Metabolism of Demoxepam, a Chlordiazepoxide Metabolite, in the Dog

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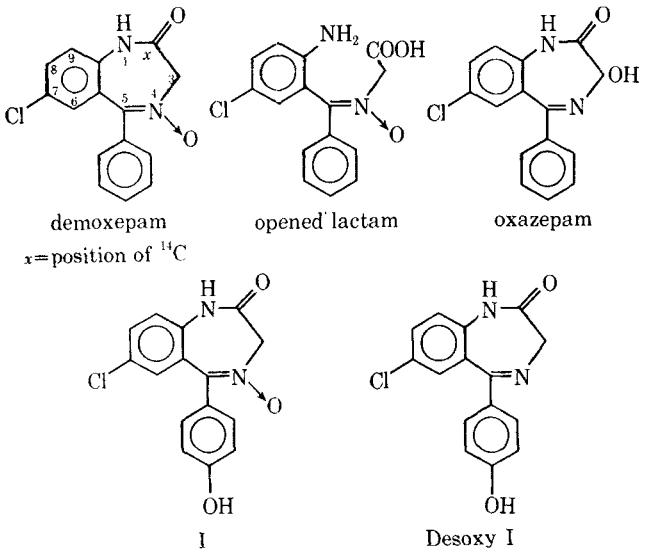
Abstract □ The pharmacokinetic evaluation of the disposition of intravenous and oral doses of demoxepam, 7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide, in the dog revealed that: (*a*) the disposition was adequately described by a two-compartment open-system model; (*b*) oral demoxepam was apparently well absorbed; (*c*) the rate of elimination (β) varied between dogs with a half-life of 10–20 hr., but within an individual dog, it did not appear to be appreciably influenced by the route of administration; and (*d*) elimination proceeded primarily by biotransformation, with the excretion of intact drug limited to 10% in the urine and less than 2% in the feces. Pathways of biotransformation were studied with the aid of demoxepam-2-^{14}C. Two oxidative pathways led to the formation of two phenolic metabolites, the 5-(4-hydroxyphenyl) derivative (I) and the 9-hydroxy derivative (II) of demoxepam. Another pathway involved the removal of the *N*-oxide function resulting in the formation of *N*-desoxydemoxepam (III), oxazepam, and the *N*-desoxy phenols, Desoxy I and Desoxy II. Finally, the hydrolysis of demoxepam to *N*-(2-amino-5-chloro-α-phenylbenzylidene)glycine *N*-oxide ("opened lactam") was also demonstrated. The finding of *N*-desoxy metabolites in the feces and the corresponding *N*-oxides in the bile suggested that the site of reduction of the *N*-oxide function was the intestinal tract.

Keyphrases □ Chlordiazepoxide metabolite—demoxepam □ Demoxepam and demoxepam-2-^{14}C—metabolism, dog □ Biotransformation, metabolite formation, excretion—demoxepam □ Metabolites, demoxepam—separation, identification □ TLC—separation, identification, estimation □ Scintillometry—analysis □ NMR—structure □ Mass spectrometry—structure

Demoxepam (formerly designated Ro 5-2092 or "lactam"), 7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide, was first shown to be a plasma metabolite of chlordiazepoxide[1] in man and dog by Koechlin and D'Arconte (1). In further studies, Koechlin *et al.* (2) confirmed this finding and presented evidence that the urinary metabolites of ^{14}C-chlordiazepoxide in man included demoxepam, *N*-(2-amino-5-chloro-α-phenylbenzylidene)glycine *N*-oxide ("opened lactam") and an unidentified derivative of either demoxepam or opened lactam. While the urinary metabolites of ^{14}C-chlordiazepoxide in the dog were not subjected to detailed investigation, they appeared on paper chromatography to be the same as those in man.



In addition to opened lactam, three more chlordiazepoxide metabolites had to be considered as possible metabolites of demoxepam. One was oxazepam[2], which was reported by Kimmel and Walkenstein (3) to be excreted in very small amounts (1% of the dose in the urine and in the feces) by a dog given oral ^{14}C-chlordiazepoxide. The other two were phenols, I and Desoxy I[3], which were found (4) to be metabolites of chlordiazepoxide in the rat. The structures of these compounds and of the labeled demoxepam used in the present study are shown.

The current experiments were designed to gain pharmacokinetic information on the disposition of demoxepam in the dog in terms of absorption, distribution, and elimination. Unlabeled demoxepam was used for this purpose, and a small portion of this pharmacokinetic data was included in the study of chlordiazepoxide disposition in the dog (5). A second objective was to elucidate the biotransformation of demoxepam in the dog. For this purpose, demoxepam-2-^{14}C was employed.

EXPERIMENTAL

Experimental Design—Three apparently normal, nonfasted, male dogs weighing 8–11 kg. were administered intravenous and oral doses of unlabeled demoxepam. At least 1 month elapsed before a dog was redosed with the drug. Dogs 1 and 2 received oral and intravenous doses of 10 mg./kg., while Dog 3 received a 5-mg./kg. i.v. dose. Demoxepam was dissolved in propylene glycol for intravenous injection and was weighed into a gelatin capsule for oral administration. At zero time and at frequent intervals after drug administration, 10 ml. of heparinized blood was drawn and centrifuged, and the separated plasma was stored frozen until analyzed.

Dog 3 also received oral and i.v. 5-mg./kg. doses of ^{14}C-demoxepam (specific activity of 1.57 μc./mg.). This experiment is designated Dog 3-^{14}C to denote the administration of the labeled compound. In addition to frequent blood sampling, a complete collection of urine and feces was made until the excretion of ^{14}C was no longer measurable. Urine collection intervals were 0–2, 2–5, 5–12, 12–24, 24–30, and 30–48 hr. and daily thereafter, while feces were collected daily. Feces were homogenized in 50% ethanol prior to counting.

[1] Chlordiazepoxide hydrochloride, 7-chloro-2-methylamino-5-phenyl-3*H*-1,4-benzodiazepine 4-oxide hydrochloride, is the active ingredient in Librium; Hoffmann–La Roche Inc., Nutley, N. J.

[2] Oxazepam, 7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2*H*-1,4-benzodiazepin-2-one, is the active ingredient in Serax; Wyeth Labs., Inc., Radnor, Pa.

[3] I is 7-chloro-1,3-dihydro-5-(4-hydroxyphenyl)-2*H*-1,4-benzodiazepin-2-one 4-oxide. Desoxy I differs from I only in that the 4-oxide (*N*-oxide) is absent.

An additional dog, Dog 4, was anesthetized with pentobarbital sodium[4] and used in a study of the biliary excretion of drug and metabolites. Prior to ^{14}C-demoxepam administration, the cystic duct was ligated and the bile duct was cannulated. Additional ligatures were placed at the pylorus and at the juncture of the small and large intestine. ^{14}C-Demoxepam (specific activity of 0.62 μc./mg.) in 0.1 *N* NaOH was injected intravenously at a dose of 5 mg./kg. The drug was injected within 3 min. after solution so as to prevent alkaline hydrolysis. Thirty-minute collections of bile were made throughout the 5-hr. experiment; at the end, urine was collected from the bladder.

Determination of Plasma Demoxepam—The fluorometric method of Koechlin and D'Arconte (1) was used to determine plasma levels of demoxepam after the administration of demoxepam or ^{14}C-demoxepam. The specificity of this assay was confirmed on finding that the phenolic derivative of demoxepam, Compound I, gave no fluorescence under the conditions of the analysis.

Labeled Demoxepam and Counting Techniques—Demoxepam-2-^{14}C was synthesized by Kaegi and Bader[5]. Its radiochemical purity was confirmed by TLC with Systems 1 and/or 2 (described later). On counting silica gel segments of the chromatoplate, 99% of the ^{14}C was found to have migrated as authentic demoxepam. Periodic checking of the radiochemical purity revealed that there was no decomposition with time.

All the counting was done in a liquid scintillation spectrometer[6], and the counts were converted to disintegrations per minute (d.p.m.) by the external standard-channels ratio procedure for quench correction. Aliquots of urine (0.2–1.0 ml.) and extracts of plasma, urine, bile, and feces were counted in Phosphor I (6). Aliquots of plasma (0.2–1.0 ml.), bile, homogenates of feces in 50% ethanol, aqueous homogenates of stomach and intestinal contents, and silica gel from chromatoplates were counted as suspensions in Phosphor II (6).

Extraction of Labeled Metabolites—The radioactivity excreted in the urine, feces, and bile was fractionated by extraction with ethyl acetate. The bile samples and the fecal homogenates were diluted 1:5 with water prior to extraction. All pH adjustments were accomplished by the addition of dilute acid or alkali. At each designated pH, two consecutive volumes of ethyl acetate, each equaling the aqueous volume, were shaken with the aqueous phases for approximately 10 min. each. The combined extract was counted and, if sufficient ^{14}C was present, was concentrated and examined by TLC.

Following the initial extraction of each sample at pH 7.0, the aqueous phase was incubated with a commercial preparation of β-glucuronidase and arylsulfatase[7], which was added to a final concentration of 1% (v/v). This incubation at pH 5.5 and 37° for 2–3 hr., which previously yielded maximum hydrolysis of conjugated benzodiazepine metabolites (6), was followed by serial extractions first at pH 7.0 and finally at pH 2.0.

TLC for Metabolite Separation, Identification, and Estimation—Silica gel containing a fluorescent indicator[8] was used for TLC with the following solvent systems: 1, chloroform–acetone–ethanol (80:5:5), 2, ethyl acetate–ethanol (90:10); 3, heptane–chloroform–ethanol–acetic acid (5:5:1:1); 4, heptane–chloroform–ethanol–concentrated ammonia (5:5:2:0.1); 4*a*, solvents of 4 in proportions of 5:5:4.5:0.1; 4*b*, solvents of 4 in proportions of 5:5:1:0.03; 5, isopropanol–concentrated ammonia (20:5); 6, heptane–chloroform–ethanol (5:5:2); 6*a*, solvents of 6 in proportions of 2:2:1; 7, chloroform–ethanol (97:3); 8, chloroform–methylene chloride–ethanol (75:25:5); and 9, heptane–ethyl acetate–ethanol (40:80:10).

The procedure utilizing TLC for metabolite identification and estimation was essentially the same as that used previously (6) in the investigation of the deconjugated urinary metabolites of ^{3}H-diazepam. In brief, identification consisted of demonstrating that, on two-dimensional TLC with two different solvent system pairs, a consistent amount of extracted ^{14}C migrated as a known compound added as an internal standard. Also obtainable from such consistent data was quantitation of labeled metabolite. The reference compounds were located by viewing the chromatoplate under shortwave UV light, and the silica gel containing each reference compound was counted as a suspension. The solvent system pairs were designated by the number of each system in the order used; for example, System 1–4 signifies that the plate was run in System 1 and then, after a turn of 90°, in System 4.

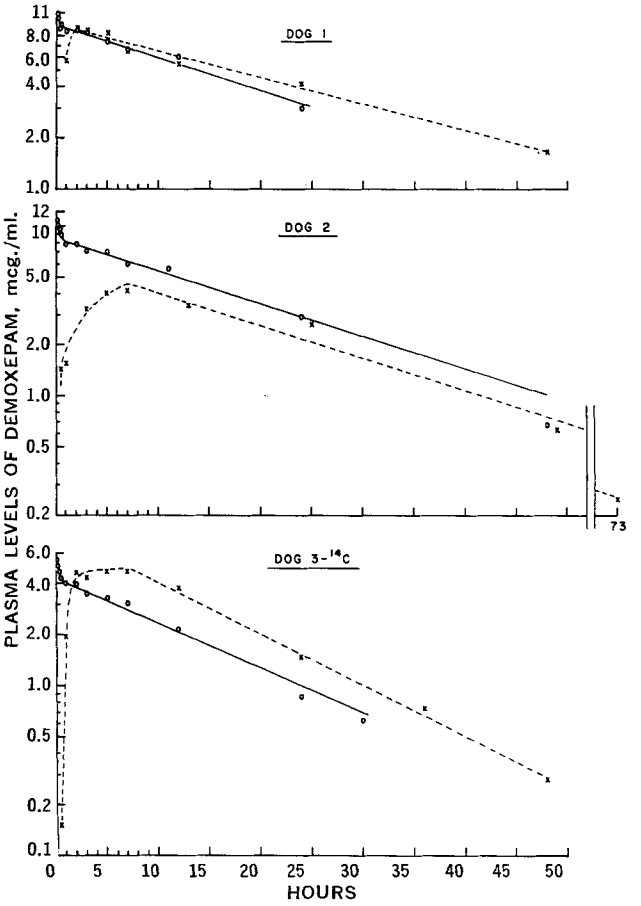


Figure 1—*Semilogarithmic plot of the plasma levels of demoxepam after oral and intravenous drug administration to three dogs. Dogs 1 and 2 received unlabeled doses of 10 mg./kg., and Dog 3-^{14}C received 5 mg./kg. of ^{14}C-demoxepam. Key: O—O, intravenous drug; and X- - -X, oral drug.*

Estimation of Urinary Opened Lactam—To facilitate the development of an assay, ^{14}C-opened lactam was synthesized from labeled demoxepam by an alkaline hydrolysis procedure similar to those previously described (7, 8). The crystallized product melted at 148–152° and was radiochemically pure by TLC.

Human control urine containing 20 mcg./ml. and 5 mcg./ml. of this ^{14}C-opened lactam was extracted with ethyl acetate at pH 7.0 and 2.0. While less than 5% of the ^{14}C was removed at pH 7.0, 93% was extracted at the acidic pH. To produce clearly defined spots on TLC, the extracted opened lactam was converted into its methyl ester (7). To accomplish this, the acidic extract was dried under nitrogen; the resulting oil, dissolved in 2 ml. of ethanol, was mixed with 2 ml. of ethereal diazomethane. After standing 15 min., the solution was evaporated to dryness and the residue dissolved in 0.2 ml. of ethanol. The methyl ester, separated by TLC in Systems 7 and 8, was located by spraying with the Bratton–Marshall reagents (9) and was scraped from the plate and counted. Recovery of opened lactam was approximately 50% by this procedure.

The assay of ^{14}C-opened lactam consisted of adding carrier opened lactam to the urine, which was extracted first at pH 7.0 and then at pH 2.0 with two equal volumes of ethyl acetate. The latter combined extract was concentrated, treated with diazomethane, and chromatographed. The ^{14}C migrating as opened lactam methyl ester was determined. A correction was made for incomplete recovery by the concomitant analysis of control urine to which ^{14}C-opened lactam was added at a concentration equaling that of carrier opened lactam added to the experimental urine.

[4] Nembutal.
[5] This synthesis by H. H. Kaegi and G. Bader has not as yet been published.
[6] Model 3380, Packard Instrument Co., Downers Grove, Ill.
[7] Glusulase, Endo Labs., Garden City, N. Y.
[8] SilicAR 7-GF-5, Mallinckrodt Chemical Works, St. Louis, Mo.

Table I—Pharmacokinetic Evaluation of Demoxepam Disposition in the Dog

Parameter	Parameter Value—Dog 1	Dog 2[a]	Dog 3-^{14}C	Dog 3
Dose (i.v. and oral), mg./kg.	10	10	5	5
Intravenous Demoxepam				
Dog wt., kg.	8.4	9.5	9.5	7.6
A, mcg./ml.	3.4	3.1	1.7	2.1
B, mcg./ml.	9.2	8.4	4.3	3.6
α, hr.$^{-1}$	8.35	3.4	4.6	4.2
α, half-life, hr.	0.083	0.20	0.15	0.17
β, hr.$^{-1}$	0.043	0.044	0.061	0.050
β, half-life, hr.	16	16	11	14
Rate constants of the two-compartment model				
k_{12}, hr.$^{-1}$	2.21	0.89	1.25	1.48
k_{21}, hr.$^{-1}$	6.12	2.48	3.34	2.69
k_{el}, hr.$^{-1}$	0.059	0.060	0.084	0.078
Compartment distribution of demoxepam				
V_p[b], % of body wt.	80	87	83	88
$F_c \times 100$[c]	73	73	73	64
Oral Demoxepam				
Dog wt., kg.	8.3	10.7	10.1	Not done
Peak plasma level, mcg./ml.	8.6	4.1	4.6	
Apparent elimination rate (β), hr.$^{-1}$	0.035	0.044	0.069	
Half-life of above, hr.	20	16	10	
Percent of dose absorbed[d]	99	70	134	

[a] The intravenous data of Dog 2 were previously reported (Dog 1 of *Reference 5*). [b] V_p in percent of body wt. = 100 × dose (mg./kg.)/A + B (mg./l.). [c] This is the percent of total body drug present in the central compartment during the elimination phase. Its calculation is described in the text. [d] Absorption was estimated from the areas under the plasma level curves; % absorption = 100 × oral dose area/i.v. dose area.

Further Metabolite Identification Procedures—The use of TLC for metabolite isolation and high-resolution mass spectrometry and/or NMR spectroscopy for identification was previously described (4, 10)[9].

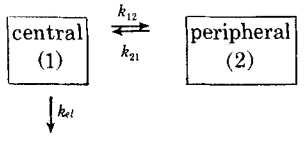
RESULTS

Pharmacokinetic Analysis of Demoxepam Disposition—The plasma levels of drug after intravenous administration exhibited a biexponential decline (Fig. 1). By means of an 'N-LIN' computer program (5, 11), all intravenous plasma level data were fitted to the biexponential equation

$$C_t = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{(Eq. 1)}$$

where C_t is the plasma level at time t, A is the zero-time intercept and α the rate constant of the fast disposition rate, and B and β are the corresponding parameters of the slow disposition rate. This equation may also be derived from the two-compartment open system (12) in which k_{12} is the rate of transfer from the central to peripheral compartment, k_{21} is the reverse transfer rate, and k_{el} is the rate at which the drug is eliminated from the central compartment (Scheme I).

The values for the parameters of Eq. 1 were used in the calculation, described by Riegelman *et al.* (12), of the transfer rates and elimination rate of the model. All results obtained from pharmacokinetic analysis of the plasma level data are shown in Table I. The half-life of the fast disposition rate (α) ranged from 0.1–0.2 hr., indicating a very rapid transfer of a portion of the drug to the peripheral compartment. The elimination rate (β) was markedly slower, with a half-life ranging from 11–16 hr. This rapid distribution and slow elimination were also reflected by the difference in magnitude of k_{12} and k_{el}. The relatively large central compartment volume (V_p), which ranged from 80–88% of the body weight, indicated uptake of demoxepam by readily accessible tissues. Nagashima *et al.* (13) showed that the fraction of total body drug present in the central compartment (F_c) during the elimination phase remains constant and is equal to β/k_{el}. From this relationship, 64–73% of the body demoxepam was calculated to be present in the central compartment during the elimination phase (Table I). The peripheral compartment, therefore, was smaller in that it contained 36–27% of the drug. It should be noted that Dogs 3 and 3-^{14}C, which were essentially duplicate experiments in the same animal, yielded results that indicated fairly good reproducibility.



Scheme I

Table II—Plasma Levels of Demoxepam in Dog 3-^{14}C by *Fluorometric* and Radiometric Assay

Hours	Intravenous 5-mg./kg. Dose—Plasma Level by Radio. Assay[a], mcg. equiv./ml.	Intravenous 5-mg./kg. Dose—Ratio of Fluoro.[b] to Radio. Assay	Oral 5-mg./kg. Dose—Plasma Level by Radio. Assay[a], mcg. equiv./ml.	Oral 5-mg./kg. Dose—Ratio of Fluoro.[b] to Radio. Assay
0.083	5.92	0.92	No sample	
0.17	5.27	0.96	Nil	
0.33	4.90	0.94	No sample	
0.50	4.75	0.91	0.13	1.15
1	4.47	0.89	1.70	1.14
2	4.31	0.90	4.49	1.02
3	3.90	0.89	4.57	0.94
5	3.83	0.84	5.03	0.94
7	3.69	0.82	5.16	0.92
12	2.53	0.83	4.20	0.89
24	1.09	0.79	1.78	0.83
30	0.79	0.80	No sample	
36	No sample		0.80	0.91
48	0.11	—[c]	0.35	0.80
72	0.02	—[c]	0.06	—[c]

[a] The d.p.m./ml. of plasma were divided by the specific activity of administered ^{14}C-demoxepam to obtain mcg. equivalents of drug/ml. [b] The plasma levels obtained by fluorometric assay were shown in Fig. 1. [c] Fluorometric assay yielded no measurable levels.

Data obtained from the oral administration of demoxepam are also shown in Fig. 1 and Table I. Although Dogs 1 and 2 both received 10 mg./kg., the peak plasma level of demoxepam in Dog 1 was about twice that of Dog 2. This appears to be related to the faster rate of absorption of drug by Dog 1 (Fig. 1). In addition, a difference in the extent of absorption was also evident. Absorption, estimated from a comparison of the areas under the plasma level curves (*per os* area/intravenous area), was found to be virtually complete in Dog 1 and 70% in Dog 2 (Table I). In Dog 3-^{14}C, however, the absorption of labeled demoxepam was calculated to be 134%. A comparison of the areas under the plasma level curves obtained after intravenous administration of nonlabeled and labeled drug to this dog (Dog 3 and Dog 3-^{14}C) revealed that these areas were almost identical, and, therefore, reproducible. Apparently, the plasma levels found after oral dosing of Dog 3-^{14}C were markedly higher than would be expected for complete absorption. Although no explanation for this anomalous result can be given at the present time, other evidence (presented later) does indicate that absorption was complete. It is further seen from Table I that the rate of elimination, β, in each dog was virtually independent of the route of administration of demoxepam.

Fate of ^{14}C-Demoxepam—Table II gives the plasma levels of ^{14}C-demoxepam determined radiometrically in Dog 3-^{14}C and compares them with those shown in Fig. 1 which were determined fluorometrically. The ratio shown is a measure of how much plasma ^{14}C is accounted for as demoxepam. After both routes of administration, the ratios are close to 1.0 at the early times and show a tendency toward lower values with time. This finding indicates that essentially all the plasma ^{14}C was present as demoxepam

[9] The mass spectra, run on a Consolidated Electrodynamics Corp. 21-110 mass spectrometer, were interpreted by Dr. W. Benz. The NMR spectra, obtained with a Varian HA-100 spectrometer using acetone-d_6 as the solvent and internal tetramethylsilane as a reference, were interpreted by Mr. R. Pitcher.

Table III—Excretion of Radioactivity by Dog 3-^{14}C following 5-mg./kg. Oral and Intravenous Doses of ^{14}C-Demoxepam

Day	Urinary Excretion, % of Dose	Fecal Excretion, % of Dose	Total Excretion, % of Dose
	Oral Dose		
1	27.7	24.3	52.0
2	12.2	15.3	27.5
3	2.3	5.6	7.9
4	0.4	0.9	1.3
5	0.1	—[a]	0.1
	42.7	46.1	88.8
	Intravenous Dose		
1	27.5	26.6	54.1
2	10.3	16.3	26.6
3	0.9	2.2	3.1
4	0.1	0.2	0.3
5	Nil	Nil	Nil
	38.8	45.3	84.1

[a] Feces were not collected.

in the first few hours and that a slight amount of labeled metabolite(s) entered the plasma with time.

The total excretion of fecal radioactivity was greater than that of urinary radioactivity after both routes of administration (Table III). Moreover, the magnitude of the daily excretion of ^{14}C in urine and feces was not materially influenced by whether labeled drug was given orally or intravenously. This finding suggests that ^{14}C-demoxepam was completely absorbed (but does not explain the 134% absorption shown in Table I).

The nature of the urinary radioactivity was investigated by solvent extraction and TLC. As shown in Table IV, the percent of urinary ^{14}C extracted before and after glusulase treatment was not influenced significantly by the route of drug administration. Sizable amounts of ^{14}C were extracted at pH 7.0 (pre- and post-glusulase) with very little ^{14}C extracted at pH 2.0. After both routes of administration, roughly two-thirds of the directly extractable radioactivity was intact demoxepam and 12–13% was Metabolite I. Following deconjugation with glusulase, about one-half of the extracted metabolites was I, 6–7% was Desoxy I, and 3–4% was oxazepam. The validity of these estimates is supported by the good agreement obtained with both solvent system pairs.

Urinary opened lactam was also determined, even though the excretion of acidic metabolites was relatively slight. This metabolite accounted for 2.1% of the urinary ^{14}C excreted in 48 hr. after intravenous drug and 1.7% of that excreted following oral drug.

Table V—Urinary Excretion of Labeled Demoxepam and Metabolites by Dog 3-^{14}C Given Oral and Intravenous 5-mg./kg. Doses of ^{14}C-Demoxepam[a]

Compound Form[b]	Urinary Excretion in Percent of Dose: Demoxepam	I	Desoxy I	Oxazepam	Opened Lactam	Unknown
After intravenous drug:						
Nonconjugated	9.3	1.8	Nil	Nil		2.9
Conjugated	0.2	8.2	1.1	0.5		7.2
Acidic					0.6	1.9
Unextracted						4.2
After oral drug:						
Nonconjugated	9.6	1.7	Nil	Nil		2.7
Conjugated	0.3	7.7	0.8	0.6		5.7[c]
Acidic					0.8	2.2
Unextracted						7.9

[a] The excretion of drug and metabolites in the first 48 hr. is presented. The radioactivity excreted in this period is shown in Table III and represents the excretion of over 90% of the total amount of ^{14}C excreted in the urine after both intravenous and oral drug. [b] The nonconjugated compounds were those extractable directly at pH 7.0, the conjugated metabolites were those extracted at this pH after glusulase treatment, and the acidic metabolites were those extracted at pH 2. [c] About one-third of this radioactivity, or 2% of the dose, was subsequently identified (see text) as Metabolite II.

Table V summarizes the urinary excretion of demoxepam and metabolites. After both routes of administration, the following is evident: (*a*) 10% of the dose was excreted as intact drug (the 0.2–0.3% found in the conjugated fraction probably represented demoxepam that was not removed in the prior extraction); (*b*) Metabolite I was the major metabolite (9–10% of the dose) and was excreted predominantly as a conjugate; (*c*) very small amounts of conjugated Desoxy I, oxazepam, and opened lactam were excreted; and (*d*) approximately 17% of the dose was excreted as unknown metabolites. The lack of influence of the route of administration on the magnitude of excretion of drug and metabolites further supports the contention that demoxepam was completely absorbed by Dog 3-^{14}C.

That portion of the unknown urinary radioactivity excreted as conjugates was further investigated. Starting with 85 ml. of a pool of the 0–48-hr. urine excreted after oral ^{14}C-demoxepam, 37.5% of the radioactivity was extracted after glusulase treatment; 15% of this ^{14}C migrated between demoxepam (R_f 0.57) and Desoxy I

Table IV—Extractability of the Urinary Radioactivity and Estimation of the Extracted Metabolites by Two-Dimensional TLC

Extract[a]	Percent of Urinary ^{14}C Extracted	System	Composition of the Extracted Radioactivity[b]: Demoxepam, %	I, %	Desoxy I, %	Oxazepam, %	Unaccounted for, %
			After Intravenous Drug				
Preglusulase pH 7, EtAc	37.1	1–5	66.5	12.9	0.6	1.2	18.8
		1–6	66.0	12.5	1.5	0.7	19.3
			66.3	12.7	N.S.	N.S.	19.1
Postglusulase pH 7, EtAc	45.4	1–5	1.1	51.0	5.0	3.0	39.9
		1–6	0.8	44.9	8.1	2.5	43.7
			N.S.	48.0	6.6	2.8	41.8
pH 2, EtAc	6.2						
			After Oral Drug				
Preglusulase pH 7, EtAc	35.2	1–3	66.3	11.9	<0.6	<0.6	21.8
		1–4	70.5	12.0	0.7	<0.6	16.8
			68.4	12.0	N.S.	N.S.	19.3
Postglusulase pH 7, EtAc	37.9	1–3	1.9	52.5	5.5	4.3	35.8
		1–4	1.9	49.1	5.7	3.4	39.9
			N.S.	50.8	5.6	3.9	37.9
pH 2, EtAc	6.8						

[a] A pool of 0–48-hr. urine excreted by Dog 3-^{14}C after intravenous or oral ^{14}C-demoxepam was extracted serially as shown in the table. EtAc = ethyl acetate. [b] The percent of extracted ^{14}C migrating with the designated internal standard is shown for each solvent system, and the average value is shown below the line. N.S. signifies that less than 2% of the ^{14}C migrated as an internal standard and the results are not considered to be significant.

Table VI—Extractability of the Fecal Radioactivity[a] of a Dog Given ^{14}C-Demoxepam Orally and Estimation of the Directly Extractable Metabolites by Two-Dimensional TLC

Extract	Percent of Fecal ^{14}C Extracted	System	Composition of the Extracted Radioactivity[b] Demoxepam, %	I, %	Desoxy I, %	Oxazepam, %	Unaccounted for, %
Preglusulase							
pH 7, EtAc	44	1–4*b*	9.6	7.4	20.6	3.4	59.0
		1–3	10.6	7.3	20.4	3.2	58.5
			10.1	7.4	20.5	3.3	58.7
			(1.1)[c]	(0.8)[c]	(2.2)[c]	(0.4)[c]	(6.3)[c]
Postglusulase							
pH 7, EtAc	9.0						
pH 2, EtAc	3.0						
Aqueous remaining	40						

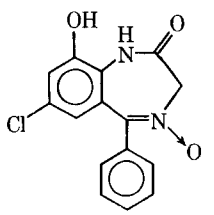
[a] An aliquot of the 1st day's feces representing the excretion of 24.3% of the dose was extracted. [b] The percent of extracted ^{14}C migrating with the designated internal standard is shown for each solvent system, and the average value is shown below the line. [c] Excretion in terms of the percent of dose administered is shown in parenthesis.

(R_f 0.36) with System 1, while 19% barely moved off the origin with System 4. From these results, it was estimated that this new metabolite comprised 6% of the urinary ^{14}C (37.5% × 17%) or roughly 2% of the dose. This is about one-third of the 5.7% of the unidentified conjugated radioactivity (Table V).

The new metabolite, purified by TLC in System 1 and rechromatographed in System 4, was analyzed by high-resolution mass spectrometry. Its empirical formula was found to differ from that of demoxepam ($C_{15}H_{11}ClN_2O_2$) by the addition of one oxygen atom. The location of this oxygen atom, however, could not be determined from the mass spectrum.

A definitive structure was obtained from a greater amount of this metabolite which was isolated in a second experiment. The extract, obtained after glusulase treatment of pooled urine which contained 6.9 × 10^7 d.p.m. (20 mg. equivalents of ^{14}C-demoxepam), was first evaporated to dryness. Then the residue was dissolved in ether–ethanol (10:1) which was extracted with an equal volume of 0.1 *N* NaOH. The alkaline phase, after adjustment to pH 7.0, was then extracted twice with ethyl acetate. This preliminary purification, based on the known ability of alkali to remove demoxepam from ether (1), was required to separate the metabolite from urinary constituents that would interfere in subsequent TLC. The final combined ethyl acetate extract, dried over anhydrous sodium sulfate, was concentrated and subsequently chromatographed with System 4*a*. The metabolite, eluted with ethanol followed by methanol, was rechromatographed with System 1. It was eluted this time with 0.1 *N* NaOH which was adjusted to pH 7.0 and extracted with ethyl acetate. The residue resulting from evaporation of the solvent was dissolved in chloroform and filtered through a cotton plug. The filtrate was again dried, and 0.65 mg. of purified metabolite was dissolved in acetone-d_6 for NMR spectroscopy.

A single scan showed the CH_2 at δ 4.71 and indicated that the oxygen was not added at the C-3 position of demoxepam. On time averaging for 144 scans, an *AB* pattern was obtained in the aromatic region (δ 6.52 and δ 7.08), with a typical *meta*-coupling of 2.5 Hz. This clearly showed that substitution had occurred at the C-9 position and that the metabolite was II, 7-chloro-1,3-dihydro-9-

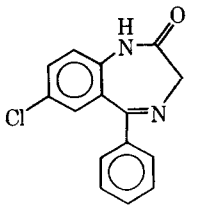


II

hydroxy-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide. Confirmation of structure was obtained following the synthesis of II; the isolated and synthetic compounds were identical on examination by NMR spectroscopy and TLC.

The nature of the fecal radioactivity was also investigated by solvent extraction and TLC. It is seen in Table VI that, in contrast to the urinary ^{14}C (Table IV), relatively little fecal ^{14}C was excreted as conjugates. TLC of the major fraction revealed that 21% of the ^{14}C directly extractable at pH 7.0 was Desoxy I, 10% was intact demoxepam, 7.4% was I, and 3.3% was oxazepam (Table VI). A striking difference in the urinary and fecal excretion of I and Desoxy I was noted. Metabolite I predominated in the urine with little Desoxy I present (Table V), while in the feces Desoxy I was excreted to a much greater extent than was I. The very low extraction of ^{14}C at pH 2 (Table VI) indicated that no significant fecal excretion of opened lactam occurred.

Further investigation of the directly extractable radioactivity resulted in the identification of two more fecal metabolites. The high-resolution mass spectrum of one, purified by TLC with System 1 (R_f 0.67) followed by rechromatography with System 4*b* (R_f 0.40), clearly showed that the metabolite was III, 7-chloro-1,3-dihydro-5-

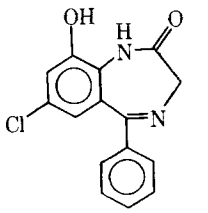


III

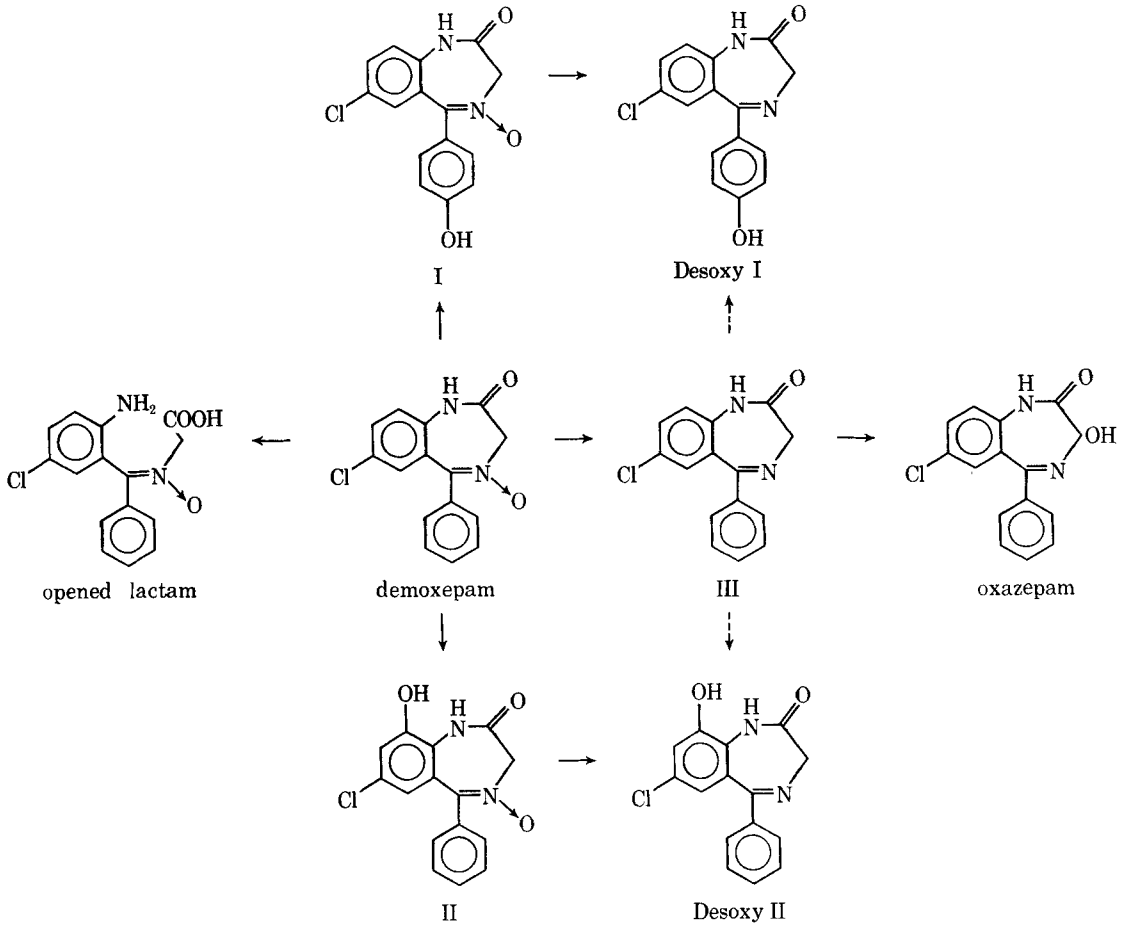
phenyl-2*H*-1,4-benzodiazepin-2-one, which is the *N*-desoxy derivative of demoxepam. Confirmation of structure and an estimate of the fecal excretion of III were obtained by two-dimensional TLC. Cochromatography of an aliquot of the directly extractable radioactivity with authentic III, a known compound (7, 8), revealed that a consistent amount of ^{14}C was recoverable as III. With System 3–4*b*, 17.9% of the radioactivity migrated as III; with System 1–4*b*, 16.0 and 18.6% migrated as III in two separate experiments. From the average distribution (18%), it was estimated that 1.9% of the dose was excreted in the feces on the 1st day as III.

The second metabolite was purified *via* System 1 (R_f 0.63), System 6*a* (R_f 0.65), and System 4*b* (R_f 0.29). The high-resolution mass spectrum showed that the metabolite no longer retained the *N*-oxide function and that it had the same empirical formula as Desoxy I ($C_{15}H_{11}ClN_2O_2$). However, TLC with System 4*b* clearly separated the metabolite and Desoxy I, thereby demonstrating that they were not identical. The subsequent isolation and identification of II as a urinary metabolite suggested that this fecal metabolite, which was an isomer of Desoxy I, might be 7-chloro-1,3-dihydro-9-hydroxy-5-phenyl-2*H*-1,4-benzodiazepin-2-one (Desoxy II).

TLC of purified metabolite and authentic Desoxy II with System



Desoxy II



Scheme II

6 revealed that 83% of the ^{14}C migrated as the reference compound. This evidence, together with that from mass spectrometry, was considered sufficient to designate the metabolite as Desoxy II. It was estimated from the recovery of the metabolite on successive chromatographic purification that the amount of Desoxy II excreted in the feces was about one-half that of III, *i.e.*, roughly 1% of the dose. This identification of two new *N*-desoxy metabolites, together with the threefold greater excretion of Desoxy I than I (Table VI), indicated that the *N*-desoxy metabolites predominated in the feces.

The mode of secretion of labeled metabolites into the gastrointestinal (GI) tract and their identity were studied in the bile duct-cannulated Dog 4. Five hours after an intravenous dose, 3.9% of the dose was found in the urine, 4.3% in the bile, less than 0.1% in the stomach contents, and 0.3% in each (small and large) intestinal contents. Most of the radioactivity excreted in the feces after intravenous ^{14}C-demoxepam entered the GI tract *via* the bile.

Table VII shows that the major portion of the biliary ^{14}C could not be extracted with ethyl acetate. However, with respect to the ^{14}C extracted, the major components were demoxepam, conjugated I, and conjugated II. Conjugated oxazepam was also present but the other *N*-desoxy metabolites, Desoxy I, Desoxy II, and III, were not found in significant amounts. Since the latter three metabolites were definitely present in the feces, these results suggest that they were formed in the GI tract from the corresponding *N*-oxides secreted in the bile.

DISCUSSION

The pharmacokinetic data presented in this study support the previously reported (5) conclusion that a two-compartment open-system model adequately describes the disposition of demoxepam in the dog. In addition, evidence was also obtained that oral demoxepam was well absorbed. The rate of demoxepam elimination by an individual dog appeared to be independent of the route of drug administration. However, in different dogs, this rate was quite variable, with a half-life ranging from 10 to 20 hr. in these studies, and as high as 28 hr. in a previous dog (5).

^{14}C-Demoxepam was eliminated by both excretion and biotransformation. Ten percent of the labeled demoxepam administered either orally or intravenously was excreted as intact drug in the urine. Since 45% of the intravenously injected radioactivity was excreted in the feces, secretion of labeled products into the GI tract was evident. In the bile duct-cannulated dog, this radioactivity was shown to enter the GI tract mainly *via* the bile. Furthermore, about 10% of this biliary ^{14}C was found to be present as intact drug. This suggests that an enterohepatic circulation of demoxepam may be in operation in the dog. The absence of appreciable fecal excretion of demoxepam was demonstrated after oral ^{14}C-demoxepam administration. It is estimated that less than 2% of the dose was excreted as intact drug in the feces.

Since the total excretion of intact drug was less than 12% of the dose, demoxepam was eliminated primarily by biotransformation. The postulated metabolic pathways shown in Scheme II involve hydroxylation of each aromatic ring, the complete reduction of the *N*-oxide function, and the hydrolysis of the lactam bond. At least two pathways resulted in phenolic metabolites. *para*-Hydroxylation of the demoxepam 5-phenyl ring was apparently preferred over hydroxylation of the benzodiazepine aromatic ring since 10% of the oral ^{14}C-demoxepam was excreted as urinary I (free and conjugated) and only about 2% was excreted as urinary conjugated II.

The reduction of demoxepam to III in the GI tract was evident from the finding that intact drug, but not III, was detectable in the bile while a small amount of III (roughly 2% of the dose) was excreted in the feces. Although a reexamination of dog urine failed to detect III, the reduction of demoxepam in the tissues cannot be entirely excluded. III, a prominent metabolite of the tranquilizer diazepam[10] in dog and man (6), is not excreted in the urine to any

[10] Diazepam, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one, is the active ingredient in Valium; Hoffmann–La Roche Inc., Nutley, N. J.

Table VII—Extractability of the Biliary Radioactivity[a] and Estimation of Metabolites in a Dog Given a 5-mg./kg. i.v. Dose of ^{14}C-Demoxepam

Extract	Percent of Biliary ^{14}C Extracted	Composition of Extracted Radioactivity[b]								
		System	Demoxepam, %	I, %	Desoxy I, %	II, %	Desoxy II, %	III, %	Oxazepam, %	Unknown, %
Preglusulase										
pH 7, EtAc	10.4	1–4	81.5	2.2	N.M.	N.M.	N.M.	N.M.	N.M.	16.3
Postglusulase										
pH 7, EtAc	17.7	1–4	5.9	29.0	1.7	33.9	1.4	N.M.	4.9	23.2
		1–9	5.3	28.5	1.1	28.8	(1.4)[c]	N.M.	(5.0)[c]	29.9
			5.6	28.8	1.4	31.4	1.4	N.M.	5.0	26.6
pH 2, EtAc	5.1									
Remaining aqueous	64.5									

[a] A 1-ml. aliquot of each bile sample was combined to form a 0–5-hr. pool which was extracted. [b] Metabolites were estimated with the designated two-dimensional solvent system. N.M. signifies that measurable amounts of ^{14}C were not found as the reference compound. The limit of detection was 2% of the radioactivity extracted at pH 7 preglusulase and 1% of that extracted postglusulase. [c] In System 1–9, 6.4% of the extracted ^{14}C migrated as unresolved Desoxy II plus oxazepam. This total was readily separated into the individual values shown in parenthesis by using the amounts found for each on TLC with System 1–4.

appreciable extent by the dog (6, 14). On the other hand, oxazepam, which has been shown (15, 16) to be a metabolite of III, is a major urinary metabolite (conjugated) of diazepam in dog and man (6, 14). In the present study, less than 1% of oral or intravenous ^{14}C-demoxepam was excreted as urinary conjugated oxazepam, thus indicating that relatively little III was metabolized in the tissues to oxazepam.

The deconjugation and reduction of biliary conjugated I and II in the GI tract to yield fecal Desoxy I and Desoxy II were also evident from the difference in composition of biliary and fecal radioactivity. However, the endogenous hydroxylation of III to Desoxy I and Desoxy II cannot be entirely excluded. Therefore, these reactions are included as possible minor (broken arrow) pathways in Scheme II.

The hydrolytic pathway did not appear to be of major significance in the dog, because less than 1% of oral and intravenous labeled demoxepam was excreted as urinary opened lactam. In a previous study (2) of ^{14}C-chlordiazepoxide metabolism in two human subjects, urinary opened lactam accounted for 4 and 8% of the dose. This suggests that the hydrolytic pathway is of greater significance in man.

Evidence that chlordiazepoxide was metabolized to demoxepam by a pathway involving Metabolite DM (Ro 5-0883/1), the *N*-desmethyl derivative of chlordiazepoxide, as an intermediate was previously reported (17). Recently, a pharmacokinetic evaluation of chlordiazepoxide disposition in the dog revealed (5) that the drug was quantitatively biotransformed to Metabolite DM and that up to 50% of this metabolite was converted to demoxepam. These results, together with the conversion of demoxepam to oxazepam shown in the present study, provide a series of metabolic reactions which explain the slight conversion of chlordiazepoxide to oxazepam observed by Kimmel and Walkenstein (3).

Present studies of demoxepam disposition and biotransformation are concerned with determining whether information obtained in the dog is applicable to man.

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ACKNOWLEDGMENTS AND ADDRESSES

Received September 28, 1970, from *Hoffmann-La Roche Inc., Nutley, NJ 07110*

Accepted for publication November 10, 1970.

The authors are indebted to Dr. W. Benz and Mr. R. Pitcher for obtaining and interpreting the high-resolution mass spectra and NMR spectra, respectively, and to Dr. E. Garcia for the synthesis of Metabolites II and Desoxy II.