Mass Spectrometric Identification of the Metabolites of Methyl N-(o-Aminophenyl)-N-(3-dimethylaminopropyl)anthranilate[1]

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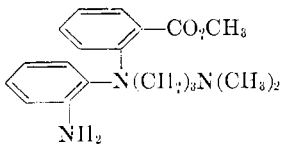
Four metabolites of the title drug, isolated by thin layer chromatography from dog and human urine, have been identified by mass spectrometry. Metabolite A is 5-(3-methylaminopropyl)-5,10-dihydro-11H-dibenzo-[*b,e*][1,4]diazepin-11-one, metabolite B is the methyl ester of N-(*o*-aminophenyl)-N-(3-methylaminopropyl)-anthranilic acid, metabolite C is 5-(3-dimethylaminopropyl)-5,10-dihydro-11H-dibenzo[*b,e*][1,4]diazepin-11-one, and metabolite D is 5-(3-aminopropyl)-5,10-dihydro-11H-dibenzo[*b,e*][1,4]diazepin-11-one. No other metabolites were detected. The structures of two of the metabolites were confirmed by mass spectrometric, infrared, and chromatographic comparison to the synthetically prepared compounds. After oral administration of 350 mg of the title drug (as the dihydrochloride) to five human volunteers, the urinary excretion of the drug and the above metabolites totaled 46% of the dose; about one-tenth of the total urinary output was intact drug. After intravenous administration of 10 mg/kg to a dog, only 19% was recovered in the urine as drug and metabolites (compared to 16% of a 10-mg/kg oral dose), indicating that biliary excretion of the drug and/or its metabolites may be extensive.

Methyl N-(*o*-aminophenyl)-N-(3-dimethylaminopropyl)anthranilate (I) is an orally active diuretic agent



I

in the rat and dog.[2] In the present work, the metabolic fate of the drug was studied in the dog and also in the human, thus affording qualitative and quantitative comparison of absorption and metabolism in two species.

Thin layer chromatography of chloroform extracts of dog and human urine samples revealed four drug-related zones (designated metabolites A, B, C, and D), in addition to a zone corresponding to intact drug. The present paper describes the mass spectrometric identification of the drug and its metabolites from microgram samples eluted from thin layer chromatograms and the quantitation of these compounds in human and dog urine samples. The kinetics of the excretion of the drug and its metabolites will be the subject of a future communication.[3]

Results and Discussion

Isolation of Drug and Metabolites.—The urine was adjusted to pH 10 and extracted with chloroform. A measured volume of the latter was evaporated to a smaller volume and quantitatively transferred to a silica gel G thin layer plate. After development, the compounds were eluted from the silica gel support with pH 10 buffer, which was then extracted with $CHCl_3$ to yield solutions for colorimetric measurement and mass and infrared spectrometry. Three thin layer systems were required to resolve completely the drug and its four metabolites. The chromatographic R_f values are presented in Table I.

After extraction at pH 10, the urine was adjusted to pH 2 with HCl and was extracted again with chloroform. Thin layer chromatography revealed no additional drug-related material. Hydrolysis of the urine with β-glucuronidase and sulfatase prior to alkaline or acidic extraction also did not reveal additional metabolites or increase the yield of metabolites A, B, C, and D, indicating that these metabolites are not conjugated with glucuronic or sulfuric acids.

The drug was shown to be stable to hydrolysis and cyclization under the conditions employed in the isolation procedure, indicating that all of the observed transformations occurred *in vivo*.

TABLE I

THIN LAYER CHROMATOGRAPHIC R_f VALUES[a]

Compd	System A	System B	System C
Drug	*0.61*	0.96	0.20
Metabolite A	0.24	*0.48*	0.02
Metabolite B	0.30	*0.79*	0.04
Metabolite C	*0.49*	0.88	0.20
Metabolite D	0.24	0.48	*0.45*
IV	0.23	0.07	...

[a] The zones used for mass spectrometry and colorimetric assay are in italics.

Mass Spectrum of the Drug.—The mass spectrum of the drug is reproduced in Figure 1. The spectrum of I exhibits major peaks at *m/e* 242, 85, and 58 and a parent ion at *m/e* 327, which corresponds to the molecular weight of the free base.[4] A comparison of the mass spectra of the drug and its metabolites (Figures 1–5, Table II) revealed that three principal fragmentation modes (cleavages 1, 2, and 3) were important for the identification of the metabolites.

TABLE II

PRINCIPAL PEAKS IN THE MASS SPECTRA

		Metabolite			
Ion	Drug	A	B	C	D
M^+	327	281	313	295	267
a^+	242	...	242	...	...
b^+	85	71	71	85	57
c^+	58	44	44	58	30
d^+	...	249	...	249	249

(1) Presented in part before the Medicinal Division of the 152nd National Meeting of the American Chemical Society, New York, N. Y., Sept 1966.

(2) B. E. Graham, unpublished results.

(3) D. L. Smith, A. L. Pulliam, and H. Ko, *J. Pharm. Sci.*, in press.

(4) The mass spectrum produced from about 50 μg of I, eluted from a thin layer chromatogram as the free base, was identical in all respects with the spectrum produced from the dihydrochloride of I as the crystalline solid.

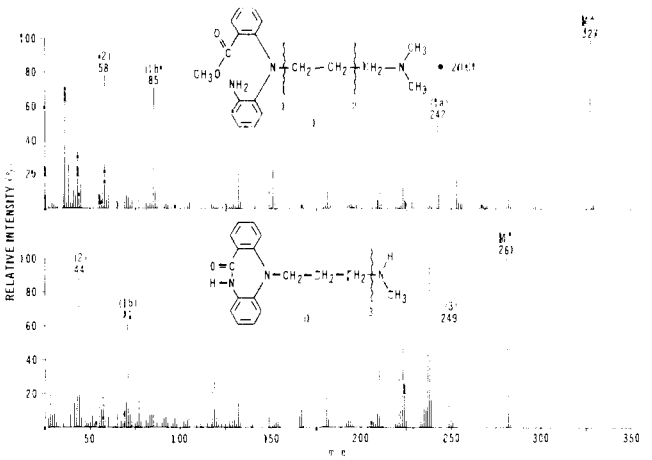


Figure 1.—Mass spectrum of methyl N-(o-aminophenyl)-N-(3-dimethylaminopropyl)anthranilate (dihydrochloride).

Figure 2.—Mass spectrum of metabolite A.

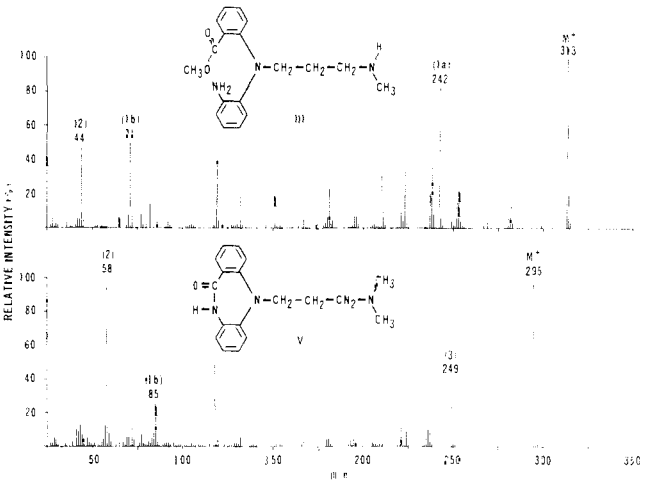


Figure 3.—Mass spectrum of metabolite B.

Figure 4.—Mass spectrum of metabolite C.

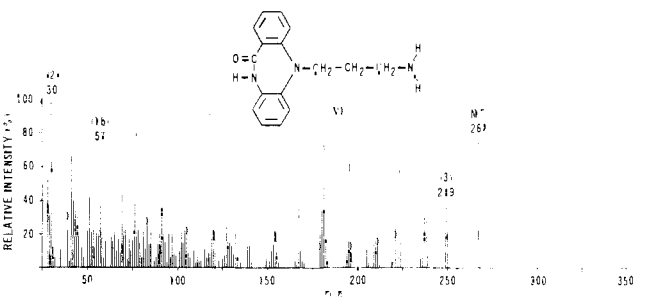


Figure 5.—Mass spectrum of metabolite D.

Cleavage 1 (Figure 1) gives rise to two major fragment peaks at m/e 242 and 85. The fragmentation process leading to the m/e 242 ion can be represented as proceeding through molecular ion M^+ and involving a loss of the entire alkyl group with a hydrogen transfer from this alkyl group to the oxygen atom of the carbonyl. Rearrangements of this type have been reported frequently and have been discussed in detail.[5-8]

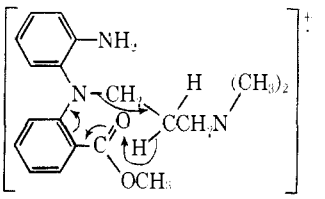
(5) F. W. McLafferty, *Anal. Chem.*, **31**, 82 (1959).

(6) K. Biemann, "Mass Spectrometry," McGraw-Hill Book Co., Inc., New York, N. Y., 1962, Chapter 3.

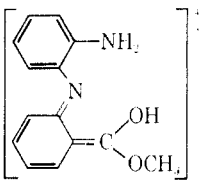
(7) H. Budzikiewicz, C. Djerassi, and H. H. Williams, "Interpretation of Mass Spectra of Organic Compounds," Holden-Day, Inc., San Francisco, Calif., 1964, Chapter 1.

(8) G. Spiteller and M. Spiteller-Friedmann, *Monatsh.*, **95**, 257 (1964).

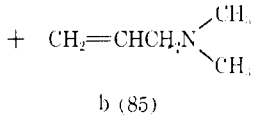
The origin of the hydrogen involved in the hydrogen transfer would have to be established by deuterium-labeling experiments. A Dreiding model of this compound shows that the hydrogens on either the α or β carbon to the nitrogen atom can easily attain the 1.8-A interatomic distance requirement proposed by Djerassi and co-workers[9] for this hydrogen transfer.



M^+ m/e 327



a$^+$, m/e 242

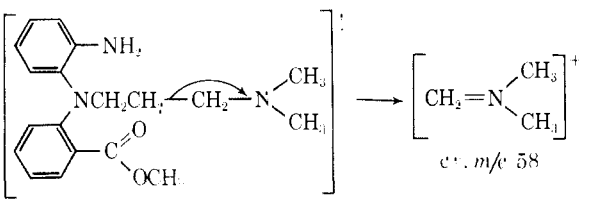


b (85) (1)

The mechanism involving the transfer of a hydrogen on the β carbon is probably preferred as it involves the loss of the stable molecule b.

The fragmentation process leading to the m/e 85 ion is essentially the same as that involved in the formation of the m/e 242 ion. However, in this case the charge resides on the smaller amine fragment. This process can be visualized as proceeding through molecular ion M^+ leading to ion b^+ and the neutral fragment a.

Cleavage 2 gives rise to the most abundant fragment ion at m/e 58. This ion arises from rupture of the C–C bond β to the nitrogen atom of the aliphatic amine portion.



M^+, m/e 327 (2)

c$^+$, m/e 58

Cleavages 1 and 2 therefore offer a means of examining two distinct portions of the drug molecule for changes which might occur upon metabolism. Ions analogous to ion a^+ reflect changes in the aromatic amine portion, whereas those analogous to b^+ and c^+ reflect the alkyl amine portion.

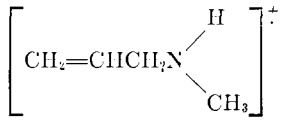
Cleavage 3, which was important for the assignment of the diazepinone structure, will be discussed below under metabolite A.

Mass Spectrometric Identification of the Metabolites.—The silica-metabolite mixture from the thin layer chromatogram could not be assayed directly in the inlet system of the mass spectrometer, since the crucible would not hold a sufficient amount of material to give a

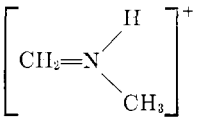
(9) C. Beard, J. M. Wilson, H. Budzikiewicz, and C. Djerassi, *J. Am. Chem. Soc.*, **86**, 269 (1964); D. H. Williams, J. M. Wilson, H. Budzikiewicz, and C. Djerassi, *ibid.*, **85**, 2091 (1963); C. Djerassi, G. von Mutzenbecher, J. Fajkos, D. H. Williams, and H. Budzikiewicz, *ibid.*, **87**, 817 (1965); D. H. Williams and C. Djerassi, *Steroids*, **3**, 259 (1964).

satisfactory spectrum and the dimensions of the cartridge were such that a larger crucible could not be accommodated. The metabolite (50–150 μg) was therefore eluted from the silica gel with pH 10 buffer;[10] a chloroform extract of the latter was injected with a microsyringe into the crucible, which was maintained at 50° to evaporate the chloroform.[11]

Metabolite A.—The molecular ion in the mass spectrum of metabolite A (Figure 2) was observed at *m/e* 281, which is 46 mass units less than the molecular weight of I. Demethylation was indicated by the ions observed at *m/e* 71 and 44 which are the demethyl analogs of ions b^+ and c^+ in the mass spectrum of I (Table II). The loss of 46 mass units suggests the loss of the elements of methanol in addition to the loss of the elements of methanol in addition to the loss of the methyl group.

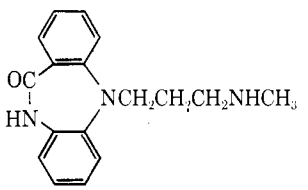


b'^+, *m/e* 71



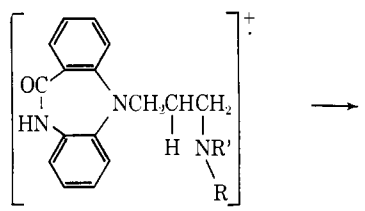
c'^+, *m/e* 44

The absence of an ion at *m/e* 242 suggested that the loss of the elements of methanol involved the methoxy group of the ester and a hydrogen of the amino group. The N-demethyldiazepinone (II) was therefore proposed as the structure of metabolite A. When a

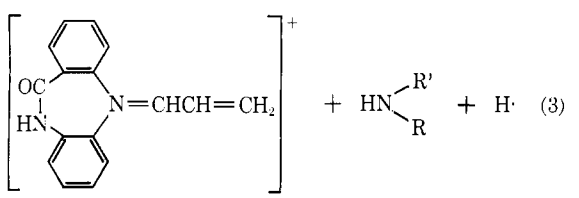


II (metabolite A)

synthetically prepared sample of this compound was obtained, its mass and infrared spectra and chromatographic mobilities in three systems (Table I) were found to be identical with that of metabolite A. The mass spectrum of metabolite A shows a significant fragment ion at *m/e* 249, which is also present in the spectra of metabolites C and D (*vide infra*). This ion (d^+) seems to be characteristic of the cyclic diazepinone structure. Since it is independent of thealkyl amine group, it very likely arises through cleavage 3 (Figure 2). A neutral amine molecule and a hydrogen atom are lost from the molecular ion. This fragmentation is energetically favorable because of the formation of the highly conjugated ion d^+. Unequivocal proof of this fragmentation mechanism would require high-resolution mass spectrometry and/or deuterium labeling experiments.

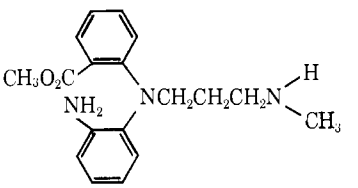


M^+, R=CH_3 or H; R'=CH_3 or H



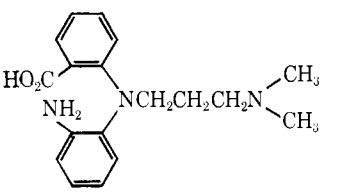
d^+, *m/e* 249

Metabolite B.—The mass spectrum of metabolite B (Figure 3) exhibited a molecular ion at *m/e* 313 corresponding to a molecular weight 14 mass units less than the molecular weight of I. Major peaks were observed at *m/e* 242, 71, and 44, but not at *m/e* 249, indicating that metabolite B was structure III, the N-demethylated analog of the intact drug. The structure was further supported by converting metabolite B to II by refluxing in methanol.



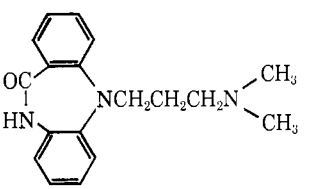
III (metabolite B)

A loss of 14 mass units could also correspond to the acid IV, although peaks *m/e* 71 and 44 would then be difficult to rationalize. Compound IV, was subsequently synthesized and its mass and infrared spectra and chromatographic mobility were shown to differ from metabolite B.



IV

Metabolite C.—The mass spectrum of metabolite C (Figure 4) exhibited a molecular ion at *m/e* 295 corresponding to a molecular weight 32 mass units less than the molecular weight of I. Major peaks were observed at *m/e* 85 and 58 (b^+ and c^+), indicating that the alkylamine group was unchanged from that of the drug and at *m/e* 249, indicating the formation of the diazepinone structure. The 32 mass unit difference could be explained by hydrolysis and cyclization of the drug *in vivo* to yield the diazepinone V, or, less likely, by complete dehydration within the mass spectrometer of IV if it



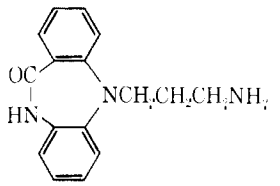
V (metabolite C)

had been the metabolite. When synthetic samples of IV and V were obtained, their mass and infrared spectra and chromatographic mobilities were found to differ considerably. Compound V was identical with metabolite C by these criteria.

(10) Recovery was very poor when the silica gel was eluted directly with $CHCl_3$.

(11) We are grateful to R. J. Wnuk of The Upjohn Co. for this technique.

Metabolite D.—The mass spectrum of metabolite D (Figure 5) exhibited a molecular ion at *m/e* 267, which is 60 mass units less than the molecular weight of I and 14 mass units less than the molecular weight of II. The peaks observed at *m/e* 242, 57, and 30 (ions a^+, b^+, and c^+) and the similarity of the rest of its spectrum to that of metabolite A indicated that it was structure VI, the N-demethylated analog of metabolite A.



VI (metabolite D)

Summary of Mass Spectrometry.—Table II summarizes the mass spectrometric results and illustrates how the metabolites were identified by their mass spectra prior to comparison with the synthetically prepared compounds. The *m/e* 242 ion (a^+) designated the noncyclic methyl ester structure, whereas the absence of this ion together with the presence of the *m/e* 249 ion (d^+) indicated the cyclic structure. Changes in the alkylamine portion were apparent upon examination of the ions b^+ and c^+.

Quantitation of Drug and Metabolites.—A method was developed for the simultaneous determination in urine of microgram per milliliter concentrations of the drug and its metabolites. The principal steps in the procedure are: (1) extraction of pH 10 urine with chloroform, (2) quantitative thin layer chromatographic separation of the compounds from each other and from background material, and (3) measurement of the 410-mμ absorption resulting from complexation in $CHCl_3$ of the isolated compounds with brom cresol purple reagent. When present at concentrations above 10 μg/ml, the compounds can be determined within a relative standard deviation of about 10%.

The drug and metabolites could be quantitatively extracted from pH 10 urine with 5 vol of chloroform. Since the spectral properties of these compounds were unsuitable for direct spectrophotometric determinations at microgram concentration levels, the brom cresol purple procedure of Woods, *et al.*,[12] was investigated. Both the drug and its metabolites responded in this sensitive procedure. Compounds I, II, and V gave very similar molar responses, and the absorbance at 410 mμ was linearly concentration dependent from 0.0 to 100 μg/ml. Thin layer chromatography was added to the procedure, since the brom cresol purple procedure is a nonspecific procedure for amines. The drug and metabolites were visualized by fluorescence quenching. The compounds were quantitatively eluted from the silica gel with pH 10 buffer. Extraction of the latter with chloroform gave a solution suitable for complexation with brom cresol purple.

Human Urine.—Individual and average 32-hr excretion data for I and metabolites A, B, and C in the five subjects are presented in Table III. So little metabolite D was produced that its quantitation was not attempted except to establish the amount excreted by a single subject. Approximately 0.05% of the drug-related material in the urine of subject no. 5 was metabolite D. Of the 350-mg oral dose an average (±SEM) of 162.0 ± 15.3 mg, *i.e.*, 46.3 ± 4.4% of the dose, was recovered in the 0–32-hr urine of the five subjects. Therefore, at least this fraction of the drug was absorbed. The distribution of drug and metabolites A, B, and C in the 0–32-hr urine collection of each subject is presented in Table IV.

TABLE III

DISTRIBUTION OF THE DRUG AND ITS METABOLITES IN THE 0–32-HR URINE OF EACH SUBJECT[a]

	mg of drug					
Subject	Metabolite A[b]	Metabolite B	Metabolite C	Drug	Total	% of dose
1	42.3	129.3	9.8	18.5	199.9	57.1
2	19.0	76.7	8.3	23.9	127.9	36.5
3	21.8	85.6	11.1	15.5	134.0	38.3
4	42.4	138.2	6.6	9.4	196.6	56.2
5	25.3	103.6	6.6	16.1	151.6	43.3
Mean	30.2	106.7	8.5	16.7	162.0	46.3
Std dev	±11.3	±26.7	±2.0	±5.2	±34.2	±9.8
% std dev	37.4	25.0	23.5	31.1	21.1	21.2
SEM[c]	±5.1	±11.9	±0.9	±2.3	±15.3	±4.4

[a] Dose ≈ 350 mg of I (as the dihydrochloride). [b] These values include a small amount (≈0.4 mg) of metabolite D. [c] Standard error of mean.

TABLE IV

DISTRIBUTION OF DRUG AND ITS METABOLITES IN THE 32-HR URINE OF EACH SUBJECT

	% of urinary oral			
Subject	Metabolite A[a]	Metabolite B	Metabolite C	Drug
1	21.2	64.7	4.9	9.3
2	14.8	60.0	6.5	18.7
3	16.3	63.9	8.3	11.6
4	21.6	70.3	3.4	4.8
5	16.7	68.3	4.4	10.6
Mean	18.1	65.4	5.5	11.0
Std dev	±3.1	±4.0	±1.9	±5.0
% std dev	17.0	6.1	34.5	45.4
SEM	±1.4	±1.8	±0.6	±2.2

[a] These values include a small amount (≈0.1 mg) of metabolite D.

Dog Urine.—After both oral and intravenous administration, the dog excreted metabolites whose thin layer mobilities in three systems corresponded to the human metabolites. The distribution of drug and metabolites in the urine was essentially independent of the route of administration (Table V). Also, the total

TABLE V

METABOLISM OF 105-MG DOSE IN THE DOG

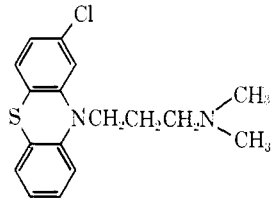
	mg excreted (0–24 hr)		% of total as each metabolite (0–24 hr urine)	
Metabolite	Iv dose	Oral dose	Iv dose	Oral dose
A	3.85[a]	2.64[a]	18.9	16.3
B	6.95	5.77	34.2	35.5
C	3.69	3.09	18.2	19.0
Drug	5.81	4.74	28.6	29.2
Total	20.30	16.24	100.0	100.0
% of dose	19.3	15.5		

[a] These values include a small amount (≈0.1–0.2 mg) of metabolite D.

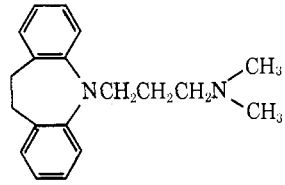
(12) L. A. Woods, J. Coelho, E. J. Fornefeld, F. G. McMahon, and M. H. Seevers, *J. Pharmacol. Exptl. Therap.*, **101**, 188 (1951).

recovered in the urine 24 hr after the oral route of administration was about 80% of that recovered after the intravenous route, indicating that at least 80% of the drug is absorbed in the dog. Extensive biliary excretion is indicated since only 19.3% of the dose was recovered in the urine after intravenous administration.

Metabolism of Structurally Related Drugs.—The investigated drug, although noncyclic, has some structural similarity to the phenothiazine drugs (*e.g.*, chlorpromazine, VII) and imipramine (VIII). Like I,



VII



VIII

both of these compounds are mono- and didemethylated *in vivo* by several species.[13] However, both of these drugs are also ring hydroxylated and then conjugated with glucuronic acid. Even after hydrolysis with β-glucuronidase, no evidence was found for ring-hydroxylated or conjugated metabolites of I. Although Fishman, *et al.*,[14] have reported the isolation of the N-oxides of chlorpromazine (0.7% of the dose) and imipramine (~2% of the dose), no evidence was found for N-oxide metabolites in the present study.

Experimental Section

Human Studies.—A single dose of 350 mg of I as the dihydrochloride was administered to each of five male volunteer subjects after an overnight fast. Food was allowed 1 hr after drug administration. Water intake was limited to 240 ml at the following times relative to the time of dosing: −1, 0, 1.5, 4, 6.5, 10, 14, 23, 24, 25.5, 28, and 30.5 hr. Urine was collected quantitatively during the intervals 0–1, 1–3, 3–5, 5–8, 8–12, 12–16, and 16–32 hr after drug administration.

Dog Studies.—Oral and intravenous 105-mg doses of I as the dihydrochloride were administered on successive weeks to a 10.5-kg female beagle, fasted overnight. Water and food were allowed *ad libitum* after administration of the drug. Urine was collected for 24 hr after drug administration.

Isolation of Metabolites (Thin Layer Chromatography).—The urine was adjusted to pH 10 and extracted with $CHCl_3$, and the latter was evaporated to dryness in a conical centrifuge tube. The residue was transferred with a small amount of $CHCl_3$ to a silica gel G thin layer plate which had been impregnated with phosphors. The following three solvent systems were employed for development: system A, C_6H_6–CH_3OH–NH_4OH (50:50:1); system B, EtOAc–DMF–NH_4OH (50:50:5); system C, CH_3OH–$(CH_3)_2CO$–C_6H_6–7 *M* NaOH (50:50:10:1). System A was used for the isolation of I and metabolite C, system B for metabolites A and B, and system C for metabolite D. The silica gel containing each of the zones was scraped from the plate and eluted with pH 10 buffer. The latter was then extracted with $CHCl_3$ to yield a solution for analysis.

Procedure for Determination of Drug and Metabolites in Urine.—To 5.0 ml of urine in a 35-ml centrifuge tube was added 3.0 ml of pH 10 buffer (20.0 g/l. of Na_2CO_3 + 12.4 g/l. of $Na_2B_4O_7$) followed by 25.0 ml of $CHCl_3$. The mixture was shaken vigorously and allowed to stand for 30 min. The phases were separated by centrifugation (2000 rpm for 10 min) and the aqueous layer was removed by aspiration. A 20.0-ml aliquot of the $CHCl_3$ was transferred to a 50-ml open-mouth centrifuge tube and evaporated to dryness under a stream of nitrogen. The tube was washed down with 5, 3, and 1 ml of $CHCl_3$, evaporating to dryness between each washing. The residue was dissolved in a few drops of $CHCl_3$, and quantitatively transferred to a silica gel G thin layer plate (250-μ thickness, impregnated with $ZnSiO_3$ and $(ZnCd)_2S$ phosphors). Standards were spotted, the plate was developed with the appropriate system (Table I), and the zones were located with a mercury lamp (254 mμ). The drug and metabolite zones were individually scraped from the chromatogram and transferred to 15-ml centrifuge tubes. Five milliliters of pH 10 buffer was added to each tube and, after shaking, the mixtures were centrifuged to settle the silica gel. Four-milliliters aliquots of the clear, aqueous solutions were transferred to another set of 15-ml centrifuge tubes, 4.0 ml of $CHCl_3$ was added, and the mixtures were shaken vigorously and centrifuged at 2000 rpm for 5 min. The aqueous phases were aspirated and 3.0 ml of the $CHCl_3$ phases were transferred to 10-ml volumetric flasks containing 0.3 ml of brom cresol purple reagent (50 mg/100 ml of $CHCl_3$). The absorbances were measured at 410 mμ with a Cary spectrophotometer, employing $CHCl_3$ in the reference cell. A zero-hour urine sample was subjected to the same procedure in order to correct for background material. The concentrations of drug and metabolites were calculated using previously prepared calibration curves, having the following slopes (microgram of drug equivalents per milliliter per absorbance unit): drug, 32.9; metabolite A, 30.7; metabolite C, 28.1. Since samples of metabolites B and D were not available for calibration, an estimated slope of 30.0 was used.

Mass Spectrometry.—The mass spectra were recorded employing an Atlas CH_4 mass spectrometer, ionizing potential 70 ev, ionizing current 20 μa. The solid samples were ionized by electron bombardment after sublimation directly into the electron beam from a small graphite crucible heated by a tungsten coil. A cathode with a tungsten wire of 0.15-mm diameter was used.

Materials.—Brom cresol purple (5′,5″-dibromo-*o*-cresolsulfonphthalein indicator) was obtained from Eastman Organic Chemicals. The preparation of compounds I and V has been described previously.[15] Compound IV was prepared by Dr. A. R. Hanze of The Upjohn Co. *via* hydrolysis of I with $Ca(OH)_2$. Compound II was prepared by Dr. R. S. P. Hsi of The Upjohn Company using the procedures described by Hanze, *et al.*[15]

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