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GAS–LIQUID CHROMATOGRAPHIC ANALYSIS OF PHENOLIC AND NON-PHENOLIC CHLORPROMAZINE METABOLITES IN THE URINE OF CHRONIC SCHIZOPHRENIC PATIENTS

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SUMMARY

The distribution of chlorpromazine and its phenolic and non-phenolic metabolites was studied in the urine of schizophrenic patients by gas–liquid chromatography on OV-17, using a flame ionization detector. Unconjugated phenolic metabolites, 7-hydroxychlorpromazine and 7-hydroxydemethylchlorpromazine in the crude extract were determined as their trimethylsilyl derivatives using N-trimethylsilylimidazole in the presence of pyridine. Trimethylsilyl 7-hydroxychlorpromazine and trimethylsilyl 7-hydroxydemethylchlorpromazine have greatly improved chromatographic properties and enable the investigator both to separate and identify the 7-hydroxy derivatives easily by gas–liquid chromatography. In addition to the phenolic metabolites, the major non-phenolic derivatives identified in urine include chlorpromazine N-oxide, chlorpromazine, demethylchlorpromazine, chlorpromazine sulfoxide and demethylchlorpromazine sulfoxide.

INTRODUCTION

Studies concerned with the analysis of psychoactive drugs from biological fluids and tissues are important in order to be able to determine whether or not there is a relationship between the metabolism of drugs and their pharmacological effects on individual patients. The phenothiazine derivatives constitute a significant member of the broad class of neuroleptic drugs now available, and chlorpromazine (CPZ) was chosen for this preliminary study because it is widely used in the treatment of mental illness. In addition, the metabolites of CPZ are easily obtained[1], thus permitting the study of many of the possible metabolites[2] of this drug in biological fluids such as blood and urine. Although there are many methods of analysis suitable for phenothiazines, gas–liquid chromatography (GLC)[3] appears to be the most satisfactory method for separating and identifying the parent compounds and their metabolites. Several investigators[4,5] have reported the separation of CPZ and its metabolites in plasma by GLC using electron capture detection, but they did not observe any phenolic CPZ derivatives. Johnson *et al.*[6] have studied the GLC properties of CPZ metabolites in the urine of psychiatric patients using a microcoulometric

detector. The only phenolic metabolite found was 7-hydroxychlorpromazine (7-OH-CPZ), which was chromatographed as its acetate derivative.

As a continuation of our interest in phenothiazine metabolism[7], we now report on a simple GLC method for the analysis of unconjugated phenolic metabolites of CPZ as their trimethylsilyl (TMSi) ethers as well as several non-phenolic derivatives in the urine of schizophrenic patients.

METHODS AND MATERIALS

Phenothiazine standards

The chlorpromazine metabolites used in this study include CPZ, demethylchlorpromazine (nor_1CPZ), didemethylchlorpromazine (nor_2CPZ), chlorpromazine N-oxide (CPZNO), chlorpromazine sulfoxide (CPZSO), demethylchlorpromazine sulfoxide (nor_1CPZSO), didemethylchlorpromazine sulfoxide (nor_2CPZSO), 7-OH-CPZ, 7-hydroxydemethylchlorpromazine (7-OH-nor_1CPZ), 7-hydroxydidemethylchlorpromazine (7-OH-nor_2CPZ), 7-hydroxychlorpromazine sulfoxide (7-OH-CPZSO) and trifluoperazine (TFP), which is used as an internal standard. All of the above standards were supplied either by Smith, Kline and French Laboratories (Philadelphia, Pa., U.S.A.) or Dr. A. Manian of the Psychopharmacology Branch of the National Institute of Mental Health.

Preparation of free base standards

An example of the preparation of free base standards for GLC analysis consists in dissolving 10 mg of the hydrochloride of the base in 2 ml of water, adjusting the pH to 12.0 with concentrated ammonia solution and extracting the free base three times with 20 ml of chloroform[8]. After evaporation of the pooled organic layers at 40° *in vacuo*, the weights are determined, and a suitable standard solution is prepared using methanol as the solvent. All methanolic solutions are stored in the dark at 4° until required for use, and appropriate aliquots are used for the standardization of the flame ionization detector (FID) of the gas chromatograph.

All reference compounds were judged to be pure by thin-layer chromatography (TLC) in three solvent systems (see *Thin-layer chromatographic methods*) and GLC on two phases (see *Gas–liquid chromatographic conditions* below), with the exception of 7-OH-nor_2CPZ and 7-OH-CPZSO, which appeared to have suffered degradation in methanol at 4° within 24 h after being prepared. However, little or none of these was detected in any of the urines examined.

Reagents

N-Trimethylsilylimidazole (TSIM) is a reagent which forms the TMSi ether of hydroxyl groups only[9] and does not attack amines such as those found in the side-chain of CPZ and its metabolites. This reagent is used to prepare the TMSi ethers of phenolic CPZ metabolites for suitable analysis by GLC. Pyridine is used as a solvent in the silylation reaction, and both reagents were purchased from Pierce (Rockford, Ill., U.S.A.).

Amberlite XAD-2 resin

Amberlite XAD-2 resin (Mallinckrodt, St. Louis, Mo., U.S.A.) was originally

washed according to a procedure described by Weissman *et al.*[10]. However, we have recently found spurious peaks in our chromatograms (see Discussion) and are currently extracting 20–25 g of resin overnight with 1000 ml of hot nanograde methanol using a Soxhlet apparatus. The resin is then re-suspended in 1000 ml of water four times so as to remove the methanol and fines and is stored in water at 4° prior to use.

Thin-layer chromatographic methods

The TLC of CPZ metabolites was carried out on pre-coated, pre-scored Anal-Tech GF plates (0.25-mm thick layer), which were developed in the following solvent systems: (A) benzene–dioxan–diethylamine–water (70:17.5:7.5:1) (ref. 1); (B) toluene–methanol–dimethylformamide (9:6:4) (ref. 1); (C) acetone–diethylamine (90:10) (ref. 11).

Relative R_F (RR_F) values were determined by dividing the R_F of a given metabolite by that observed for CPZ.

Preparative layer chromatography (PLC) was carried out on silica gel G (0.25 mm) plates, which were developed in solvent system B. The band corresponding to 7-OH-CPZ (rendered visible with UV light) was scraped into a filter-beaker (Ace Glass, Vineland, N.J., U.S.A.) and was eluted with methanol. After evaporation of the solvent, the metabolite was analyzed by GLC as its TMSi ether.

Gas–liquid chromatographic conditions

A Hewlett-Packard Model 5750 gas chromatograph equipped with dual FID was used. Two 6-ft. coiled glass columns (Supelco, Bellefonte, Pa., U.S.A.) were packed as follows. One column was packed with 3% OV-3 (Applied Science Lab., State College, Pa., U.S.A.) coated on re-silanized Gas-Chrom Q (100–200 mesh, Applied Science Lab.) using the filtration method of Horning *et al.*[12]. A second column was packed with pre-coated 3% OV-17 on Gas-Chrom Q (100–200 mesh, Applied Science Lab.). However, for routine urine analysis, two OV-17 columns were used for reasons which will be discussed later (see Results). The conditions used were as follows: injection port temperature, 250°; column oven, 235°; FID, 310°; helium was used as the carrier gas at flow-rates of *ca.* 50 ml/min (OV-3) and 75 ml/min (OV-17).

Peak areas were measured by triangulation, and the amount of each compound present was calculated using the area of the internal standard (TFP) and relative detector responses (RDR).

Extraction of CPZ and its metabolites

A sample of urine from patients receiving 600–1800 mg of CPZ daily is collected for 24 h in polyethylene containers with screw-tops and is kept tightly stoppered and refrigerated (4°) between collections until analyzed. If the volume is less than 500 ml, the urine is diluted to that volume prior to analysis. A 20-ml aliquot of the sample is adjusted to pH 9.0 with concentrated ammonia solution according to a modification of the method described by Bastos *et al.*[13], and is passed through a 1×10 cm column of Amberlite XAD-2 resin which has been previously washed as described by Weissman *et al.*[10].

More recently, we have found that increased recoveries of metabolites are

obtained by not adjusting the pH of urines that have a pH range from 5.0 to 7.0 (see Results).

After the urine has drained into the resin bed, the column is washed with 10 ml of 0.2 *N* ammonia solution and 10 ml of deionized water. Excess water is removed from the resin by gently aspirating the column with a water aspirator, and the phenothiazines and other urinary pigments are eluted with 20 ml of methanol, which is collected in a 100-ml pear-shaped flask. After removal of the solvent *in vacuo* at 40°, the residue is extracted with 20 ml of chloroform–methanol (3:1) and is mixed for 30 sec on a Vortex-Geni with 2 ml of 0.2 *N* ammonia solution, which removes most of the pigments as previously described[7]. The ammonia solution is washed with an additional 20 ml of chloroform, and the organic layers are combined and evaporated at 40° under reduced pressure. The residue obtained is transferred into a 5-ml centrifuge tube with methanol and is evaporated to dryness under nitrogen at 40°, after which it is re-dissolved in 400–1200 μl of methanol containing TFP (165 ng/μl minimum concentration) depending upon the CPZ dosage. An aliquot of 1.0–1.5 μl is injected for analysis by GLC under conditions described previously (see *Gas–liquid chromatographic conditions*).

Gas–liquid chromatographic analysis

Non-phenolic CPZ metabolites. The FID is standardized first by dissolving a mixture (16–130 μg) of CPZ and its metabolites in a total volume of 200 μl of methanol containing TFP and injecting 1.0–1.4 μl into the gas chromatograph. Both the relative retention time (RRT) and the RDR of each metabolite compared with the internal standard can be calculated as follows:

$$\text{RRT} = \frac{\text{retention time of CPZ metabolites}}{\text{retention time of TFP}}$$

$$\text{RDR} = \frac{\text{peak area/}\mu\text{g metabolite}}{\text{peak area/}\mu\text{g TFP}}$$

A suitable aliquot of the urinary extract containing CPZ metabolites and TFP is then injected into the gas chromatograph, and the identity and amount of each metabolite is determined by the RRT and the RDR, respectively. This method is not suitable for the determination of phenolic derivatives.

Phenolic CPZ metabolites. An aliquot of 100 μl of the mixture of standards is evaporated to dryness at 40° under nitrogen and is silylated at room temperature for 20 min in a tightly capped vial containing 5 μl of TSIM and 95 μl of pyridine*. An aliquot of *ca.* 1.2 μl of this mixture is injected for analysis of the phenolic derivatives only, and the RRT and RDR of the TMSi ethers are determined. A suitable aliquot of the urinary extract is then evaporated to dryness in a small vial containing TFP and is silylated with TSIM and pyridine as described above. An aliquot of this extract is injected into the gas chromatograph under identical operating conditions, and the identity and amount of phenolic derivatives are determined from the RRT and RDR, respectively.

* Recently, we have observed incomplete silylation in a few samples during conditions of high humidity and have obtained complete derivatization with 10 μl of TSIM reagent.

TABLE I

RETENTION TIMES OF CHLORPROMAZINE AND ITS METABOLITES

Metabolite	*OV-17*		*OV-3*	
	Free base	*OTMSi**	*Free base*	*OTMSi**
CPZNO	0.40	0.40	0.34	0.34
CPZ	0.67	0.68	0.61	0.61
nor$_1$CPZ	0.84	0.85	0.69	0.68
nor$_2$CPZ	0.89	0.90	0.61	0.61
CPZNOSO	1.76	1.77	—	—
7-OH-CPZ	2.33	1.41	1.69	1.43
CPZSO	2.64	2.65	1.82	1.79
7-OH-nor$_1$CPZ	3.07	1.77	1.93	1.61
7-OH-nor$_2$CPZ	—	1.89	2.59	2.16
nor$_1$CPZSO	3.38	3.41	2.11	2.09
nor$_2$CPZSO	3.89	—	2.30	—
7-OH-CPZSO	4.80	4.77	—	—

* RRT determinations were obtained after derivatization with TSIM.

RESULTS

The relative retention times on OV-3 and OV-17 of selected metabolites of CPZ are summarized in Table I, as the free base and after trimethylsilylation of the mixture of standards. As OV-3 cannot separate CPZ and nor$_2$CPZ, it was not used in the routine examination of urinary metabolites. However, OV-17 separated the non-phenolic and phenolic derivatives very well, and relative retention times were reproducible within each set of analyses.

Fig. 1 indicates a typical response of the FID to each of the metabolites in a mixture of standards relative to TFP. As the detector exhibits a wide range of sensitivity of each of these compounds (from 0.25 for CPZNO to 2.50 for CPZ), it is

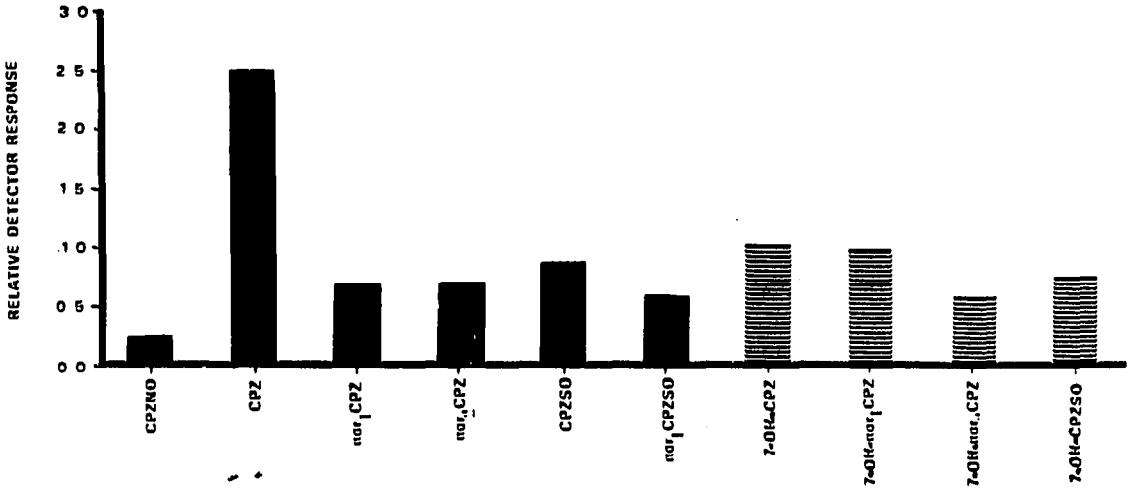

Fig. 1. Relative detector responses of non-phenolic (solid bars) and TMSi-phenolic (striped bars) metabolites of CPZ.

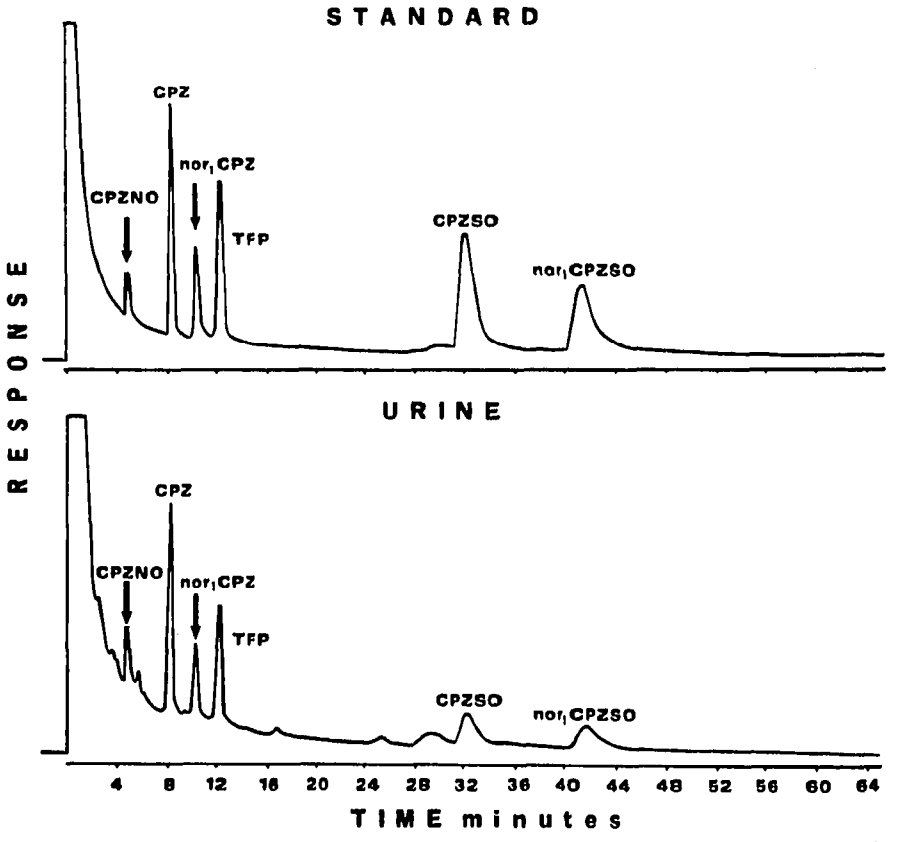

Fig. 2. Gas–liquid chromatographic profile on 3% OV-17 of a mixture of standards (above) and urinary extract (below) containing CPZ and its metabolites. Conditions are given in the text.

necessary to standardize the gas chromatograph daily prior to quantitative analysis of urinary extracts.

Fig. 2 shows a comparison of a mixture of standards of CPZ metabolites (above) and a typical urinary extract (below) of a patient receiving 600 mg of CPZ daily. The non-phenolic urinary metabolites identified in the order of increasing retention times include CPZNO, CPZ, nor_1CPZ, CPZSO and nor_1CPZSO. Phenolic derivatives are discernible but not measurable under these conditions.

Preparation of the TMSi derivatives of an aliquot of the mixture of standards and the urinary extract leads to clear identification of both 7-OH-CPZ and 7-OH-nor_1CPZ as the major phenolic metabolites of CPZ in Fig. 3. Although the non-phenolic derivatives in both samples retain their respective retention times, CPZNO and nor_1CPZ appear to undergo some degradation in the preparation of the TMSi compounds. Therefore, separate analyses of non-phenolic and phenolic derivatives are necessary for proper quantification.

Recovery experiments performed on 20 ml of control urine of a drug-free patient have been carried out in duplicate at pH 9.5 and 5.5 in order to determine possible pH effects. A mixture of 12 metabolites ranging in amounts from 1.1 μg (CPZ) to 8.3 μg (nor_2CPZSO) was added as a methanolic solution (100 μl) to the

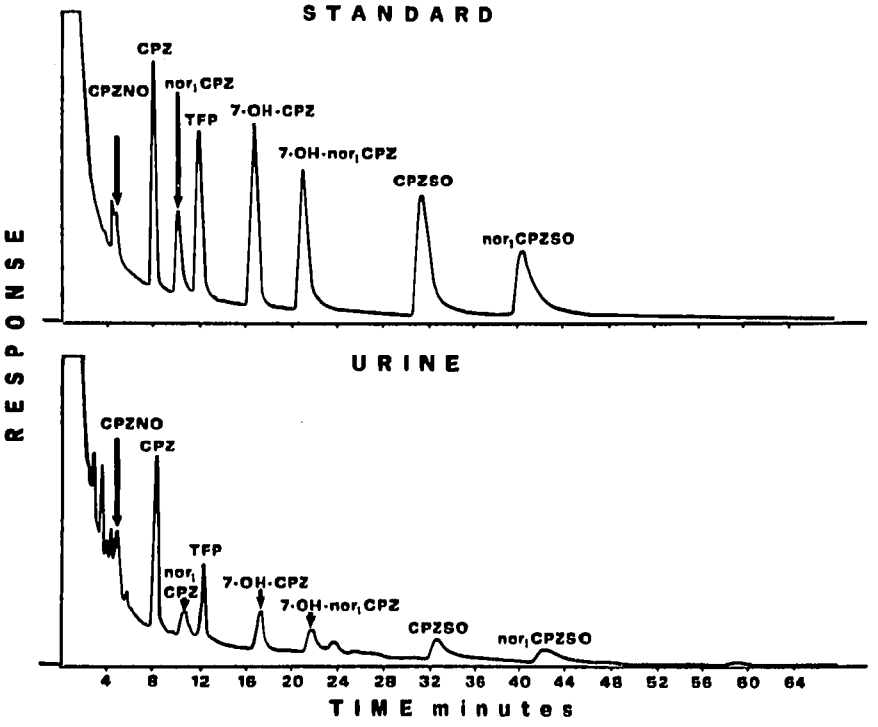

Fig. 3. Gas–liquid chromatographic profile of phenolic and non-phenolic derivatives prepared with TSIM. The upper trace represents a mixture of standards and the lower trace a urinary extract containing CPZ metabolites. Conditions are given in the text.

urine, which was extracted at the two pH values as described above. Fig. 4 summarizes the results of these recovery experiments and indicates that the acidic pH gives higher recoveries with a smaller standard deviation from the mean. The nor_2CPZ is not extracted at the alkaline pH, and nor_2CPZSO does not appear to be extracted at either pH. In general, the extraction procedure appears to be reasonably good for the more polar derivatives (with the exception of nor_2CPZSO) and appears particularly useful for extracting unconjugated 7-OH-CPZ and its metabolites.

Although long-term studies of urinary excretion of CPZ and its metabolites have only just begun, preliminary results for two patients are summarized in Table II. The results suggest that the amount of each metabolite excreted is directly related to the dose administered, as the percentages dose of each metabolite excreted are approximately the same for both the low- and high-dose patients. This trend is also reflected in the excretion of each metabolite relative to CPZ. The non-phenolic oxygenated derivatives predominate, with nor_1CPZSO eliminated in the largest amounts in both cases. This observation has also been characteristic of a number of patients examined so far.

DISCUSSION

Amberlite XAD-2 resin has been used for extracting narcotic analgesics in urine by a number of investigators[13–15] but the assay methods usually consist of

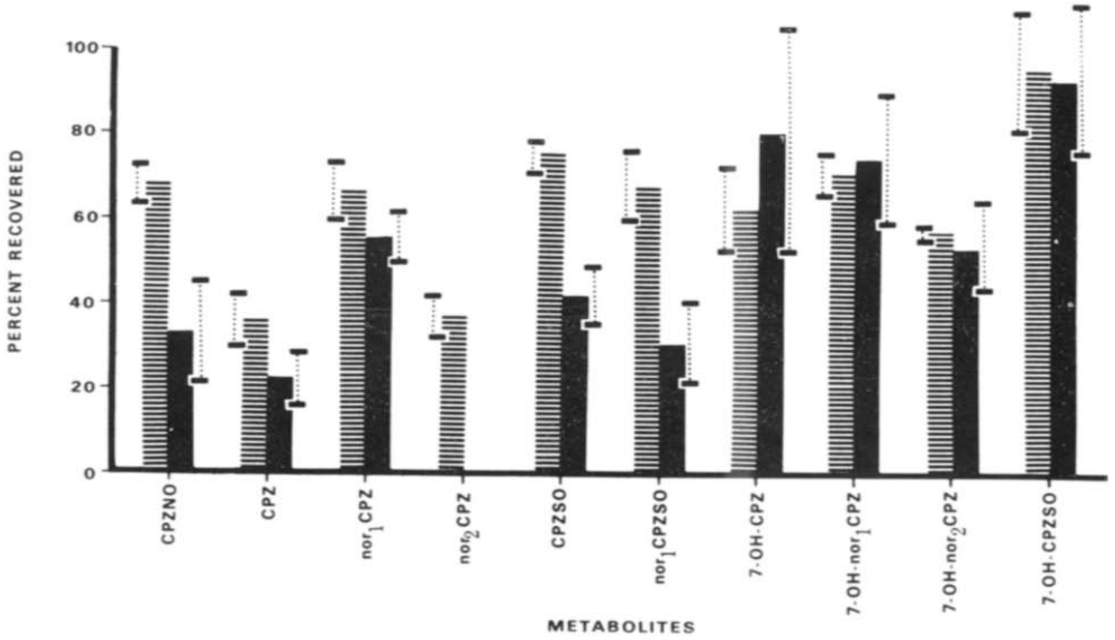

Fig. 4. Recovery of chlorpromazine and its metabolites from control urine performed at pH 9.5 (solid bars) and pH 5.5 (striped bars). Standard deviations are represented by dotted lines. The concentrations (μg/ml urine) of each of the metabolites in the mixture used in the recovery were: CPZNO 2.0, CPZ 1.1, nor$_1$CPZ 3.4, nor$_2$CPZ 5.5, CPZSO 6.3, nor$_1$CPZSO 6.4, nor$_2$CPZSO 8.3, 7-OH-CPZ 2.0, 7-OH-nor$_1$CPZ 4.0, 7-OH-nor$_2$CPZ 5.0 and 7-OH-CPZSO 6.0. Different concentrations of each metabolite were used owing to the variation in the individual RDR values of the compounds used in this study.

TABLE II

SUMMARY OF URINARY EXCRETION OF CPZ AND ITS METABOLITES

Metabolite	*Amount excreted (mg/24 h)* *		*Dose excreted (%)*		*Metabolite/CPZ*	
	Low dose	*High dose*	*Low dose*	*High dose*	*Low dose*	*High dose*
CPZNO	7.44	23.33	1.24	1.24	1.87	2.29
CPZ	3.97	9.76	0.66	0.54	1.00	1.00
nor$_1$CPZ	8.21	21.48	1.37	1.19	2.07	2.20
CPZSO	6.25	18.05	1.04	1.00	1.57	1.85
nor$_1$CPZSO	9.43	38.90	1.57	2.16	2.38	3.99
7-OH-CPZ	3.01	6.44	0.50	0.36	0.76	0.66
7-OH-nor$_1$CPZ	3.95	12.14	0.66	0.67	1.00	1.24

* Data from one low-dose (600 mg/day) and one high-dose (1800 mg/day) patient.

screening by TLC. However, the GLC assay of control urine extracted with this resin occasionally produces extraneous peaks that coincide with the retention times of CPZ and nor$_1$CPZ, respectively, and thus interfere in the assay. By extracting the Amberlite resin as supplied by the manufacturer overnight with hot methanol in

a Soxhlet apparatus, these "peaks" are removed, thus giving an essentially blank chromatogram of the free compound or the TMSi derivative.

Although recoveries of the individual metabolites vary with this procedure, presumably owing to differences in their polarities, we feel that the method is sufficiently reproducible in the acidic pH range to be used on a routine basis. As the recovery experiments were carried out in duplicate only, it is premature to assess the correct standard deviation in recoveries at present. In addition, it is difficult to design a meaningful recovery experiment, which may not reflect the micro-environment of the metabolites *in vivo*. Our experience has shown a resonable consistency in the excretion of each metabolite in individual patients from week to week, which supports this hypothesis.

At the time we first prepared the TMSi derivatives of the 7-hydroxy compounds, we were unaware of an earlier report by Craig and Kray[16], in which they prepared the TMSi derivatives of a number of phenolic metabolites of CPZ using hexamethyldisilazane, which is a less specific silylating agent than is TSIM. Silylation with hexamethyldisilazane, which these investigators applied to pure phenolic compounds, would not afford the selective preparation of TMSi derivatives of phenolic compounds in a crude urinary extract, as in the procedure reported here.

The formation of the TMSi ether of the 7-hydroxy derivatives appears to be quantitative, as indicated by the disappearance of the free non-TMSi phenolic peaks and the consistency of the RDR in routine standardizations. Recently, combined gas chromatography–mass spectrometry* confirmed the formation of the mono-TMSi-7-OH-CPZ with a molecular ion of $m/e=406$ and base peak at $m/e=58$. The remaining fragmentation pattern was consistent with that described by Duffield *et al.*[17,18].

A number of isomeric phenolic metabolites have been found in human urine, but the major phenolic derivative, 7-OH-CPZ, has been reported by several investigators[19–21]. To confirm the presence of 7-OH-CPZ in urine, an extract of a patient excreting 0.5 mg/day of 7-OH-CPZ was assayed by TLC in solvent systems A, B and C. In all instances, the RR_F values agreed with those in the literature and ruled out the possibility of 8-OH-CPZ, which has a significantly different RR_F in all three solvent systems. Unfortunately, authentic 8-OH-CPZ was not available for characterization by GLC or TLC. Additional evidence confirming the identity of 7-OH-CPZ was obtained by PLC purification of a urinary extract in solvent system B. The partially purified residue was silylated with TSIM and pyridine and gave a peak whose RRT was identical with that of 7-OH-CPZ on OV-17.

Excretion values for the urinary CPZ metabolities studied so far agree fairly well with the values obtained by other investigators[1,19,20,22], considering the different assay methods used and the variations expected within a given population of patients. Although this procedure does not extract nor$_2$CPZSO (see Fig. 4), which is one of the major urinary metabolites[22], it is especially useful in assaying the phenolic derivatives, which are probably of greater pharmacological importance[23,24]. The sensitivity of the present method will permit the detection of CPZ metabolites in concentrations as low as 500 ng/ml in urine.

We hope that the improved clinical assay for phenolic derivatives described

* We are grateful to Dr. William Sherman of Washington University for this analysis, which was performed on an LKB Model 9000 gas chromatograph–mass spectrometer.

here will lead to the demonstration of significant correlations between clinical behavior and drug metabolism.

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