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CHLORDIAZEPOXIDE METABOLITES IN THE RAT.
CHARACTERIZATION BY HIGH RESOLUTION MASS SPECTROMETRY

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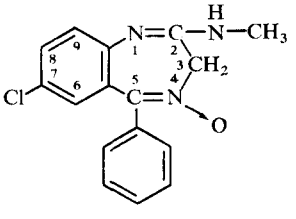
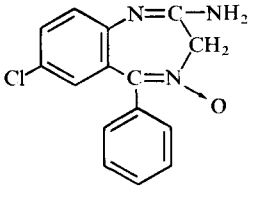
Abstract—Four rat urinary metabolites of chlordiazepoxide (7-chloro-2-methylamino-5-phenyl-3H-1,4-benzodiazepine 4-oxide) labeled at C-2 with ^{14}C were separated by solvent extraction and thin-layer chromatography (TLC) and characterized by high resolution mass spectrometry. Each metabolite was found to have a hydroxyl function in the C-5 phenyl ring, and two-dimensional TLC of metabolites with reference compounds indicated that the most probable location of the phenolic function was *para* to the diazepine ring. The four metabolites were: 7-chloro-1,3-dihydro-5-(4-hydroxyphenyl)-2H-1,4-benzodiazepin-2-one 4-oxide (Metabolite 1); 2-amino-7-chloro-5-(4-hydroxyphenyl)-3H-1,4-benzodiazepine 4-oxide (Metabolite 2); 7-chloro-5-(4-hydroxyphenyl)-2-methylamino-3H-1,4-benzodiazepine 4-oxide (Metabolite 3); and 7-chloro-1,3-dihydro-5-(4-hydroxyphenyl)-2H-1,4-benzodiazepin-2-one (Metabolite 4), which differs from the others in that it no longer retains the N-oxide function).

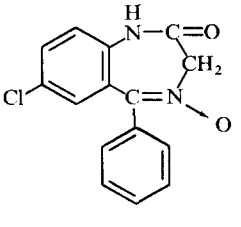
CHLORDIAZEPOXIDE,* 7-chloro-2-methylamino-5-phenyl-3H-1,4-benzodiazepine 4-oxide, was reported[1, 2] to be metabolized in dog and man to 7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one 4-oxide ("lactam"), which is further metabolized to "opened lactam" metabolites. In the rat other metabolites, which were not identified, were found. Recently, N-desmethyl chlordiazepoxide (metabolite D-M), 2-amino-7-chloro-5-phenyl-3H-1,4-benzodiazepine 4-oxide, has been detected in the blood of rats, squirrel monkeys, and humans treated with chlordiazepoxide and has been demonstrated to be formed *in vitro* by rat and dog liver.[3]



Chlordiazepoxide



Metabolite D-M



Lactam†

The recent application of thin-layer chromatography (TLC) for separation and purification combined with high resolution mass spectrometry for identification yielded the characterization of four metabolites of diazepam‡ (another compound in the 1,4-benzodiazepine series) in the intestinal contents of one rat treated with 25 mg of

* Chlordiazepoxide hydrochloride is the active ingredient in Librium marketed by Hoffmann-La Roche Inc., Nutley, N. J.
† "Opened lactam" results from hydrolysis of the lactam bond.
‡ Diazepam is the active ingredient in Valium marketed by Hoffman-La Roche Inc., Nutley, N. J.

^{3}H-labeled drug.[4] This encouraged us to employ these techniques for the identification of chlordiazepoxide metabolites in the rat. A brief account of the high resolution mass spectrometry of these metabolites has been presented.[5]

EXPERIMENTAL

Counting of samples. Urine, extracts of urine, and silica gel segments from thin-layer chromatoplates were all counted in a liquid scintillation spectrometer (Packard Instruments, La Grange, Ill.) by procedures previously described.[6]

Dosage. Chlordiazepoxide-2-^{14}C (0·77 μc/mg) was freshly sublimed and suspended in 0·9% saline as previously described.[2] Two 280-g male rats (Charles River) designated No. 1 and No. 2 were each injected intravenously with 1 ml saline containing $4{\cdot}31 \times 10^{6}$ dpm (2·6 mg) of ^{14}C-chlordiazepoxide.

Urine extraction. Urine was collected from each rat for 3 days and all subsequent treatment of the urine samples was done in either semidarkness or under a red light in order to avoid photochemical degradation of chlordiazepoxide and metabolites.[2] Essentially all the urine excreted by each rat during the first 30 hr was lyophilized and dissolved in 10 ml water. Then each lyophilate was adjusted to pH 1·0 with dilute hydrochloric acid and extracted once with 2 vol. ethyl acetate. The organic phases were washed with 0·1 N hydrochloric acid and the resulting acid washes combined with the aqueous phases were adjusted to pH 7·0 with dilute sodium hydroxide and extracted twice with 1 vol. ethyl acetate. These latter ethyl acetate extracts were washed with 0·1 M potassium phosphate buffer, pH 7·0, and the washes were added to the neutral aqueous phases. The pH of the aqueous phases was then brought to 5·5, Glusulase (Endo Labs., Garden City, N.Y.) was added to a final concentration of 1% (v/v), and after a 3-hr incubation at 37° the ethyl acetate extractions at pH 1·0 and then at pH 7·0 were repeated.

Metabolite isolation. The general procedure for separation and purification of labeled metabolites previously used[4] was applied again. The labeled components of the ethyl acetate extracts were first separated by TLC on silica gel containing a fluorescent indicator (Camag Kieselgel DF-5) with system 1, heptane:chloroform:ethanol (2:2:1), for development. Pilot plates were first run with small aliquots of the extracts, and the fluorescence-quenching bands seen under short-wave u.v. light were scraped from the plates and counted in order to determine the distribution of radioactivity. This was then used as a guide for the major isolation experiments in which the extracts were applied as streaks across 20 × 20 cm plates and the labeled components separated by TLC were eluted from the silica gel with ethanol. These eluates, after concentration, were subjected to further TLC either in system 2, ethyl acetate:ethanol (80:20), or in system 3, ethyl acetate:ethanol (90:10), to effect purification of the labeled metabolites. Samples of 2–4 μg of purified metabolites were prepared for mass spectral analysis as previously described.[4]

High resolution mass spectrometry. A brief description of this technique and of the instrument employed in these studies has been reported.[4]

Two-dimensional TLC. Final elucidation of metabolite structure was obtained by two-dimensional TLC of a mixture of labeled metabolite and synthetic reference compound. Five reference compounds (described in detail under 'Results') with structures suggested by the mass spectral analysis of the isolated metabolites were synthesized by A. Stempel and L. H. Sternbach (Hoffmann-La Roche Inc., Nutley, N.J.). The following four solvent system pairs were used in this phase of the

study: system 2D-1, heptane:chloroform:ethanol:concentrated ammonia (5:5:3:0·3) followed by heptane:chloroform:ethanol:acetic acid (5:5:3:1); system 2D-2, chloroform:ethanol (90:10) followed by ethyl acetate:ethanol (95:5); system 2D-3, heptane-chloroform-ethanol (2:2:1) followed by ethyl acetate:ethanol (90:10); and system 2D-4, chloroform:ethanol (90:10) followed by isopropanol:ethanol (90:10). After development in one of these systems, the silica gel containing the reference compound (located under short-wave u.v. light) was scraped from the plate and counted to determine whether the labeled metabolite had migrated the same as had the reference compound.

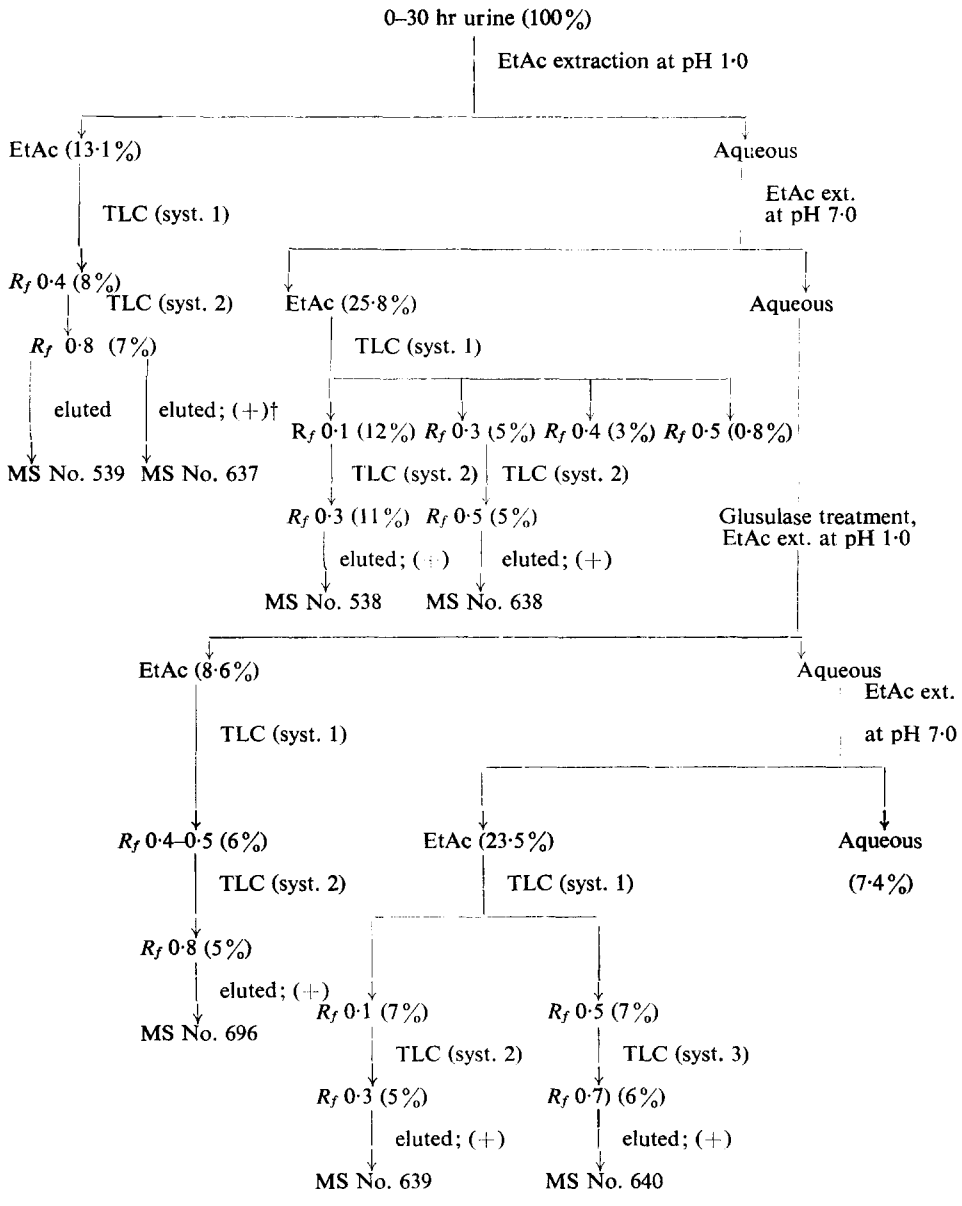
RESULTS

Isolation of metabolites. Rat 1 excreted 31·6 per cent of the administered radioactivity in the urine in 3 days, with 29·8 per cent of the dose excreted in 0–30 hr and only 0·6 per cent excreted during 48–72 hr. The ^{14}C excretion by rat 2 was very similar; 32·1 per cent of the dose was excreted in 3 days, with 28·5 per cent of the dose excreted in 0–30 hr and only 0·5 per cent during 48–72 hr. Since the 0–30 hr urine from each rat contained roughly 90 per cent of the total urinary ^{14}C, these samples were taken for metabolite isolation.

Table 1 represents a flow diagram of the isolation of metabolites from the urine of rat 1 according to procedures described in Experimental. The urine of rat 2 was processed in the same manner as that of rat 1 and yielded only slightly different amounts of the same metabolites. The identical metabolites from each rat (recognized by their TLC migration) were pooled and 7 samples representing 6 metabolites were prepared for high resolution mass spectrometry. It is seen from Table 1 that two samples represented one metabolite extracted at pH 1·0 into ethyl acetate, the metabolite from the urine of rat 1 alone (MS No. 539) and the same metabolite combined with that from the urine of rat 2 (MS No. 637). From the R_f values of the metabolites, it is apparent that the metabolite extracted at pH 1·0 before Glusulase treatment (MS No. 539 and No. 637) may be identical to the one extracted at this pH after Glusulase (MS No. 696). Furthermore, while one metabolite extracted at pH 7·0 before Glusulase (MS No. 538) may be the same as one extracted at this pH after Glusulase (MS No. 639), the other 2 metabolites (MS No. 638 and No. 640) are apparently not identical.

High resolution mass spectrometry. The possible identity of certain metabolites with one another (described above) was confirmed by mass spectral analysis. Therefore the results of this analysis (Table 2) are presented in terms of the four individual metabolites (designated Metabolites 1–4) which were isolated. Shown first in Table 2 is the fragmentation pattern of chlordiazepoxide, and the following discussion illustrates the structural implications of this fragmentation. The N-oxide oxygen was readily lost giving rise to a fragment which differed from the molecular ion by the loss of O or OH (column 5). A fragment resulting from the loss of the C-3-N-4 moiety (loss of O + H + HCN) was also observed. The loss of H + HCN was not seen, since chlordiazepoxide could not lose the N-4 nitrogen without also losing the attached oxygen. The loss of H + CO and of O + H + CO was also not observed, because this type of fragmentation is characteristic of benzodiazepines which contain a carbonyl function at C-2. Finally, the HOC_6H_4CN fragment was not seen, because this fragment indicates the presence of a hydroxyl function in the 5-phenyl ring.

TABLE 1. ISOLATION OF THE URINARY METABOLITES OF RAT 1*



* This scheme shows the isolation procedures used to obtain purified metabolites for analysis by high resolution mass spectrometry. The final purified fractions from which aliquots were taken for mass spectrometry are denoted by MS No. The values in parentheses give the per cent of the original urinary ^{14}C which was separated by ethyl acetate extraction and by subsequent TLC of the extracts.
† The designation (+) indicates that the purified metabolite from rat 1 was combined with the identical one from rat 2 prior to mass spectral analysis.

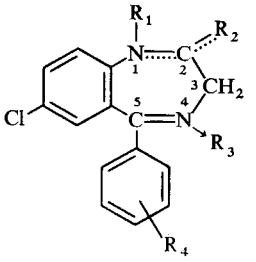
Metabolite 1 (MS No. 539, No. 637 and No. 696) had a molecular ion composition of $C_{15}H_{11}N_2O_3Cl$, which differed from that of chlordiazepoxide by the subtraction of CH_3N and addition of 2 oxygens. The fragmentation resulting in loss of O + H + CO

TABLE 2. HIGH RESOLUTION MASS SPECTRAL ANALYSIS OF CHLORDIAZEPOXIDE AND METABOLITES

Sample (MS No.)	Metabolite designation	Molecular ion composition		Fragmentation by loss of:						Structure*			
		Empirical formula	Difference from chlordiazepoxide	O or OH	O + H + HCN	H + HCN	H + CO	O + H + CO	HOC_6H_4CN fragment detected	R_1	R_2	R_3	R_4
Chlordiazepoxide†		$C_{16}H_{14}N_3OCl$		yes	yes	no	no	no	no	none	$HNCH_3$	O	H
539,637,696	Metabolite 1	$C_{15}H_{11}N_2O_3Cl$	$-CH_3N, +2O$	yes	yes	no	no	yes	yes	H	O	O	OH
538,639	Metabolite 2	$C_{15}H_{12}N_3O_2Cl$	$-CH_2, +O$	yes	yes	no	no	no	yes	none	NH_2	O	OH
638	Metabolite 3	$C_{16}H_{14}N_3O_2Cl$	$+O$	yes	yes	no	no	no	yes	none	$HNCH_3$	O	OH
640	Metabolite 4	$C_{15}H_{11}N_2O_2Cl$	$-CH_3N, +O$	no	no	yes	yes	no	yes	H	O	none	OH

† Authentic chlordiazepoxide was used as a reference compound.

* The following benzodiazepine structure is convertible into the specific structures of chlordiazepoxide and metabolites by insertion of each appropriate R-group. A lactam bond is formed when $R_2 = O$ and $R_1 = H$.



demonstrated that a carbonyl function had replaced the methylamino moiety at C-2; this accounted for the subtraction of CH_3N and addition of one oxygen. The appearance of an HOC_6H_4CN fragment indicated that the second O was added to the 5-phenyl ring. Metabolite 2 differed from chlordiazepoxide by the subtraction of CH_2 and the addition of O. The presence of the HOC_6H_4CN fragment showed that the O was added as a phenolic function to the 5-phenyl ring. Since in other respects the fragmentation did not differ from that of chlordiazepoxide, it is evident that the loss of CH_2 represents N-demethylation at C-2 leaving a primary amino group on this carbon. Metabolite 3 differed from chlordiazepoxide by the addition of one O, which was added to the 5-phenyl ring. Metabolite 4 differed from chlordiazepoxide by the loss of CH_3N and the gain of one O. The fragmentation pattern indicated that this metabolite was different from all the others in that it no longer retained the N-oxide oxygen (no loss of O or OH). In other respects the structure was identical to that of Metabolite 1, with a carbonyl function at C-2 (—CH_3N,+ O) and an oxygen added to the 5-phenyl ring.

Comparative two-dimensional TLC. Mass spectral analysis could not supply the specific location of the hydroxyl group present in the 5-phenyl ring of each metabolite. This was accomplished by comparative two-dimensional TLC, a technique available to us because A. Stempel and L. H. Sternbach synthesized five reference compounds. The structures of four of these compounds, the *para*-hydroxy isomers of the structures obtained by mass spectrometry, are included in Table 3; the fifth reference compound, 7-chloro-1,3-dihydro-5-(2-hydroxyphenyl)-2H-1,4-benzodiazepin-2-one, was the *ortho*-hydroxy isomer of Compound R-4. When this isomer was co-chromatographed with Compound R-4 in the two-dimensional systems, 2D-1, 2D-2, and 2D-3, it was found that each solvent system was capable of completely resolving the isomers. It is seen from Table 3 that the major portion of the ^{14}C of each metabolite migrated fairly consistently with the reference compound with which it was compared. Therefore, since the two-dimensional systems were capable of distinguishing between at least two of the three possible isomers, it was concluded that the most probable location of the 5-phenyl hydroxyl group of each metabolite was *para* to the diazepine ring.

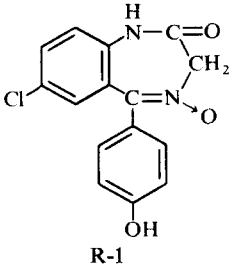
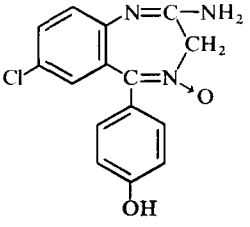
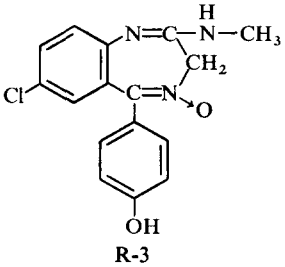
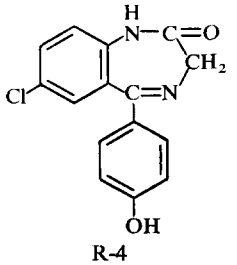
The excretion of the four metabolites in quantitative terms is summarized in Table 4. Metabolites 1 and 2 (conjugated and unconjugated) were each excreted to over twice the amount of Metabolite 3 (unconjugated only) and of Metabolite 4 (conjugated only). The amounts of each metabolite were estimated very conservatively without provision for losses sustained during the isolation procedures. Therefore, the urinary radioactivity not identified represented both the amounts of Metabolites 1–4 lost during the isolation and other metabolites which were present in such small amounts as to preclude isolation and identification.

DISCUSSION

Roughly one-third of the injected radioactivity was excreted in the urine by both rats. The other two-thirds was presumably excteted in the feces, since it has been shown previously[2] that radioactivity from chlordiazepoxide-2-^{14}C injected intraperitoneally was secreted into the stomach and intestinal tract.

Certain properties of the rat urinary metabolites of chlordiazepoxide-2-^{14}C were also previously reported.[2] One fraction, which was extractable at pH 1, migrated as one component on paper chromatography; this most likely was Metabolite 1, which

TABLE 3. COMPARATIVE TWO-DIMENSIONAL TLC OF THE METABOLITES WITH REFERENCE COMPOUNDS

Reference compound*	^{14}C-metabolite No.	Solvent system†	Per cent of ^{14}C migrating with reference compound
 R-1	1 (MS No. 637)	2D-1	90
	1 (MS No. 637)	2D-2	82
	1 (MS No. 637)	2D-3	86
	1 (MS No. 696)	2D-1	88
	1 (MS No. 696)	2D-2	76
	1 (MS No. 696)	2D-3	92
 R-2	2 (MS No. 538)	2D-3	79
	2 (MS No. 538)	2D-4	72
	2 (MS No. 639)	2D-3	87
	2 (MS No. 639)	2D-4	82
 R-3	3 (MS No. 638)	2D-1	81
	3 (MS No. 638)	2D-2	83
	3 (MS No. 638)	2D-3	92
 R-4	4 (MS No. 640)	2D-1	79
	4 (MS No. 640)	2D-2	68
	4 (MS No. 640)	2D-3	87

* The chemical name of each reference compound follows:
R-1 = 7-chloro-1,3-dihydro-5-(4-hydroxyphenyl)-2H-1,4-benzodiazepin-2-one 4-oxide;
R-2 = 2-amino-7-chloro-5-(4-hydroxyphenyl)-3H-1,4-benzodiazepine 4-oxide;
R-3 = 7-chloro-5-(4-hydroxyphenyl)-2-methylamino-3H-1,4-benzodiazepine 4-oxide;
R-4 = 7-chloro-1,3-dihydro-5-(4-hydroxyphenyl)-2H-1,4-benzodiazepin-2-one.

† The solvent systems employed for two-dimensional TLC are described under Experimental.

in the present study was found to account for most of the ^{14}C extracted at pH 1·0 into ethyl acetate. Of the two distinct components extractable at pH 7 in the previous study, the major one was found to have u.v. absorption maxima at 260 and 330 mμ, a 260 mμ/330 mμ u.v. absorption ratio of 3·2, a relatively small amount of primary aromatic amine liberated by acid hydrolysis (as measured by the Bratton–Marshall reaction), and no methylamino moiety at C-2. Metabolite 2, the major metabolite extracted at pH 7·0 in the present study, exhibited u.v. absorption maxima at 260 and 330 mμ, a 260 mμ/330 mμ ration of 3·4, only ½ the Bratton–Marshall molar extinction after acid hydrolysis as that produced by chlordiazepoxide, and it has no

TABLE 4. ESTIMATED QUANTITIES OF EACH METABOLITE EXCRETED UNCONJUGATED (BEFORE GLUSULASE TREATMENT) AND CONJUGATED (AFTER GLUSULASE TREATMENT) IN THE 0–30 HOUR RAT URINE

		Amount of urinary metabolite*			
		% of Urinary ^{14}C		% of Dose	
Urine extract	Metabolites	Rat 1	Rat 2	Rat 1	Rat 2
Before Glusulase treatment:					
EtAc (at pH 1·0)	1	7	9	2	3
EtAc (at pH 7·0)	2	11	15	3	4
	3	5	6	2	2
After Glusulase treatment:					
EtAc (at pH 1·0)	1	5	6	2	2
EtAc (at pH 7·0)	2	5	5	2	2
	4	6	5	2	2

* The amount of each was estimated from the ^{14}C which separated as each metabolite on TLC (as shown in Table 1 for rat 1).

methylamino function at C-2. Therefore, from the general agreement of properties it appears that the major basic metabolite previously described was Metabolite 2. Although the properties of the minor metabolite were not investigated thoroughly,[2] this metabolite could have been Metabolite 3, which was found above to be present to a lesser extent than Metabolite 2 in the pH 7·0, ethyl acetate extracts.

An outline of chlordiazepoxide metabolism in the rat in the light of metabolites so far identified is shown in Fig. 1. Metabolite D-M, although not found in rat urine in the present study, is included in this scheme because previously[3] it was found in rat blood after chlordiazepoxide administration and rat liver did produce this metabolite from chlordiazepoxide *in vitro*. It is seen that Metabolite 2 formation may involve Metabolite D-M or Metabolite 3, or both as precursors.

As was found for diazepam,[4] *para*-hydroxylation of the 5-phenyl ring is a major reaction in the metabolism of chlordiazepoxide in the rat. The metabolism of the two 1,4-benzodiazepines differed in that diazepam was also hydroxylated at C-3 while this pathway was not seen with chlordiazepoxide; instead a stepwise elimination of the methylamino moiety at C-2 occurred.

It is of interest that one metabolite in the rat common to both drugs was found.

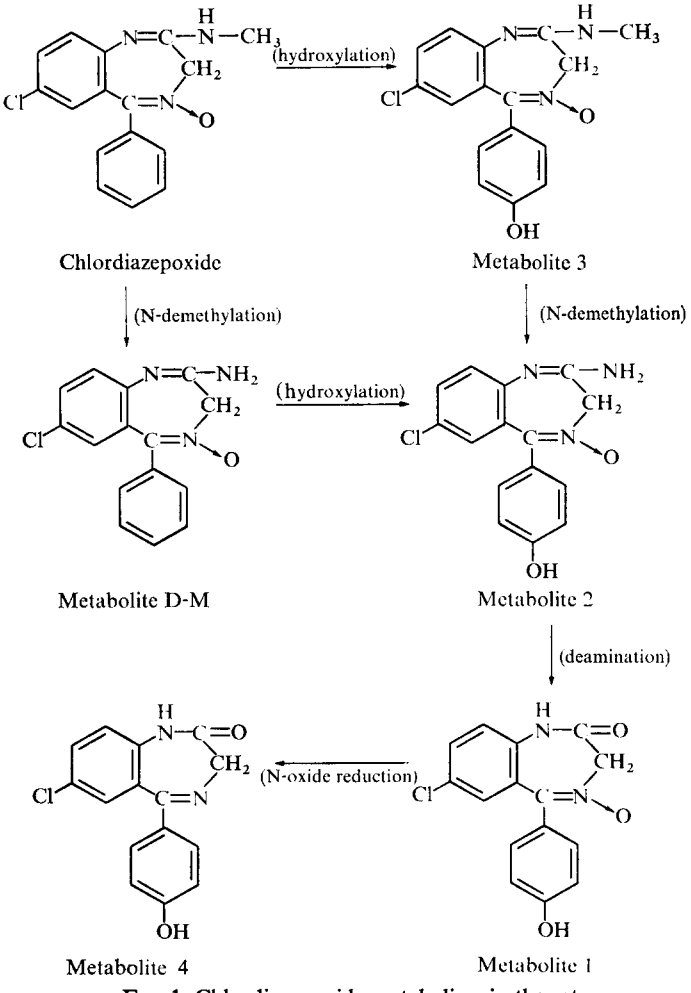


FIG. 1. Chlordiazepoxide metabolism in the rat.

Metabolite 4 of chlordiazepoxide, the only metabolite which no longer retained the N-oxide function, had been previously found[4] to be a diazepam metabolite (designated Metabolite D) in the rat. In the dog another metabolite, oxazepam, has been reported to be produced by both diazepam[6, 7] and chlordiazepoxide.[8]

Acknowledgments—We gratefully acknowledge our indebtedness to Dr. P. Bommer for continued interest in the high-resolution mass spectrometry of the metabolites, to Mrs. A. Goetz for operation of the mass spectrometer, and to Drs. A. Stempel and L. H. Sternbach for the chemical synthesis of those compounds suspected of being metabolites.

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