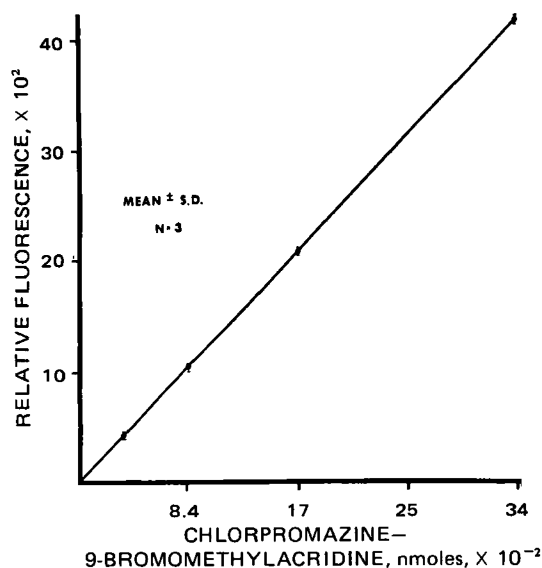

Figure 5—*Linear correlation between the quantity of chlorpromazine quaternary salt photolyzed on a silica gel TLC plate and the relative fluorescence generated. Each point represents the mean of three values* ± SD.

products with those of the synthesized authentic samples (23), the major component was found to be 9-methylacridine. The other two spots were 9-acridinemethanol and 9-acridinecarboxaldehyde, respectively, in the order of the magnitude of their fluorescence contribution.

Table IV shows that 90% of the maximum elutable fluorescence was eluted from the TLC spot by vortexing the cutout spot in 20% methanolic sulfuric acid (0.01 *N*) for 5 sec. There was no significant difference ($p > 0.05$) in the relative elutions at 10, 20, and 30 sec. A 20-sec vortexing time was chosen for the elution procedure.

Optimum Assay Procedure—To 0.063–3.13 nmoles (20 ng–1 μg) of chlorpromazine and/or its sulfoxide as base in a 1.5-ml stoppered polyethylene tube are added 94 nmoles of 9-bromomethylacridine and 0.22 μmole of 2-chlorophenothiazine in 100 μl of acetonitrile and 50 mg of 200-μm glass beads. The tube is stoppered and incubated at 50° for 18 hr. The reaction mixture is evaporated to dryness under nitrogen and reconstituted with 100 μl of acetonitrile. An aliquot of 10 μl is spotted on a 100-μm silica gel TLC plate with terephthalate backing and developed twice in acetonitrile–water (9:1).

The plate is dried at 80° for 15 min after each development and then placed at a distance of 12–15 cm under a UV lamp (λ_{max} 254 nm) for 3 min. The spot containing the generated fluorescent products is marked, cut out, transferred to a 50-ml centrifuge tube, and vortexed for 20 sec in 10 ml of 20% methanolic sulfuric acid (0.01 *N*). Then the tube is centrifuged for 3 min at 2250 rpm. The clear supernate is subjected to fluorometric determination at excitation and emission wavelengths of 350 and 474 nm, respectively.

Precision, Blanks, and Sensitivity—Replicate analyses on sets of different amounts of chlorpromazine and its sulfoxide bases, when carried out in the absence of blood blank, showed a very high precision, with a coefficient of variation within 0–3%. The reagent blanks invariably gave a reading of 100–120 units on the fluorometer, while the TLC plate blanks read 60–80. The fluorescence value relative to the quaternary product of 2 ng of chlorpromazine on a TLC spot read 250. Thus, as low as 60–100 pmoles of the drug and/or its sulfoxide can be determined by the assay.

Table IV—Time Required to Elute Fluorescent Products from Silica Gel Plates

Elution Time, sec	Maximum Eluted Fluorescence, %
5	91 ± 1.2
10	95 ± 2.3
20	97 ± 3.8
30	98 ± 2.2

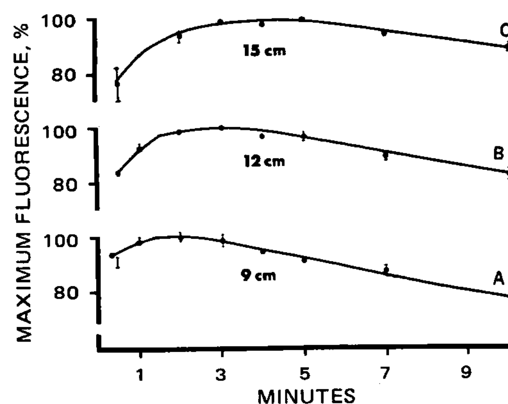

Figure 6—*Time-dependent photolysis of the quaternary product of chlorpromazine on a silica gel TLC plate with the UV lamp heights at 9 (A), 12 (B), and 15 (C) cm. Each point represents the mean of three values ± coefficient of variation.*

In the experiments carried out in the presence of blank blood extracts, the values of the total blank (including the contributions due to blood extractables, the reagent, and the TLC plate) ranged from 100 to 200, depending upon the source of blood. Although these blanks varied from blood to blood, the replicate values within a blood varied less than 5%.

Specificity—The method is specific for tertiary amine drugs. Primary and secondary amine metabolites of the drugs, as well as nonamine substances, do not interfere. Mixtures of tertiary amines, if present in the quaternization reaction, are difficult to fractionate for individual determinations. However, by adopting specific TLC procedures with particular adsorbents and solvents, the method can become selective and specific. For example, the described methodology has been specifically adapted for assaying chlorpromazine and its sulfoxide rather accurately, even when present together.

The quaternization fluorometric method should enable the study of dose–blood level–clinical response relationships for chlorpromazine and other drugs. Furthermore, with increasing emphasis being placed on the use of generic products, the potential of the method for conducting bioavailability and bioequivalence studies on tertiary amine drugs is obvious. If a further increase in the sensitivity of the method is accomplished by spotting a larger aliquot of the reaction mixture or similar other modifications, it would perhaps allow the study of localization and biotransformation of chlorpromazine in the relative brain areas reflective of the role or mechanism of action of the drug in the therapeutic regression of schizophrenic symptomatology. The method is currently being applied to clinical studies.

SUMMARY AND CONCLUSIONS

The need for a sensitive, accurate, and precise method for the quantitative determination of picomole quantities of chlorpromazine is fulfilled by the described quaternization assay. This method offers sensitivity in the 20–30-ng range with good accuracy. The coefficient of variation of most replicate analyses was 0–3%. The methodology requires instrumental and technical capabilities well within the scope of a routine clinical laboratory. Furthermore, the number of samples that can be analyzed by an individual per day is considerably larger than is possible with any GC method matching the sensitivity of the described fluorometric assay.

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Chlorpromazine Metabolism VIII: Blood Levels of Chlorpromazine and Its Sulfoxide in Schizophrenic Patients

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Abstract ☐ A procedure was standardized for extracting chlorpromazine and its sulfoxide from the blood and for applying a recently developed fluorometric assay method to determine blood levels of these two compounds in schizophrenic patients receiving chlorpromazine therapy. The described methodology opens avenues for performing bioavailability and generic equivalence studies in humans.

Keyphrases ☐ Chlorpromazine and its sulfoxide—extraction from whole blood, fluorometric analysis ☐ Tranquilizers—chlorpromazine and its sulfoxide, extraction from whole blood, fluorometric analysis ☐ Fluorometry—analysis, chlorpromazine and its sulfoxide after extraction from whole blood

An approach to assaying chlorpromazine and its sulfoxide in relatively low concentrations was described recently (1–4). It utilizes quaternization of the tertiary amines with 9-bromomethylacridine and a photolytic process to generate fluorescent acridine derivatives in a stoichiometric fashion. The chief advantages of this methodology include a relatively high sensitivity, adequate for determining the clinically accrued blood levels; a high degree of precision, not present in any other method available for assaying chlorpromazine on a routine basis; and ease of analysis of a large number of samples simultaneously.

This paper describes the biological and clinical applications of the fluorometric methodology, preliminary to detailed clinical and pharmacokinetic studies on chlorpromazine currently in progress.

EXPERIMENTAL

Materials and Equipment—The materials and equipment were described previously (4). Isoamyl alcohol, 1-propanol, ether, toluene, *n*-heptane[1], and various chlorpromazine metabolites[2] were also used.

Assay Procedure—The optimum assay procedure, developed and standardized previously (4), was used except that chlorpromazine and its sulfoxide had to be isolated from the blood by an appropriate extraction procedure prior to application of the assay method. Therefore, the extraction procedure was standardized as to solvent, pH, and shake-time for optimum extraction.

Extraction Procedure—The use of sonicated whole blood was preferred over plasma, since chlorpromazine (5) as well as its metabolites (6, 7) is localized in the erythrocytes. The sample size was limited to 3 ml as in other assays (7), since volumes of 5 ml or less are clinically reasonable. However, larger volumes may be used if necessary when assaying chlorpromazine levels below 20 ng/ml.

Extraction Solvent—To seven sets of three tubes, each containing 3.16 nmoles of chlorpromazine in 3 ml of sonicated blood adjusted to pH 13, was added 10 ml each of various organic solvents in a 2:1 organic to aqueous volume ratio. The tubes were shaken for a constant time and centrifuged. A 9-ml aliquot of the organic solvent was removed and processed through the remainder of the procedure.

Extraction pH—A series of extraction tubes each contained a 3-ml sonicated blood sample with 3.16 nmoles of chlorpromazine and its sulfoxide. The samples were adjusted to various pH values between 9 and 14, with a set of three to five tubes per pH value. The samples were extracted for a constant time with *n*-hexane containing 1.5% isoamyl alcohol. The extracts were evaporated to dryness under nitrogen and at room temperature and processed through the assay procedure to determine the optimum pH for extraction.

Duration of Extraction—Sets of extraction tubes, with each tube containing fixed known quantities of chlorpromazine and its sulfoxide in 3-ml blood samples at pH 13 and 10 ml of an extraction solvent, were shaken horizontally in a mechanical shaker at 150 cpm for various lengths of time. The tubes were centrifuged,

[1] All nanograde or analytical reagent grade, Mallinckrodt Chemical Works, St. Louis, Mo.

[2] Obtained from the Psychopharmacology Research Branch, National Institute of Mental Health.