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DETERMINATION BY MICROCOULOMETRIC GAS CHROMATOGRAPHY OF CHLORPROMAZINE METABOLITES IN HUMAN URINE

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Abstract—The application of gas-liquid chromatography to the analysis of chlorpromazine and its metabolites is described. Several new metabolites have been identified in the urine of psychiatric patients with the microcoulometer as a detector. The method, which requires no elaborate extraction or cleanup procedures, is rapid and quantitative for eight metabolites.

CHLORPROMAZINE and related drugs have been shown to be metabolized by oxidation of the ring sulfur atom, oxidation of the amino group on the side chain, and demethylation of the aminoalkyl chain. Thus, chlorpromazine,[1, 2] promazine,[3] methoxypromazine,[4] and thioridazine[5] are oxidized to the corresponding sulfoxides. Thioridazine is oxidized to the sulfone.[5] Demethylation of chlorpromazine by rats *in vivo*[6] and *in vitro*[7] has been reported. The sulfoxides of mono-and dimethyl chlorpromazine have been found in the urine of humans receiving chlorpromazine.[8–10] Goldenberg and Fishman[11] found dealkylated promazine which had been oxidized to the sulfoxide in the urine of dogs.

Many investigators have used paper and thin-layer chromatography for separating chlorpromazine metabolites. Recent reports[12–15] have shown that phenothiazine compounds are amenable to gas-liquid chromatography. This paper reports the successful application of gas-liquid chromatography to the analysis of chlorpromazine and many of its metabolites in the urine of psychiatric patients receiving various dosages of chlorpromazine. Two new chlorpromazine metabolites were tentatively identified by means of the microcoulometer and thin-layer chromatography. The identities of eight other chlorpromazine metabolites confirmed previous reports.[2, 8–11, 15, 20–23]

MATERIALS AND METHODS

Chlorpromazine, chlorpromazine sulfoxide, chlorpromazine sulfone, demethylchlorpromazine, and didemethylchlorpromazine were obtained from Smith, Kline & French Laboratories. Chlorpromazine-N-oxide, chlorpromazine-N-oxide sulfoxide, demethylchlorpromazine sulfoxide, 1-hydroxy chlorpromazine, 8-hydroxy chlorpromazine, 7-hydroxy chlorpromazine, 2-chloro-3-hydroxy phenothiazine, and 3-hydroxy chlorpromazine were supplied by the Psychopharmacology Service Center, National Institute of Mental Health. Thioridazine was obtained from Sandoz, phenothiazine from Eastman Kodak Co., and fluphenazine from E. R. Squibb & Sons. Samples of

2-chlorophenothiazine and 2-chlorophenothiazine sulfoxide were kindly provided by Dr. J. Cymerman Craig of the University of California.

Carbowax 20 M was obtained from Union Carbide Corp. and XE-60 from F & M Scientific Co.

Extraction procedures

Most of the urine specimens were obtained from psychiatric patients at the San Antonio State Hospital. Patients with a variety of mental disorders were chosen from a large number receiving doses of 100 to 1,050 mg chlorpromazine/day. They ranged in age from 18 to 80 years. All specimens were collected early in the morning in brown glass containers, frozen within 3 hr, and thawed just before extraction. Care was taken to ensure that the samples were not exposed to sunlight. All urine samples were tested with Forrest reagent.[16] FPN reagent (nitric acid : perchloric acid : ferric chloride, 50 : 45 : 5)[17] was occasionally used as a check on the dosage of chlorpromazine given to the patient.

The standard procedure for preparing the urine for chromatography was as follows: a 25-ml sample was adjusted to pH 13 with 2 N NaOH and then extracted with two 25-ml portions of dichloromethane. The combined dichloromethane extracts were evaporated to dryness under a stream of nitrogen and the residue taken up in 0·1 ml of dichloromethane. This fraction is referred to as the DCM extract. A portion of the DCM extract was then injected into the gas chromatograph for analysis.

Chemical modification of chlorpromazine metabolites

1. *Reduction of oxides.* The following procedure was used to reduce oxides of chlorpromazine. The organic solvent containing the metabolites was evaporated, the residue taken up in 0·1 N HCl, and 0·1 g granular zinc was added. The solution was shaken vigorously for 5 min and the pH adjusted to 13. The solution was then extracted twice with 25-ml portions of dichloromethane. This procedure reduced all the sulfoxides and N-oxides of chlorpromazine tested, but it was discovered that when the reduction was carried out in the presence of individual chlorpromazine standards, a portion of the 2-chlorophenothiazine standard was lost. This presumably was because of irreversible adsorption of the compound on zinc.

2. *Acetylation.* Some of the chlorpromazine metabolites (demethyl and hydroxy compounds) require acetylation for optimum gas-liquid chromatography. The procedure used was as follows: 5 ml of a benzene solution containing 1 ml acetic anhydride was added to the extract, and the solution let stand at room temperature for 1 hr. The benzene and excess anhydride were evaporated under a stream of nitrogen, and the residue taken up in dichloromethane.

Conditions for gas-liquid chromatography

A laboratory-modified Micro-Tek model 2500R gas chromatograph was operated in conjunction with the Dohrmann microcoulometric titration system (model C-100); halogen and sulfur titration cells were used.

1. *Column-packing material.* Glass beads (60/80 mesh) were washed thoroughly with (in order) aqua regia, distilled water, concentrated ammonium hydroxide, and distilled water. The beads were dried at 100° for 2 hr, then given additional heating in a muffle furnace at 400° for 12 hr. The beads were coated with 0·15% XE-60, 0·1% Carbowax 20M, and 2·0% of particles of diatomaceous earth by a unique procedure described by Johnson *et al.*[18] The coated beads were packed into a 2-foot glass column, with gentle tapping and suction from a vacuum pump. The packed column was aged for 18 hr at 300° with a flow rate of 100 ml nitrogen/min. Aging at temperatures much above 200° for 18 hr resulted in very poor columns.

2. *Operating conditions.* It has been found that an all-glass system is required for optimum chromatography of chlorpromazine metabolites. This includes the injection port, the column, and the exit line. Glass is preferred to stainless steel, aluminum, and copper, in that order, for any portion of the chromatographic system in contact with the sample. Aluminum and copper are very poor materials to use in gas chromatography of chlorpromazine metabolites. The inlet temperature was maintained at 250°, the exit line at 250°, the combustion tube at 750° and the column temperature programmed from 130 to 230° at 10°/min. The flow rate of nitrogen through the column was maintained at 200 ml/min.

Both the halogen and sulfur modes of detection were employed, and the bias voltages normally used were 140 mV for the sulfur detector and 250 mV for the halogen titration cell. The sensitivity setting was normally maintained at 256 ohms for sulfur detection and 512 for the halogen determination.

To perform an analysis, 1 to 5 μlitre of solution containing approximately 0·01–1·0 μg of each metabolite per μliter was injected into the chromatograph and the column effluent vented to the atmosphere to prevent solvent vapor from entering the detector. After 30 sec, the column effluent was switched to the combustion tube and into the titration cell for analysis. The temperature program was initiated 1 min after injection of the sample.

Conditions for thin-layer chromatography

Silica gel or alumina was coated on glass plates (100 × 200 mm) at a thickness of 0·2 mm. The plates were not activated, but care was taken to ensure that the coated plates were kept dry prior to use.

Two solvent systems were used in this investigation—methanol : acetone : triethanolamine (1 : 1 : 0·03), and benzene : dioxane : concentrated ammonium hydroxide (12 : 5 : 1). The urine extracts and standards were applied to the thin-layer plates under a stream of nitrogen. The plates were then developed, dried, and sprayed witn FPN test solution, which produces purple to pink spots for the different chlorpromazine metabolites. Other spray reagents were used for identification of metabolites. One microgram of most chlorpromazine standards will produce detectable spots.

If samples were to be eluted from the plate, only part of the chromatogram was sprayed with FPN reagent. Areas across from zones of color development were scraped from the plates and the chlorpromazine metabolites eluted with dichloromethane or tetrahydrofuran. It was established that the materials were not altered during thin-layer chromatography by rechromatography and comparison of u.v. absorption spectra with those obtained on standards in cases where these were available.

RESULTS

Chromatography of phenothiazine standards

With the use of the column conditions described above, various phenothiazine compounds have been successfully chromatographed. Table 1 lists the retention times of these compounds (temperature program 130 to 230°). Better resolution can be obtained if a longer column is used and if the rate of temperature programming is decreased, but lower recoveries for demethylchlorpromazine (Nor_1CP) and didemethylchlorpromazine sulfoxide (Nor_2CPSO) are obtained.

TABLE 1 RETENTION TIMES OF PHENOTHIAZINE STANDARDS

Standard	Retention time (min)
Promazine	0·9
Phenothiazine	1·0
Chlorpromazine-N-oxide (CPNO)	1·5
Chlorpromazine (CP)	2·0
Demethylchlorpromazine (Nor_1CP)	3·0
2-Chlorophenothiazine (2-Cp)	4·0
Didemethylchlorpromazine (Nor_2CP)	4·1
Chlorpromazine-N-oxide sulfoxide (CPNSO)	4·8
Chlorpromazine sulfoxide (CPSO)	5·0
Acetyl-demethylchlorpromazine (acetyl-Nor_1CP)	6·0
Demethylchlorpromazine sulfoxide (Nor_1CPSO)	6·2
Acetyl-fluphenazine	6·2
Acetyl-didemethylchlorpromazine (acetyl-Nor_2CP)	6·5
Didemethylchlorpromazine sulfoxide (Nor_2CPSO)	6·7
2-Chlorophenothiazine sulfoxide (2-CpSO)	7·0
Acetyl-demethylchlorpromazine sulfoxide (acetyl-Nor_1CPSO)	7·0
Acetyl-didemethylchlorpromazine sulfoxide (acetyl-Nor_2CPSO)	8·0
Acetyl-7-hydroxy chlorpromazine (acetyl-7-OH-CP)	8·1

Standards gas chromatographed on a 2-ft glass column packed with glass beads coated with particles of diatomaceous earth, XE-60, and Carbowax 20M. The column temperature was programmed from 130 to 230° at 10° min; 1 to 2 μg of each compound was injected.

A 6-foot column packed with Gas Chrom P coated with 5% SE-30 was evaluated, but satisfactory results were not obtained. Chlorpromazine-N-oxide (CPNO) decomposed to three peaks; Nor_1CPSO and Nor_2CPSO did not chromatograph at all; and chlorpromazine sulfoxide (CPSO) came through the column in low yield with an extra peak. 2-Chlorophenothiazine (2-Cp) had an earlier retention time than chlorpromazine, Acetyl-Nor_1CP and acetyl-Nor_2CP had retention times longer than CPSO.

When chlorpromazine-N-oxide is injected into the glass column, a large peak appears. In addition, a smaller peak (less than 2% of the total) is found which corresponds to chlorpromazine. The large peak corresponds in retention time to the N-allyl derivative of 2-chlorophenothiazine which is produced by deamination. This result is in agreement with findings reported by Craig *et al.*[19] concerning the breakdown of CPNO in the injection port of a gas chromatograph to yield the allyl compound plus

a small amount of chlorpromazine. The area of the major peak represents 95% of the injected sulfur or chlorine (microcoulometric determination) so that the peak represents CPNO quantitatively, even though CPNO itself is not being chromatographed.

Demethyl and didemethylchlorpromazine do not chromatograph well without chemical modification. These two compounds emerge from the column just after chlorpromazine, but the peaks tail, and less than 50% of the injected compounds can be accounted for. After treatment with acetic anhydride the compounds come through the column in 100% yield. The conversion of the demethylated compounds to the acetyl derivatives appears to be quantitative As can be seen in Table 1, acetyl-Nor_1CP and acetyl-Nor_2CP have longer retention times than the nonacetylated forms. Chlorpromazine comes through the column in about 95% yield with no extra peaks.

About 80% of injected chlorpromazine sulfoxide comes through the column, but no other peaks are seen on the chromatogram as possible breakdown products.

Nor_1CPSO and Nor_2CPSO come through the column in high yield without chemical treatment. 2-Chlorphenothiazine sulfoxide (2-CpSO) does not chromatograph well. The percentage of compound that goes through the column is low and the peak is skewed, possibly because it is difficult to flash evaporate (m.p. 269°). This difficulty can be overcome by reducing 2-CpSO to 2-chlorophenothiazine which chromatographs without difficulty. 7-Hydroxy chlorpromazine does not chromatograph efficiently without acetylation. The retention time is long and the peak tails badly. The acetyl derivative comes through the column in high yield and gives a sharp peak at about 8 min.

All the per cent recoveries of standards are based on the microcoulometric detection system, which gives a direct reading of the quantities of sulfur or chlorine emerging from the column. Results are calculated from the following equation.

$$\text{yield} = \frac{\mu\text{g sulfur or chlorine detected}}{\mu\text{g compound injected} \times \text{per cent sulfur or chlorine}}$$

Chromatography of DCM extracts from human urine

Many DCM extracts of urine samples of male and female mental patients have been analyzed for the following reasons: (1) to guide the development of analytical techniques for measuring chlorpromazine compounds, (2) to detect and identify new metabolites, and (3) to determine the sensitivity level necessary to measure these metabolites in specimens from patients receiving different dosages of the drug. These objectives have been achieved in addition to finding distinct individual variations in the excretion patterns of chlorpromazine compounds from patients receiving the same dosage of the tranquilizer.

Figure 1 shows the results of chromatographing a DCM extract from a female patient (C.M.) receiving 1,050 mg chlorpromazine/day. The sample is a DCM extract without chemical treatment. This chromatogram represents 0·25 ml of the urine sample. The sulfur mode of detection was employed. There are two unidentified peaks which have retention times of less than 1 min. These components contain equal molar quantities of chlorine and sulfur, so it is assumed that they represent chlorpromazine metabolites. The large peak that follows has a retention time identical with that of the CPNO standard. After reduction of the extract, this peak does not appear, and the peak for chlorpromazine is increased proportionately. The peak contains equal

molar quantities of chlorine and sulfur. The next peak represents unaltered chlorpromazine. In all urine extracts examined thus far, there has been a measurable amount of the unchanged chlorpromazine. The small peak just after chlorpromazine

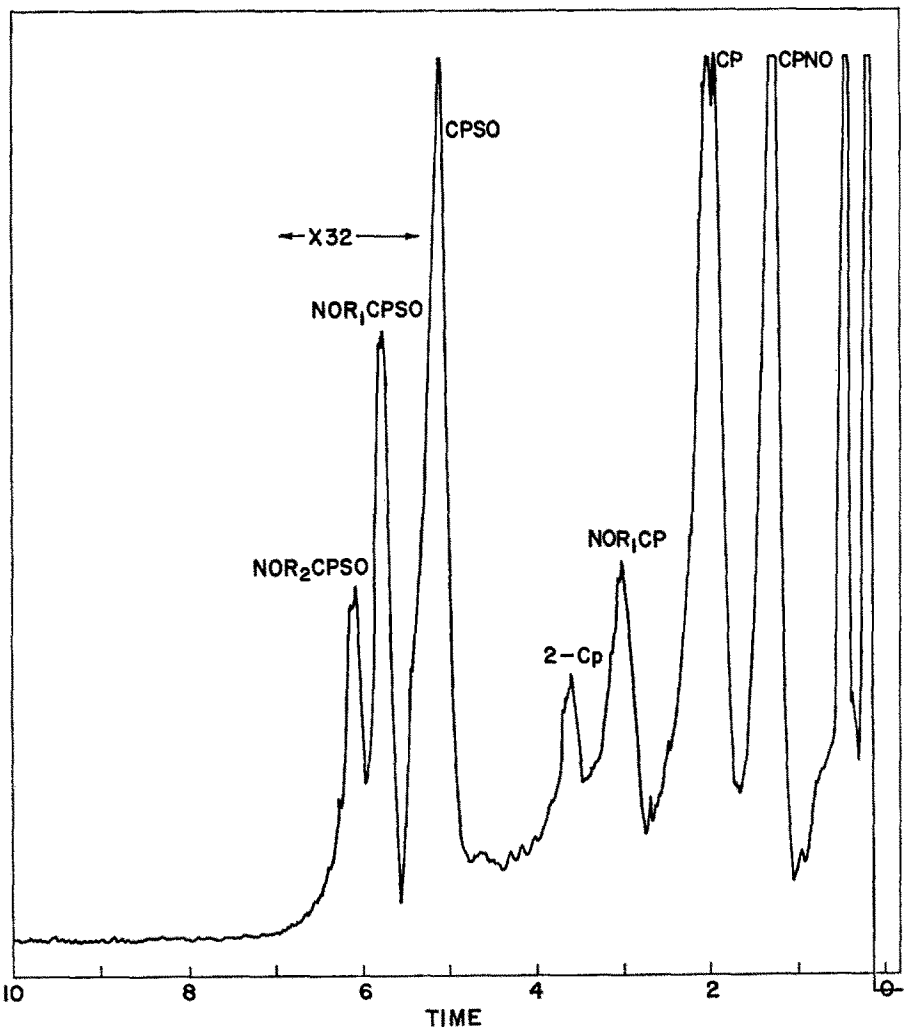

FIG. 1. Gas chromatography of 1·0 μliter of DCM extract, representing 0·25 ml of urine from a female patient receiving 1,050 mg Thorazine per day. A two-foot column packed with glass beads coated with XE-60, Carbowax 20-M, and diatomaceous earth was used. The sulfur mode of detection was employed; the solvent was vented to the atmosphere for the first 15 sec after injection.

has an identical retention time with the standard demethylchlorpromazine. This compound does not chromatograph efficiently unless it is acetylated. The next large peak represents 2-chlorophenothiazine. This peak has been found consistently in urine extracts of male and female psychiatric patients on various levels of the drug. This is the first report of this particular metabolite of chlorpromazine. A small peak with a

retention time for didemethylchlorpromazine comes off the column as a shoulder on the 2-Cp peak. Nor_2CP chromatographs poorly unless acetylated.

Chlorpromazine sulfoxide is the next large peak, followed by two very large peaks which appeared to be Nor_1CPSO and Nor_2CPSO. These three sulfoxides comprise the largest amounts of excreted metabolites in the DCM fraction. Three small un-

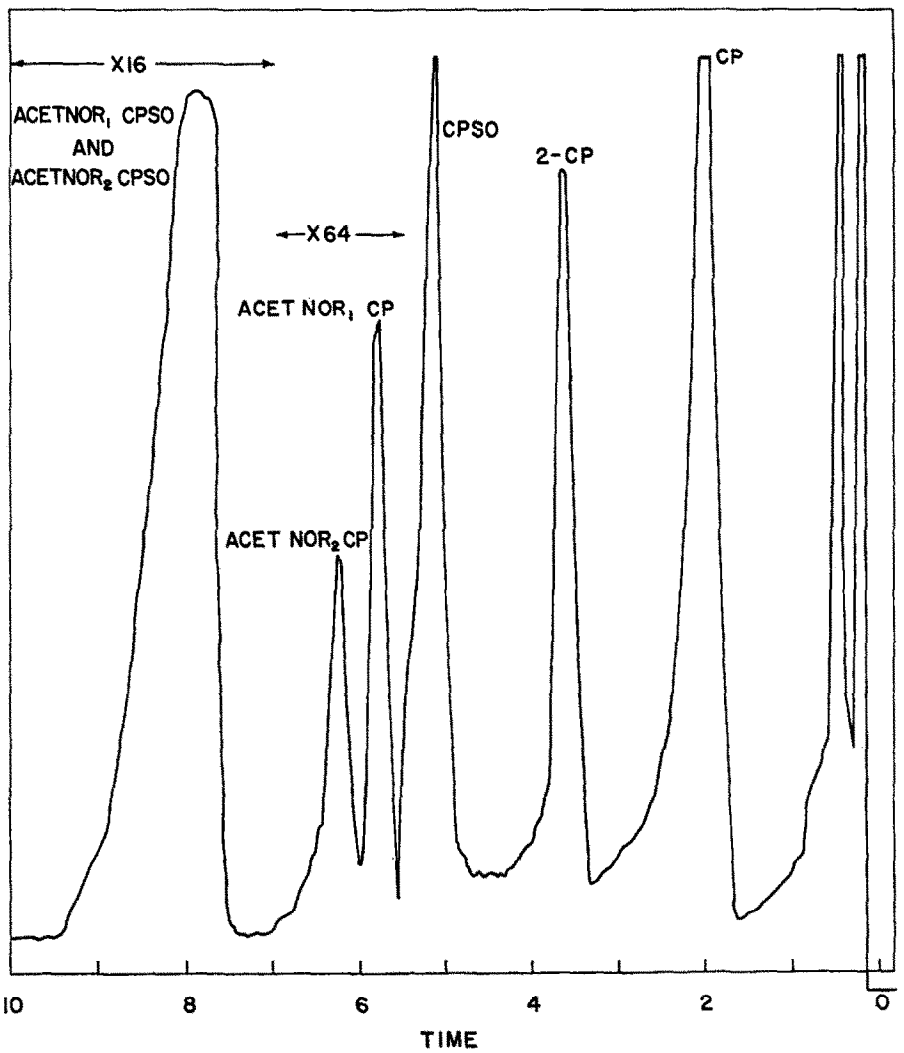

FIG. 2. An acetylated DCM extract of urine from a female patient receiving 1,050 mg Thorazine per day. Gas chromatography of 1·0 μliter of an acetylated DCM extract. All other conditions as in Fig. 1.

identified peaks occasionally appear after the sulfoxide peaks. It is possible that these small peaks represent compounds such as 2-CpSO and hydroxy chlorpromazines, all of which chromatograph poorly under these conditions.

When this extract is acetylated, it gives the chromatogram shown in Fig. 2. Two large peaks appear after CPSO, which have identical retention times with those for

acetylated Nor_1CP and Nor_2CP. The extremely large peak with the longest retention time apparently represents the sum of acetylated Nor_1CPSO and Nor_2CPSO. The relative amounts of these metabolites found in the extract of this patient are as follows: $Nor_1CPSO > Nor_2CPSO > Nor_1CP > Nor_2CP >$ CPSO > CPNO > CP > 2-Cp.

1. *Evidence for the presence of chlorpromazine-N-oxide.* Fishman *et al.*[20] have reported on the identification of the N-oxide of chlorpromazine in the urine of psychiatric patients receiving the drug Thorazine. Gas-liquid chromatography in this laboratory has shown a peak with a retention time identical with that obtained on chromatographing CPNO, although it is recognized that the N-oxide is not being chromatographed as such. This peak contains equal molar quantities of chlorine and sulfur, and it does not appear after reduction of the extract. The peak for chlorpromazine is increased proportionately after reduction. The peak representing CPNO (N-allyl-2-chlorphenothiozine) disappears after treatment of the extract with acetic anhydride, which apparently converts it to 2-Cp and an unknown compound.

Thin-layer chromatography of the DCM extract shows a spot with an R_f identical with that of standard CPNO. The color produced with FPN reagent is the same as that obtained with the CPNO standard. Chlorpromazine-N-oxide is a very unstable metabolite, as indicated by its decomposition during gas chromatography and its breakdown when treated with acetic anhydride. This compound also decomposes very rapidly in acid solution, and decomposition was noted when solutions of the compound in organic solvents were stored in the freezer for several weeks. Crystalline CPNO will decompose to CP and 2-Cp over a period of 12 to 18 months if stored at room temperature.

2. *Evidence for the presence of 2-chlorophenothiazine.* The dealkylated form of chlorpromazine has not been reported as a metabolite in human urine samples. Gas-liquid chromatography has revealed a peak which appears to be 2-chlorophenothiazine in DCM extracts of all urine specimens examined thus far. This peak has a retention time identical with that of pure 2-chlorophenothiazine, contains equal molar quantities of chlorine and sulfur, and has the same solubility characteristics as the standard compound. The use of the microcoulometric titration system has greatly facilitated the identification of this compound. When DCM extracts were chromatographed by means of the flame ionization detector, it was believed that the peak represented a normal urinary constituent rather than a chlorpromazine metabolite.

DCM extracts which have been reduced still yield peaks for 2-Cp. This is an important finding because of the possibility that sulfoxides of chlorpromazine could break down on the chromatographic column to form 2-Cp. None of the standard compounds available in this laboratory have produced a peak for 2-Cp upon injection of large quantities into the gas chromatograph. The partition of 2-chlorophenothiazine between urine and organic solvents should be unaffected by changes in the pH of the aqueous phase. A standard DCM extraction was made on the urine, following which the extract was washed with 50 ml of water adjusted to a pH of 3·0. The acid wash should remove most of the cationic chlorpromazine metabolites. Gas-liquid chromatography of the DCM phase revealed a peak for 2-Cp and a peak for chlorpromazine. The partition characteristics of all the standards were determined and compared to those of the unknown peaks. When all the available chlorpromazine

metabolites were added to control urine, and extracted as described above, the DCM extract (neutral fraction) contained chlorpromazine and 2-Cp, and the acid wash contained the remaining metabolites. Furthermore, when a neutral fraction was prepared similarly from urine samples from psychiatric patients and then reduced, a peak representing 2-Cp was obtained.

Thin-layer chromatography was used in an attempt to obtain confirming evidence for the presence of 2-Cp. No spot in the DCM extract was found to match the spot for the standard when any of the solvents described above was used. It was found, however, that if 2-Cp was added to the DCM extract and the sample then chromatographed, a spot matching the R_f of the pure compound was not obtained. It is apparent that impurities contained in the urine extract drastically affect the R_f of this compound. The added 2-Cp in the DCM extract coincides exactly with an unknown spot on the thin-layer chromatogram.

The peak seen in the DCM extract which has been identified as 2-Cp is not affected by acetylation, methylation (diazomethane), or trifluoroacetylation. The standard 2-Cp is not affected by treatment with any of these reagents.

3. *Evidence for the presence of 2-chlorophenothiazine sulfoxide.* Since 2-chlorophenothiazine is present in the DCM extract of urine it would seem logical that the corresponding sulfoxide of this compound should be present. Unfortunately, gas-liquid chromatography of 2-chlorophenothiazine sulfoxide is unsatisfactory. When 2-CpSO (5 μg) is injected into the gas chromatograph, a skewed peak is obtained, and recovery is only about 10%.

Two-dimensional thin-layer chromatography of DCM extracts produces a spot that matches the R_f given by pure 2-CpSO. The spot not only matches in R_f but also yields the same color as that given by the standard upon reaction with FPN reagent. Both the unknown and the standard give a slightly tailing spot. A thin-layer chromatogram of a DCM neutral extract reveals the same spot, and after the reduction of this extract, the spot does not appear but the spot for 2-Cp is larger.

4. *Evidence for the presence of demethylchlorpromazine and didemethylchlorpromazine.* Nor_1CP and Nor_2CP standards do not gas chromatograph efficiently without acetylation. Small peaks are indicated on unacetylated DCM extracts, but much larger peaks are observed after acetylation, as is illustrated in Fig. 2. These two peaks have been found in all urine extracts examined thus far.

Two-dimensional thin-layer chromatography has confirmed the identities of these compounds. The R_f's of standards and unknowns agree. The spot with the same R_f as Nor_1CP is positive to ninhydrin and positive to sodium nitroprusside. The spot corresponding to Nor_2CP is positive to ninhydrin and negative to nitroprusside.

5. *Evidence for the presence of demethylchlorpromazine sulfoxide and didemethylchlorpromazine sulfoxide.* These two metabolites have been reported by other investigators[15, 20] as being major excretion products in human urine, resulting from chlorpromazine administration. Gas-liquid chromatography of DCM extracts produces two very large peaks with retention times identical with those of Nor_1CPSO and Nor_2CPSO. Acetylated DCM extract yields peaks with longer retention times, which are identical with those of the acetylated standards.

Reduced DCM extract does not show the two large peaks but two peaks do appear with retention times the same as those of unacetylated Nor_1CP and Nor_2CP. When this reduced DCM extract is acetylated, two extremely large peaks appear, representing acetyl-Nor_1CP and acetyl-Nor_2CP.

A solvent extraction technique, developed by Driscoll *et al.*[15] for qualitative separation of chlorpromazine sulfides from sulfoxides, reveals that compounds yielding two large peaks with retention times longer than CPSO are present in the sulfoxide fraction, as would be predicted. Nor_1CP and Nor_2CP are found in the sulfide fraction. Two-dimensional thin-layer chromatography of the urine extracts has confirmed the presence of these two compounds by comparison of R_f's with standards.

6. *Evidence for the presence of 7-hydroxychlorpromazine (unconjugated).* Gas-liquid chromatography of 7-hydroxychlorpromazine yields a peak which tails badly. DCM extracts of urine have on occasion produced small peaks which tail with retention times similar to that of the 7-OH-CP standard. Acetylated 7-OH-CP chromatographs very efficiently but unfortunately has the same retention time as acetyl-Nor_1CPSO. Reduced and acetylated DCM extract shows a peak for acetyl-7-OH-CP (Fig. 3). During the reduction step all of the Nor_1 and Nor_2CPSO are converted to the corresponding sulfides; therefore they do not yield peaks that would interfere with acetyl-7-OH-CP.

Thin-layer chromatography has consistently shown a spot with an R_f identical with that of 7-OH-CP. The unknown spot produced the same purple color with FPN reagent that was observed when authentic 7-OH-CP was treated with the reagent.

Chromatography of DCM extracts from individual patients

The DCM extracts of urine specimens obtained from a number of individual male and female mental patients have been gas chromatographed. Figure 4 shows the results obtained from the urine of the female patient (C.M.) receiving 900 mg Thorazine/day as opposed to 1,050 mg (Fig. 1). This chromatogram shows distinct differences when compared with the results obtained earlier. For example, the two peaks that appeared early in the chromatogram are absent, and two unidentified peaks with retention times longer than Nor_2CPSO are present. The peak for CPNO is much larger. This individual (C.M.) had been receiving about 1,050 mg chlorpromazine/day for some time, and during this period urine extracts yielded chromatograms which agreed with one another very well. However, during a period when the dosage was decreased to 900 mg daily there was a significant change in the urinary excretion pattern, as can be seen in this chromatogram.

Most of the urine samples analyzed have been taken from individual patients, but pooled samples have also been analyzed (Fig. 5). The results are qualitatively similar to those seen previously except that three small unidentified peaks appear after Nor_2CPSO. The peaks for CPSO, Nor_1CPSO, and Nor_2CPSO are at one sixteenth the detector sensitivity setting of the other peaks on this chromatogram.

Quantitative aspects of method

The gas chromatographic method for the analysis of chlorpromazine metabolites present in the DCM fraction has been evaluated for quantitative analysis. It has been

shown that, for the standards tested, these compounds can be recovered from urine samples in yields greater than 80%. Standard curves have been prepared for the following compounds: CP, 2-Cp, CPNO, CPSO, Nor_1CP, Nor_2CP, Nor_1CPSO, and Nor_2CPSO. In each case the chlorpromazine compound was added to control urine

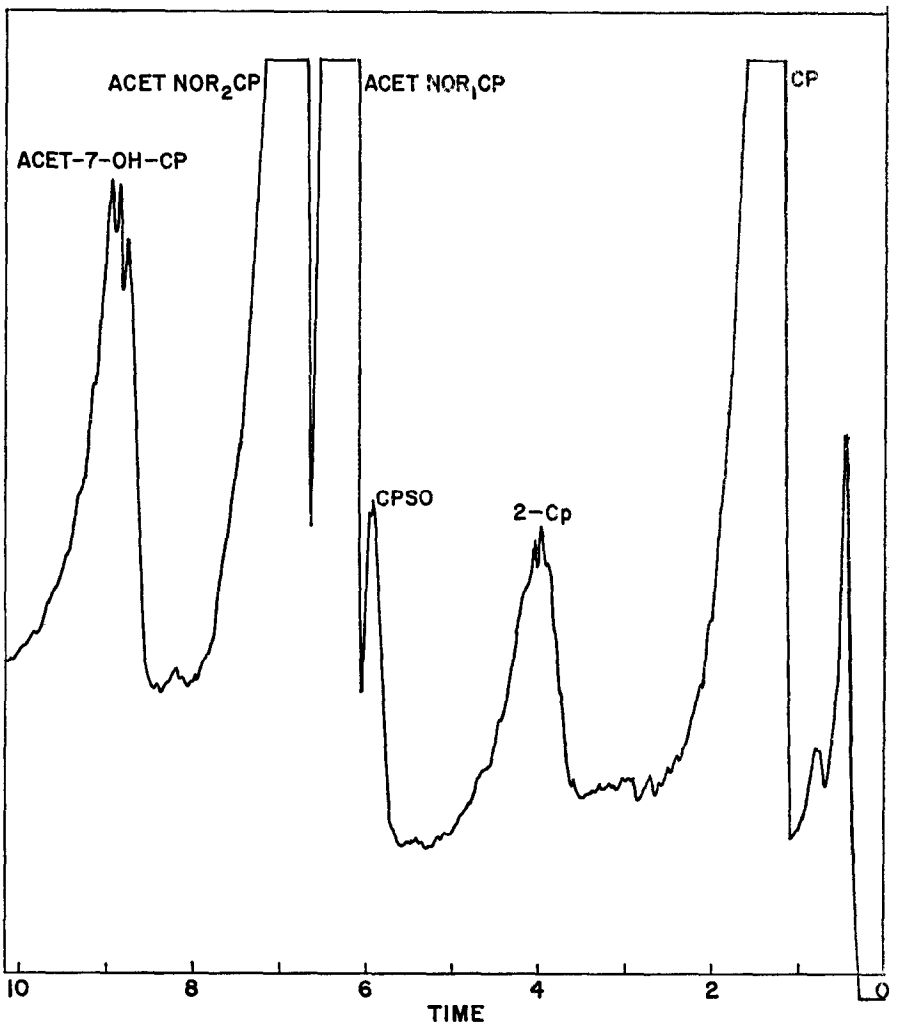

FIG. 3. Gas chromatography of 5·0 μliter of a DCM extract from urine of a patient receiving Thorazine, which has been reduced and acetylated. The aliquot represents 1·25 ml of urine. All other conditions as in Fig. 1.

at three concentrations. Triplicate analyses were made at each concentration, and the number of micrograms of material was plotted against the square inches of peak area. In all cases tested there was a straight-line relationship, and the yields of sulfur or chlorine obtained on the individual metabolites agreed closely. Figure 6 shows the results obtained with representative chlorpromazine compounds (CP and 2-Cp).

A straight-line relationship is seen in each case and the lines lie relatively close together. The average deviation between replicate analyses was $\pm 7\%$. Two extractions with dichloromethane of urine at a pH of 13·5 is sufficient to remove better than 80% of each of the chlorpromazine metabolites. Moderate shaking of the separatory funnel containing the alkaline urine and dichloromethane for a period of 2 min is necessary for complete extraction.

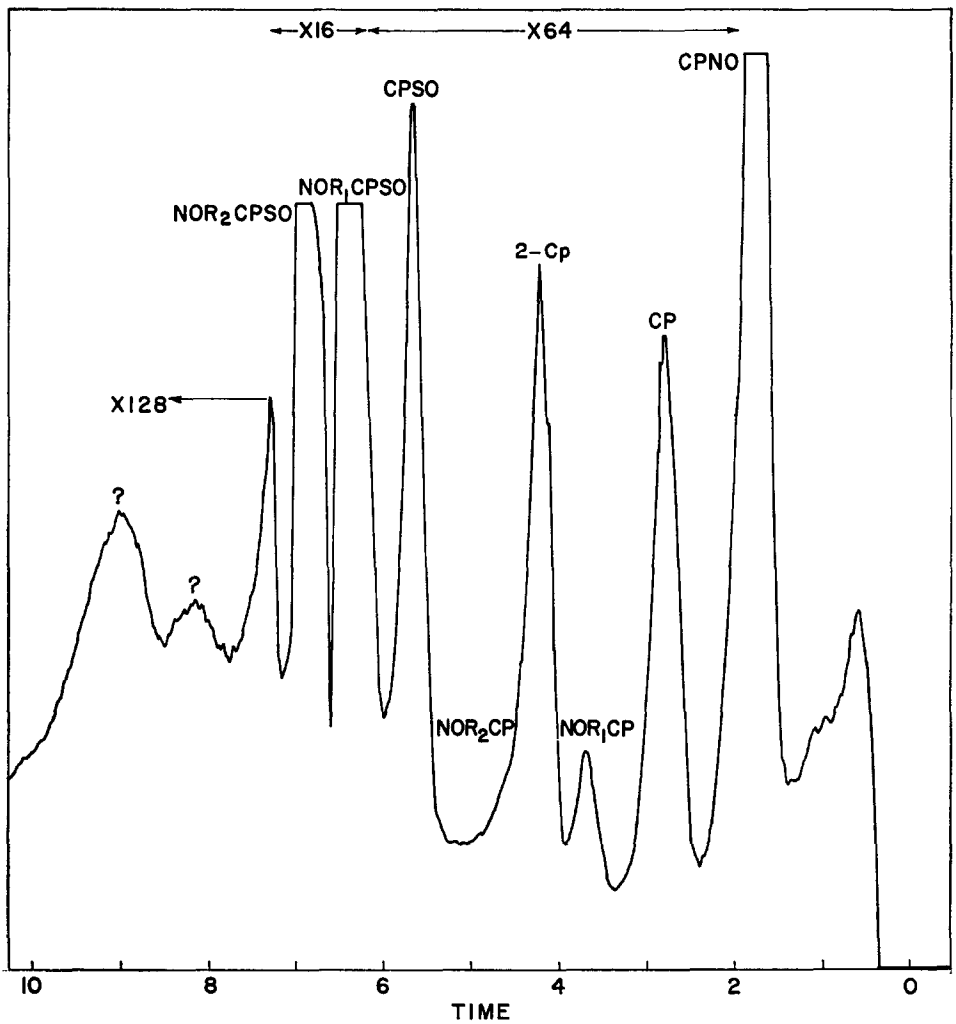

FIG. 4. Gas chromatography of 1·0 μliter of a DCM extract from 0·25 ml of urine from a female patient receiving 900 mg Thorazine per day. Other conditions as in Fig. 1.

Since the microcoulometer is an absolute detection device, it is not necessary to calibrate the instrument or the chromatographic system daily. It usually is necessary, however, to inject a standard solution containing several of the chlorpromazine compounds to facilitate identification of the metabolites in the urine extract.

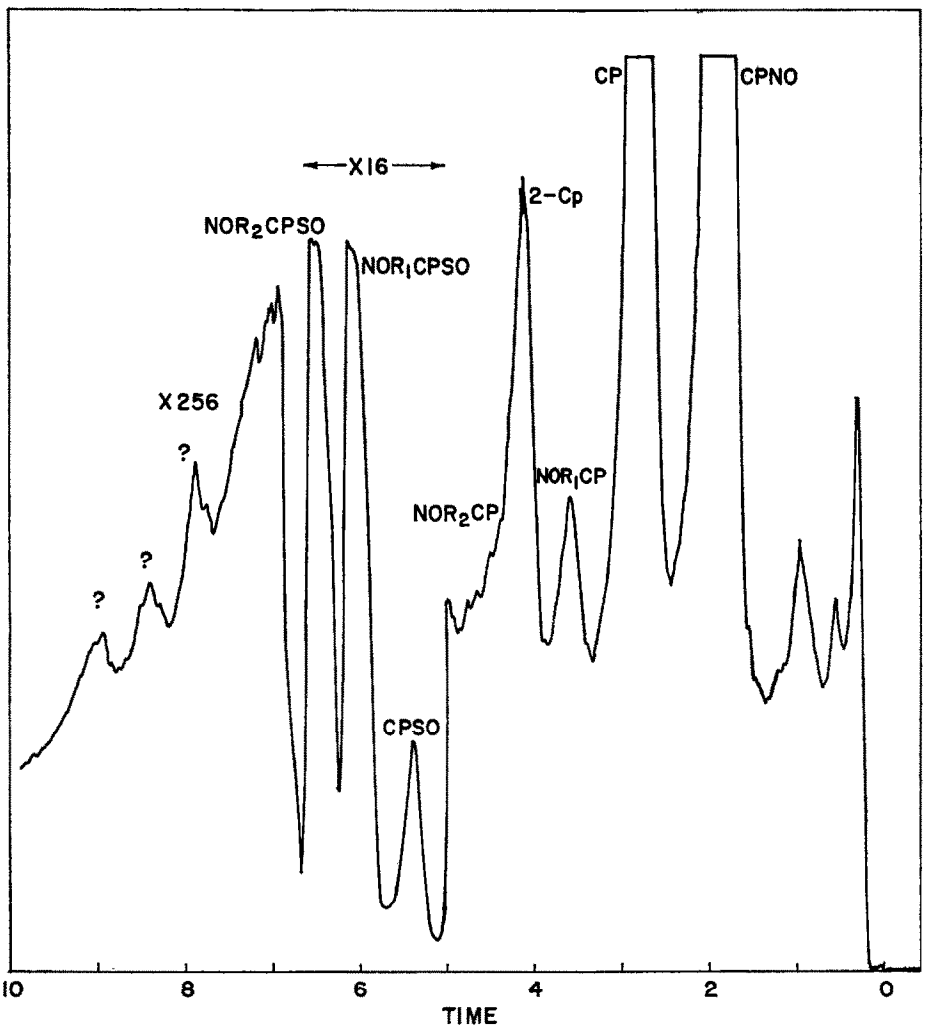

FIG. 5. Injected 1·0 μliter of a DCM extract from 0·25 ml of a pooled urine sample from male patients on high dosages of Thorazine. Other conditions identical with Fig. 1.

DISCUSSION

Advantages of the extraction procedure

Extraction of alkaline urine with dichloromethane removes a number of unconjugated chlorpromazine metabolites from the aqueous phase. Many of the extracted chlorpromazine metabolites can be gas chromatographed without further treatment of the sample. The extract does not contain large quantities of solid materials that might interfere with the final analysis. This extraction procedure is simple, requires a minimum of time, and no cleanup of the sample is required prior to analysis. The extraction removes CP, 2-CpSO, 2-Cp, CPNO, Nor_1CP, Nor_2CP, CPSO, Nor_1CPSO, Nor_2CPSO, and 7-OH-CP from fortified urine samples in yields greater

than 80%. Normally only minor problems are encountered from the formation of emulsions during extraction. The dichloromethane is easily evaporated for concentration of the sample for analysis.

Since CPNO and CPSO are known chlorpromazine metabolites, it is possible that the compound CPNOSO (chlorpromazine-N-oxide sulfoxide) is also present in urine

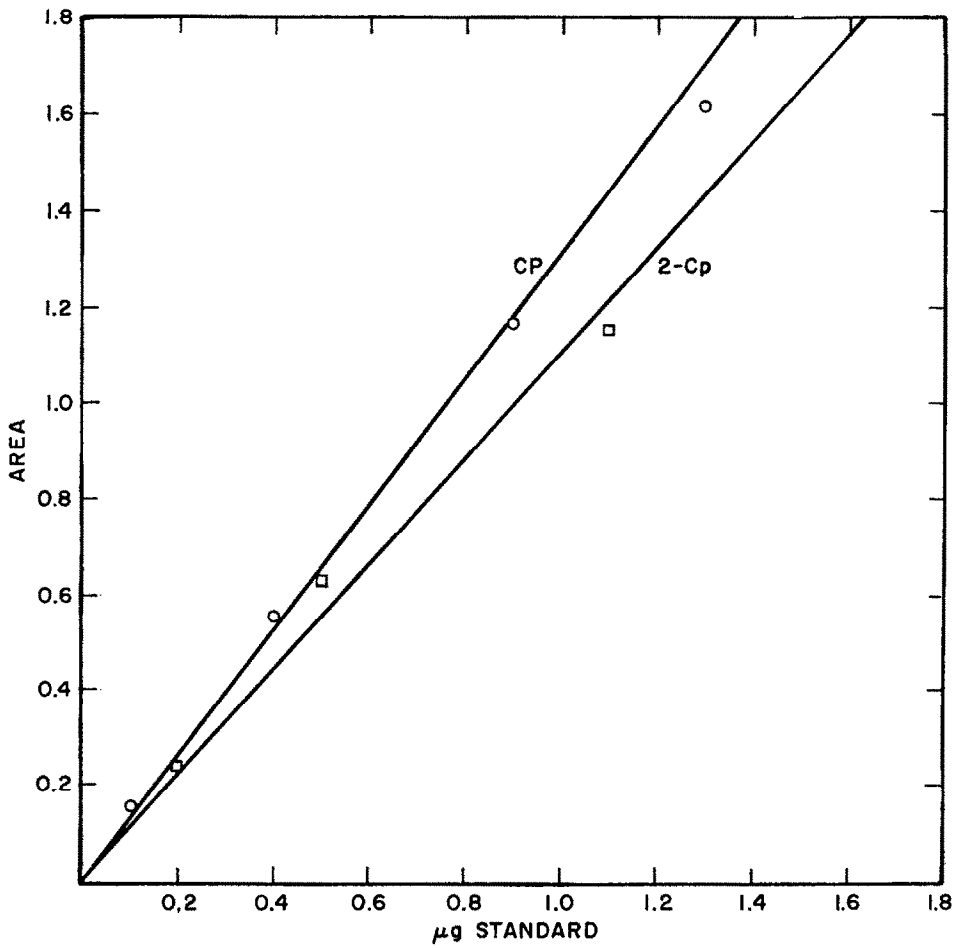

FIG. 6. Standard curve for CP and 2-Cp from control urine extracts. Data obtained by adding standards to control urine, extracting with dichloromethane, and injecting an appropriate aliquot into the gas chromatograph. Areas under the peaks were plotted against micrograms of standard added to urine.

extracts. However, CPNOSO is very water soluble and does not extract with dichloromethane or any of the other common organic solvents tested. No peaks have been seen for this compound in any of the extracts analyzed; however, when a urine sample which has been thoroughly extracted with DCM is reduced and then extracted, a peak for chlorpromazine appears. This could represent CPNOSO.

Chlorpromazine metabolites are much more stable in dichloromethane than in aqueous solution, but extracts should be analyzed as soon as possible. As a general precaution DCM extracts should be analyzed immediately or, if this is not possible, stored in a freezer until they can be analyzed.

Advantages of gas-liquid chromatography with the microcoulometric titration system

Gas-liquid chromatography combined with microcoulometry has been applied successfully to the analysis of pesticides, but little use has been made of this technique in studies of drug metabolism. Extracts of urine samples contain many naturally occurring compounds which could have retention times similar to those of chlorpromazine metabolites. Early in this investigation, the flame ionization detector was used for analyzing extracts from urine. It was possible to ascertain whether or not a particular peak represented a chlorpromazine metabolite only by comparison with control urine specimens. This procedure is not satisfactory since chlorpromazine therapy causes changes in metabolism which could result in differences in the excretion patterns of naturally occurring metabolites.

This early work demonstrated the need for a selective detector. The microcoulometric titration system is selective for compounds containing sulfur or chlorine; since chlorpromazine and its metabolites contain both elements, it is ideal for this application. Extracts of control urines have shown no peaks when we used the microcoulometric titration cells, since there are very few, if any, volatile urinary constituents containing sulfur or halogen.

This detector coupled with the high resolution of the gas chromatographic column provides an extremely selective and sensitive method of analysis. The identification of unknown peaks as chlorpromazine metabolites is greatly facilitated by the capacity of this system for detecting only sulfur or halogen compounds. For example, the peak representing 2-Cp was not recognized as a chlorpromazine metabolite with the hydrogen flame detector because of its low response, but it was quite easy to ascertain that it was a chlorpromazine metabolite with the microcoulometer. The maximal practical sensitivity of the microcoulometer for chlorpromazine metabolites is about 0·1 μg of each compound.

The electron capture detector was evaluated for use in this investigation, but it was found that it responded to a number of the nonchlorpromazine compounds. However, as little as 1 ng of chlorpromazine could be detected.

Metabolites of chlorpromazine

The following chlorpromazine metabolites have been tentatively identified in DCM extracts from urine of mental patients receiving Thorazine: CPNO, CP, CPSO, 2-Cp, Nor_1CP, Nor_2CP, Nor_1CPSO, Nor_2CPSO, 7-OH-CP, and 2-CpSO. The evidence for the presence of these metabolites was presented. Chlorpromazine, chlorpromazine sulfoxide, chlorpromazine-N-oxide, and the demethylated sulfoxide derivatives of chlorpromazine have previously been reported in human urine. There is also substantial evidence in the literature[21, 22] that 7-hydroxy chlorpromazine is present in the conjugated form in human urine. This is the first report of the finding of 2-chlorophenothiazine and its sulfoxide. It is not clear whether the unconjugated 7-hydroxy chlorpromazine[23] that was found resulted from partial hydrolysis of the conjugated form after excretion or was excreted in the free form.

Both gas-liquid and thin-layer chromatography indicate that in urine there are at least two and probably more unconjugated CP derivatives which are relatively nonpolar and volatile enough for direct gas chromatography. These compounds are present in approximately one tenth the quantities of the other metabolites.

Extraction of urine samples with dichloromethane does not reduce the color intensity of the urine with the Forrest reagent or the FPN reagent. It would appear from these data that the unconjugated metabolites of chlorpromazine represent a small proportion of the excreted metabolites. However, this may be deceiving since the unconjugated metabolites do not give as intense a color reaction with the Forrest and FPN reagents as do the conjugated metabolites.

Prospects for clinical tests

With the gas chromatographic conditions described above, it is possible to analyze for CPNO, CP, 2-Cp, CPSO, Nor_1CP, Nor_2CP, Nor_1CPSO, and Nor_2CPSO in the urine of psychiatric patients by extraction of alkaline urine with DCM, followed by acetylation and chromatography. No elaborate extraction prodecure is required nor is there any need for cleanup of the sample. The overall procedure is simple, requires 30 to 45 min, is very sensitive, and is quantitative. One of the most important aspects of this development is that the procedure is highly selective for chlorpromazine compounds.

With the developments described above, rapid clinical analysis of psychoactive drugs and their metabolites in body fluids and tissues should be possible. This would make it feasible to conduct surveys to determine whether a relationship could be established between the manner in which drugs are metabolized and their pharmacological effects on individual patients. Thus, differences in drug metabolism might explain why some patients do not respond so well to drug treatment as others or why higher doses are required to produce the same effects. If a correlation is established, this information would be useful as a guide to therapy.

Several families of psychoactive drugs are now available, many of the more important of these being derivatives of phenothiazine. The tranquilizer chlorpromazine was selected for initial study since it is commonly used in the treatment of mental diseases, and urine specimens of patients receiving it are readily available. Although the work described in this report has been centered on chlorpromazine and its metabolites, other phenothiazine drugs have been chromatographed, and it is believed that the metabolism of fluphenazine, compazine, thioridazine, and other members of this family can be studied by using modifications of the techniques developed for chlorpromazine.

During the course of the work, urine specimens from patients on chlorpromazine therapy have been used almost exclusively as a source of biological material because of availability. From a clinical standpoint, more meaningful results might be obtained from the analysis of blood and spinal fluid. We have begun such investigations, and the results indicate that with minor changes in the extraction procedures this technique can be applied to the routine clinical analysis of these specimens.

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REFERENCES

1. N. P. SALZMAN, N. C. MORAN and B. B. BRODIE, *Nature, Lond.*, **176**, 1122 (1955).
2. N. P. SALZMAN and B. B. BRODIE, *J. Pharmacol. exp. Ther.* **118**, 46 (1956).
3. S. S. WALKENSTEIN and J. SEIFTER, *J. Pharmacol. exp. Ther.* **125**, 283 (1958).
4. L. G. ALLGEN, B. JONSSON, A. RAPPE and R. DAHLBOM, *Experientia (Basel)* **15**, 318 (1958).
5. K. ZEHNDER, F. KALBERER, W. KREIS and J. RUTSCHMANN, *Biochem. Pharmac.* **11**, 535 (1962).
6. J. J. ROSS, R. L. YOUNG and A. R. MAAS, *Science* **128**, 1279 (1958).
7. R. L. YOUNG, J. J. ROSS and A. R. MAAS, *Nature, Lond.*, **183**, 1396 (1959).
8. V. FISHMAN and H. GOLDENBERG, *Proc. Soc. exp. Biol.* **104**, 99 (1960).
9. H. GOLDENBERG and V. FISHMAN, *Proc. Soc. exp. Biol. (N.Y.)* **108**, 178 (1961).
10. A. H. BECKETT, M. A. BEAVEN and A. E. ROBINSON, *Biochem. Pharmac.* **12**, 779 (1963).
11. H. GOLDENBERG and V. FISHMAN, *Proc. Soc. exp. Biol. (N.Y.)* **115**, 1044 (1964).
12. M. W. ANDERS and G. J. MANNERING, *J. Chromatog.* **7**, 258 (1962).
13. W. J. A. VANDENHEUVEL, E. O. A. HAAHTI and E. C. HORNING, *Clin. Chem.* **8**, 351 (1962).
14. K. D. PARKER, C. R. FONTAN and P. L. KIRK, *Analyt. Chem.* **34**, 757 (1962).
15. J. L. DRISCOLL, H. F. MARTIN and B. J. GUDZINOWICZ, *J. Gas Chromatog.* **2**, 209 (1964).
15. F. M. FORREST and I. S. FORREST, *Am. J. Psychiat.* **113**, 931 (1957).
17. I. S. FORREST and F. M. FORREST, *Clin. Chem.* **6**, 11 (1960).
18. D. E. JOHNSON, C. F. RODRIGUEZ and W. SCHLAMEUS, *J. Gas Chromatog.* **3**, (1965).
19. J. C. CRAIG, N. Y. MARY and S. K. ROY, *Analyt. Chem.* **36**, 1142 (1964).
20. V. FISHMAN, A. HEATON and H. GOLDENBERG, *Proc. Soc. exp. Biol. (N.Y.)* **109**, 548 (1962).
21. V. FISHMAN and H. GOLDENBERG, *Proc. Soc. exp. Biol. (N.Y.)* **112**, 501 (1963).
22. H. GOLDENBERG and V. FISHMAN, *Biochem. biophys. Res. Commun.* **14**, 404 (1964).
23. S. PRICE, H. F. MARTIN and B. J. GUDZINOWICZ, *Biochem. Pharmac.* **13**, 659 (1964).