carbamate), 1700 cm^{-1} (ureide). Anal. ($C_{20}H_{32}N_4O_{13}·2CH_3CO-OH·0.5H_2O$) C, H, N.

$N^{3''}$,$N^{3''}$-Dimethylkanamycin A (4). A solution of 1 (1.1 g, 2.0 mmol) in 6 mL of 37% aqueous formaldehyde, 10 mL of acetonitrile, and 5 mL of water was treated with sodium cyanoborohydride (0.75 g, 11.9 mmol). Acetic acid (1 mL) was added slowly as the mixture was stirred. After 2 h, another 1 mL of acetic acid was added. The mixture was concentrated under reduced pressure after 16 h and the residual solid (compound 2) was dissolved in 10 mL of water. Barium hydroxide (10 mL of a 1 M solution) was added and the mixture was heated at reflux for 48 h. It was neutralized with CO_2 and filtered, and the filtrate was concentrated to a small volume and diluted with acetone. The brown solid that formed was purified by chromatography on Amberlite CG-50 (NH_4^+) with 0–0.2 M NH_4OH as solvent. This procedure was repeated and the product was recrystallized from aqueous ethanol–acetone to give 0.244 g (23%) of 4 as the dicarbonate, a white powder that decomposed above 260 °C: $[\alpha]^{24}_{546}$ +92.0 (*c* 0.4, H_2O); NMR (D_2O) δ 2.5 [6 s, $N(CH_3)_2$]. This sample was identical in its IR spectrum and R_f value (upper layer of chloroform–methanol–27% NH_4OH, 1:1:1, or chloroform–methanol–27% NH_4OH–water, 1:4:2:1) with a sample furnished by Bristol Laboratories.[1] Anal. ($C_{20}H_{40}N_4O_{11}·2H_2CO_3$) C, H, N.

1,3,6′,3″-Tetra-*N*-acetyl-1′,2′,4″-tri-*O*-acetylkanamycin A (19). A solution of 15 (0.43 g, 0.62 mmol) in 10 mL of dry methanol was treated with 2 mL of acetic anhydride and stirred at 25 °C for 40 h. It was concentrated to dryness with the aid of added ethyl acetate. The residue was precipitated from methanol–ether to give 0.35 g (72%) of 19 as the hemihydrate, a white powder: mp 208–210 °C; $[\alpha]^{24}_{546}$ +145.1 (*c* 1.0, DMF); IR 1730 (acetate), 1640 (amide I), 1540 cm^{-1} (amide II); NMR δ 1.8–2.25 (ms, acetyl), 7.75 (6, amide). Anal. ($C_{32}H_{50}N_4O_{18}·0.5H_2O$) C, H, N.

1,3,2′-Tri-*N*-benzyloxycarbonyl-6′,4′:3″,2″-*N*,*O*-carbonyl-6″,4″-*O*-cyclohexylidenekanamycin B (21). A solution of 10 (1.05 g, 1.12 mmol) in 2 mL of dry *N*,*N*-dimethylformamide was warmed at 80 °C under reduced pressure until the volume was reduced to 10 mL. It was cooled to 50 °C and treated with *p*-toluenesulfonic acid (40 mg) and 1,1-dimethoxycyclohexane (2.5 g, excess). Then it was again warmed at 80 °C under reduced pressure for 1.5 h. The residue was neutralized with triethylamine, concentrated to a small volume, and treated with water. The solid that formed was reprecipitated from dioxane–ether to give 1.11 g (97%) of 21 as a white solid: mp 238–240 °C dec; $[\alpha]^{24}_{546}$ +53.4 (*c* 1.0, DMF); IR 1770 (five-membered cyclic carbamate), 1725 (six-membered cyclic carbamate), 1700 (amide I), 1520 cm^{-1} (amide II); NMR δ 1.45 (br, cyclohexyl), 5.0 (s, benzylic), 7.3 (br s, aromatic). Anal. ($C_{50}H_{59}N_5O_{18}$) C, H, N.

1,3-Di-*N*-(benzyloxy)-6′,4′:3″,2″-*N*,*O*-carbonyl-6″,4″-*O*-cyclohexylidenekanamycin A (22). This compound was prepared by the procedure described for 21. From 2.2 g of 12 was obtained, after reprecipitation from dioxane–ether, 2.38 g (98%) of 22 as a white solid: mp 228–230 °C dec; $[\alpha]^{24}_{546}$ +67.0 (*c* 1.0, DMF); IR 1770 (five-membered cyclic carbamate), 1720 (six-membered cyclic carbamate), 1700 (amide I), 1525 cm^{-1} (amide II); NMR δ 1.5 (br, cyclohexyl), 5.05 (br s, benzylic), 7.25 (br s, aromatic). Anal. ($C_{42}H_{52}N_4O_{17}$) C, H, N.

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Novel Application of Proton Nuclear Magnetic Resonance Spectroscopy in the Identification of 2′-Chloronordiazepam Metabolites in the Dog

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The only metabolite of 2′-chloronordiazepam, 7-chloro-1,3-dihydro-5-(2′-chlorophenyl)-2*H*-1,4-benzodiazepin-2-one (1), previously identified in the dog is lorazepam (2), which is a product of 3-hydroxylation. Two phenolic metabolites (3 and 4) in the dog corresponding to 4′-hydroxylation of the 5-phenyl ring and 9-hydroxylation of the fused benzene ring, respectively, have now been identified. The structure of the 9-hydroxy isomer 4 is deduced simply from the observed NMR spectral AB (J_{meta} = 2.5 Hz) pattern of the protons of the fused benzene ring. In contrast, since a 2′-chloro substituent is present on the 5-phenyl ring of the parent drug, the usual method of recognizing 4′-hydroxylation of this ring by observation of AA′BB′ multiplets in the proton nuclear magnetic resonance (^1H NMR) spectra is inapplicable. Hence, a novel method is introduced to identify the 4′-hydroxy isomer 3, based on attributing different sets of NMR substituent effect parameters to hydroxyl groups, depending on whether these groups are meta or para to the benzodiazepinimine function. The urinary plus fecal excretion of 2–4 by one dog given a single oral 10 mg/kg dose of ^{14}C-labeled 1 amounted to 20, 5, and 7% of the dose, respectively; the urinary metabolites were excreted predominantly as conjugates of glucuronic acid and/or sulfate.

2′-Chloronordiazepam, 7-chloro-1,3-dihydro-5-(2′-chlorophenyl)-2*H*-1,4-benzodiazepin-2-one (1), was reported by Zbinden and Randall[1] to be a potent benzodiazepine in terms of central nervous system activity in

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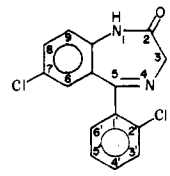
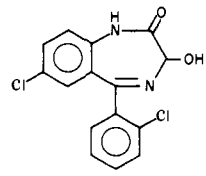
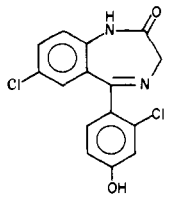


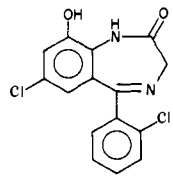
(1) 2'-CHLORONOR-DIAZEPAM



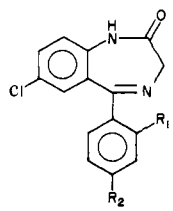
(2) LORAZEPAM (METABOLITE A)



(3) 2'-CHLORO-4'-HYDROXY-NORDIAZEPAM (METABOLITE B)



(4) 2'-CHLORO-9-HYDROXY-NORDIAZEPAM (METABOLITE C)



(5) $R_1 = R_2 = H$; NORDIAZEPAM

(6) $R_1 = H, R_2 = OH$; 4'-HYDROXYNORDIAZEPAM

(7) $R_1 = OH, R_2 = H$; 2'-HYDROXYNORDIAZEPAM

humans and animals. It is the 2'-chloro derivative of nordiazepam (5), another benzodiazepine, which is active in animals[2] and which is the major metabolite in the blood of humans treated with the antianxiety agents diazepam,[3,4] prazepam,[5] and chlorazepate dipotassium.[6]

Nordiazepam is metabolized to the 3-hydroxy derivative oxazepam,[3,7-9] and de Silva et al.[10] have reported the analogous biotransformation of 1 in the dog to lorazepam (2), which was excreted in the urine as a glucuronide and/or sulfate conjugate. Schillings et al.[11] found that lorazepam given to humans, dogs, pigs, and cats is apparently excreted in the urine primarily as the glucuronide conjugate. However, they also detected a metabolite of lorazepam in dog urine which was partially characterized by mass spectrometry as a phenol with its hydroxyl group at an unspecified position on the 5-phenyl ring.

The present study is an investigation of the metabolism of 1 in the dog with particular emphasis on the complete identification of two phenolic metabolites by ^1H NMR spectroscopy. The structures of diazepam metabolites hydroxylated in the 4' position have been deduced from the observation of symmetrical four-spin AA'BB' patterns, indicating symmetrical para-disubstituted phenyl rings.[12] However, when a parent drug possesses a substituent in the phenyl ring, a symmetrical four-spin pattern is no longer obtainable and hydroxylation occurring in either the 4' or 5' position cannot be distinguished using the symmetry considerations. Therefore, we have used NMR chemical-shift substituent effect parameters deduced from hydroxyacetophenones and from benzodiazepines 5 and 6 to elucidate the site of hydroxylation of the 5-phenyl ring of the parent drug 1.

Results

Excretion of Radioactivity by the Dog. In 6 days 60.5% of the ^{14}C administered as 10 mg/kg of labeled 1 to one dog was excreted in the urine and 28.5% in the feces. Since 58% of the dose was excreted in the urine in the first 3 days, these samples were pooled and a small aliquot was serially extracted with ethyl acetate. As shown in Table I, the major fraction of urinary ^{14}C was removed at pH 9 only after the urine was incubated with Glusulase; i.e., conjugated derivatives of 1 comprised the major metabolite fraction.

Table I. Extractability of the Urinary Radioactivity Excreted by a Dog Given [^{14}C]1

serial extract.[a]	% of urinary ^{14}C extract.
pre-Glusulase	
pH 9	9.2
pH 7	0.9
post-Glusulase	
pH 9	46.8
pH 7	1.9
pH 2	17.7

[a] Extraction with ethyl acetate as described under Experimental Procedures.

Table II. Designation of Metabolites and Amounts Found

metabolite	R_f (syst 2)	amt (mg-equiv of [^{14}C]1)
A	0.71	10.7
B	0.48	1.6
C	0.13	1.7

Isolation of the Conjugated Metabolites. A pool (1.7 L) comprised of 50% of each urine sample excreted over the first 4 days was extracted with ethyl acetate before and after incubation with Glusulase. The post-Glusulase ethyl acetate extract was concentrated and filtered. The resulting 34-mL filtrate, which contained 43% of the ^{14}C originally present in the urine, was concentrated to an oil, which was dispersed in 30 mL of ethanol. After the solution was left for 1 h in the refrigerator, a precipitate was removed, and another precipitate which formed on concentration of the ethanol to 11 mL was also removed. Only a negligible amount of ^{14}C was lost in each precipitate.

Following a number of pilot experiments, 0.5 mL of the ethanol solution was applied to each of 19 thin-layer plates (20 × 20 cm), and the labeled metabolites were separated with system 2. The silica gel segments associated with three UV-absorbing bands containing significant amounts of ^{14}C were separately scraped into three 500-mL Erlenmeyer flasks containing 200 mL of ethanol, and pools of each band were thus obtained. After swirling each flask for a short time, the contents were filtered through a filter aid (Hyflo) and the ^{14}C in each filtrate was determined. The designation of each metabolite and the amounts found are listed in Table II.

Although all of these metabolite fractions contained extraneous UV-absorbing material, 3–5 μg samples of B and C were submitted for mass spectroscopic analysis. Metabolites A and B were subsequently purified by TLC with system 3 using six and four plates, respectively. The eluted metabolite A was identified chromatographically, whereas the ethanol eluate containing metabolite B was evaporated to dryness; the residue dissolved in chloroform was filtered through a cotton plug to give a silica gel free 1-mg sample for analysis by ^1H NMR.

Further purification of the metabolite C fraction, however, did not result in a sample of suitable homogeneity for characterization by ^1H NMR. Therefore, another urine pool (30%, v/v) was extracted similarly to the first one, but this time an additional purification step was introduced. On the presumption that metabolite C was a phenol, the post-Glusulase ethyl acetate extract was

Table III. NMR Chemical Shift and Substituent Effect Parameters of Benzodiazepines

entry	compd	solvent	CH_2	H-6	H-8	H-9	H-2'	H-3'	H-4'	H-5'	H-6'
1	1[a]	Me_2SO-d_6	4.17	6.88	7.52	7.22		7.47	7.47	7.47	7.47
2	1	Me_2CO-d_6	4.29	7.00	7.46	7.31		~7.52	~7.52	~7.52	~7.52
3	1[a]	$CDCl_3$	4.36	7.04	7.52	7.07		~7.38	~7.38	~7.38	~7.38
4	3	Me_2SO-d_6	b	6.92	7.53	7.19		6.78		6.81	7.33
5	3	Me_2CO-d_6	4.23	7.06	7.49	7.30		6.86		6.92	7.40
6	3	$CDCl_3$	4.32	7.10	7.43	7.02		6.82		6.78	7.36
7	3-1	Me_2SO-d_6	b	0.04	0.01	−0.03		−0.69		−0.66	−0.14
8	3-1	Me_2CO-d_6	−0.06	0.06	0.03	−0.01		−0.66		−0.60	−0.12
9	3-1	$CDCl_3$	−0.04	0.06	−0.09	−0.05		−0.56		−0.60	−0.02
10	4	Me_2CO-d_6	4.24	7.15	6.59			~7.47	~7.47	~7.47	~7.47
11	5[a]	Me_2SO-d_6	4.09	7.14	7.53	7.23	7.42	7.42	7.42	7.42	7.42
12	5	Me_2CO-d_6	4.23	7.26	7.56	7.36	~7.45	~7.45	~7.45	~7.45	~7.45
13	5[a]	$CDCl_3$	4.32	7.31	7.55	7.09	~7.47	~7.47	~7.47	~7.47	~7.47
14	6[c]	Me_2SO-d_6	4.03	7.20	7.53	7.21	7.29	6.75		6.75	7.29
15	6	Me_2CO-d_6	4.18	7.33	7.56	7.36	7.44	6.87		6.87	7.44
16	6	$CDCl_3$	4.28	7.33	7.45	7.02	7.44	6.84		6.84	7.44
17	6, 5	Me_2SO-d_6	−0.06	0.06	0	−0.02	−0.13	−0.67		−0.67	−0.13
18	6, 5	Me_2CO-d_6	−0.05	0.07	0	0	−0.01	−0.58		−0.58	−0.01
19	6, 5	$CDCl_3$	−0.04	0.02	−0.10	−0.07	−0.03	−0.63		−0.63	−0.03
20	7	Me_2SO-d_6	4.17	7.39	7.64	7.26		6.94	7.35	6.84	7.13
21	7	Me_2CO-d_6	4.28	7.51	7.65	7.41		6.96	7.37	6.83	7.18
22	7[a]	$CDCl_3$	4.27[d]	7.49	7.53	7.12		7.04	7.36	6.80	7.12
23	7-5	Me_2SO-d_6	0.08	0.25	0.11	0.03		−0.48	−0.07	−0.58	−0.29
24	7-5	Me_2CO-d_6	0.05	0.25	0.09	0.05		−0.49	−0.08	−0.62	−0.27
25	7-5	$CDCl_3$	−0.05	0.18	−0.02	0.03		−0.43	−0.11	−0.67	−0.35

[a] In general, the chemical shifts of the NH group were not determined, since the proton was exchanged by deuterium or gave bands too broad to be observed under the conditions used. The position of this group was δ 8.68 for both 1 and 5 in $CDCl_3$, 9.70 and 9.52, respectively, in Me_2SO-d_6, and 8.20 for 7 in $CDCl_3$. [b] Not recorded. [c] H-2', -3', -5', and -6' bands comprise a symmetrical AA'BB' pattern. [d] Average value for anisochronous methylene proton bands 0.70 ppm apart.

evaporated to dryness and the resulting residue taken up in 77 mL of ether–ethanol (10:1) was extracted twice with 80 mL of 0.1 N NaOH. The alkali was then adjusted to pH 9 (with 6 N HCl) and extracted twice with ethyl acetate to give an extract which was much lighter in color but which retained over 90% of the ^{14}C present before extraction with alkali. After concentration and TLC (10 plates) with system 2, the same three labeled bands were seen as before: A (R_f 0.60), B (R_f 0.48), and C (R_f 0.13). Upon elution with ethanol, 0.74 mg-equiv of the metabolite C fraction was obtained. This was further purified by TLC with system 4 to yield a labeled component at R_f 0.68, which was eluted with ethanol and, after removal of the solvent, was dissolved in chloroform–methanol (9:1), filtered through a cotton plug, and dried. A few micrograms was analyzed by high-resolution mass spectrometry and 0.45 mg by NMR.

Metabolite Identification. Comparative TLC with system 2 (R_f 0.70) and 5 (R_f 0.12) indicated that metabolite A was lorazepam, the urinary metabolite reported by de Silva et al.[10] This was confirmed by gas chromatography (Hewlett-Packard Model 402 gas chromatograph) using 3% OV-17 as the liquid phase and the conditions reported by de Silva et al.;[10] authentic lorazepam and metabolite A both exhibited a retention time of 3.8 min.

The low-resolution mass spectrum of metabolite B exhibited a molecular ion at m/e 320, whereas the high-resolution mass spectrum of metabolite C yielded a molecular ion at m/e 320.0115 corresponding to $C_{15}H_{10}N_2O_2Cl$. These spectra indicate that oxygen was added to 1 to form both metabolites, which appeared to be phenolic isomers of metabolite A (lorazepam).

The structure elucidation of metabolite C as the 9-substituted isomer 4 was deduced in a straightforward manner from the observed NMR spectral AB multiplet with a coupling constant of 2.5 Hz typical for protons meta to each other. Thus, a single scan of metabolite C (0.45 mg) in Me_2CO-d_6 showed the CH_2 at δ 4.24 and indicated that the oxygen was not added at the C-3 position of 1, whereas a time average of 144 scans gave an AB pattern in the aromatic region at δ 6.59 and 7.15 due to H-6 and H-8 with a typical meta coupling of 2.5 Hz. This meta coupling clearly showed that substitution had occurred in the 9 position.

The ^1H NMR spectrum of metabolite B (1 mg) from a single scan in $CDCl_3$ solution gave a band at δ 4.32, indicating an intact methylene group, while the time-averaged spectrum gave chemical shifts for the aromatic protons (see Table III, entry 6). Corresponding data were obtained in Me_2SO-d_6 and Me_2CO-d_6 (Table III, entries 4 and 5).

It was clear from the two ABX patterns observed in each solvent and, in particular, the absence of a four-proton band at δ 7.47 in Me_2SO-d_6 solution that the 5-phenyl ring was oxygenated. Whereas one of the two ABX patterns was similar to that in the parent drug and indicated that the fused benzene ring was preserved, the other ABX pattern, also typical of a 1,2,4-trisubstituted benzene ring, indicated 4'- or 5'-hydroxylation and definitely ruled out 3'- or 6'-hydroxylation.

In order to decide between 4'- or 5'-hydroxylation, it was first necessary to postulate that the substituent parameters of a hydroxyl group meta to a >C=X function (X is oxygen in the model acetophenones and nitrogen in the benzodiazepines) is significantly different from that of a hydroxyl para to the same function. Thus, the success of our method in distinguishing between 4'- and 5'-hydroxylation rests on both the use of acetophenones as model compounds and the demonstration that our postulation is correct for acetophenones. This approach has not been used previously to determine the site of hydroxylation in aromatic compounds, since it is usually assumed that the substituent parameters for a hydroxyl group are independent of its relationship to other groups on the benzene ring.

First, the chemical shift and substituent effect parameters were obtained for the parent drug 1, metabolite B, and model benzodiazepines 5–7 run in various solvents (Table III). Parameters for a hydroxyl group ortho to the >C=X function are included for completeness. It is seen

Table IV. NMR Chemical-Shift Substituent Effect Parameters of Acetophenones

entry	solvent	relationship of C=O to OH	chemical shifts 2	3	4	5	6
1[a]	Me_2SO-d_6	o		−0.57 (o)	0 (m)	−0.57 (p)	−0.08 (m)
2		m	−0.58 (o)		−0.50 (o)	−0.21 (m)	−0.57 (p)
3		p	−0.13 (m)	−0.67 (o)		−0.67 (o)	−0.13 (m)
4	Me_2CO-d_6	o		−0.59 (o)	−0.05 (m)	−0.57 (p)	−0.08 (m)
5		m	−0.55 (o)		−0.50 (o)	−0.19 (m)	−0.51 (p)
6		p	−0.11 (m)	−0.60 (o)		−0.60 (o)	−0.11 (m)
7	$CDCl_3$	o		−0.51 (o)	−0.04 (m)	−0.58 (p)	−0.21 (m)
8		m	−0.41 (o)		−0.34 (o)	−0.19 (m)	−0.46 (p)
9		p	−0.03 (m)	−0.51 (o)		−0.51 (o)	−0.03 (m)

[a] Acetophenone itself gave NMR data as follows. Me_2SO-d_6: δ 2.55 (CH_3), 7.50 (H-3, -4, -5), 7.94 (H-2, -6); Me_2CO-d_6: δ 2.55 (CH_3), 7.49 (H-3, -5), 7.52 (H-4), 7.96 (H-2, -6); $CDCl_3$: δ 2.57 (CH_3), 7.46 (H-3, -5), 7.48 (H-4), 7.93 (H-2, -6).

Table V. Chemical Shifts for Ring C Protons of Metabolite B (3)

entry	solvent		chemical shifts d[a]	dd[b]	d[c]
1	Me_2SO-d_6	found	6.78	6.81	7.33
2		calcd 4′-OH	6.80	6.80	7.34
3		calcd 5′-OH	6.89	6.97	7.26
4	Me_2CO-d_6	found	6.86	6.92	7.40
5		calcd 4′-OH	6.92	6.92	7.41
6		calcd 5′-OH	6.97	7.02	7.33
7	$CDCl_3$	found	6.82	6.78	7.36
8		calcd 4′-OH	6.87	6.87	7.35
9		calcd 5′-OH	6.97	7.04	7.19

[a] J = 2 Hz. [b] J = 2 and 8.5 Hz. [c] J = 8.5 Hz.

that the difference in chemical shifts for the 5-phenyl ring protons of **5** and **6** run in three different solvents (see Table III, entries 11–16) does afford for each solvent used a set of substituent effect parameters attributable to 4′-hydroxylation (see Table III, entries 17–19).

Since a benzodiazepine model was not available for 5′-hydroxylation, *m*-hydroxyacetophenone was compared to unsubstituted acetophenone. *p*-Hydroxyacetophenone served as a check for 4′-hydroxylation, and *o*-hydroxyacetophenone afforded parameters to confirm our postulation that the substituent parameters of a hydroxyl group are dependent on its relationship to the >C=X group. The NMR data for these acetophenones run in three solvents are summarized in Table IV. The parameters obtained from acetophenone and *p*-hydroxyacetophenone in Me_2SO-d_6 (Table IV, entry 3) are identical to the parameters for the benzodiazepines **5** and **6** in the same solvent (Table III, entry 17), thus confirming acetophenones as a good model for **1** in Me_2SO-d_6 solutions as far as the effect of 4′-hydroxylation upon the δ values of the 5-phenyl ring protons are concerned. It is also seen that this solvent gave the best agreement. Apparently, the 5-phenyl ring protons of the parent drug were isochronous coincidentally only in this solvent and gave a singlet at δ 7.42, whereas in the other solvents the spectra were complex and provided less accurate chemical-shift base values.

Following this demonstration, the substituent effect parameters of Table IV (entries 2, 3, 5, 6, 8, and 9) were added to the chemical shifts of the 5-phenyl ring protons of **1** taken from Table III (entries 1–3) to give the calculated chemical shifts shown in Table V together with the chemical shifts found for metabolite B.

Clearly, metabolite B is hydroxylated in the 4′ position. Although the best correspondence is observed in Me_2SO-d_6, in both of the other two solvents a closer agreement of the experimentally determined chemical shifts with those calculated for the 4′- as opposed to 5′-hydroxy metabolite was obtained.

Table VI. Excretion of ^{14}C-Labeled **1** and Three Hydroxylated Metabolites by a Dog

compd form	excret as % of ion dose 1	2	3	4
urine				
nonconjug	1.1	0.4	1.0	1.1
conjug	nil	15.3	2.8	2.5
feces				
nonconjug	2.7	4.2	1.5	3.7
	3.8	19.9	5.3	7.3

Estimation of Metabolite Excretion. Since 55% of the ^{14}C of a 2–4-day fecal pool was extracted at pH 9 prior to Glusulase treatment and only 3% after this treatment, the nonconjugated fecal metabolites were assayed as well as the nonconjugated and conjugated urinary metabolites excreted in 4 days. The extracts were analyzed by two-dimensional TLC, using system 1 for the first dimension and system 2 for the second. Added with the extract at the origin were authentic **1** and lorazepam; in place of authentic unlabeled **4** which was not synthesized, we used 2′-dechloro (**4**),[13] which was found in preliminary experiments to migrate the same as **4**. Although no carrier was available for **3**, sufficient quantities of labeled metabolite were present in the extracts so that it could be detected as a UV-absorbing spot under shortwave UV light.

The amounts of drug and metabolites excreted, calculated from the radioactivity which migrated as each substance, are shown in Table VI. Most of the 4% of the dose excreted as unchanged **1** was in the feces. Of the 20% of the dose excreted as lorazepam, three-quarters was found as a conjugate in the urine. The phenolic metabolites were excreted to a smaller extent than was lorazepam. Roughly the same amounts of each were found in the urine as both free and conjugated metabolites, but in the feces more of **4** was found.

Discussion

The three metabolic reactions of **1** found in the dog, 3-, 9-, and 4′-hydroxylation, are not new; they have already been reported for other benzodiazepines.[14] It is of interest, however, that the 2′-chloro function did not influence the usual site of hydroxylation of the 5-phenyl ring, namely, para to the C-5 carbon. Furthermore, the finding that 4′-hydroxylation of **1** is a metabolic pathway suggests by analogy that the phenolic metabolite of lorazepam reported by Schillings et al.[11] may be a 4′-hydroxy derivative. Urinary conjugated lorazepam was reported[10] to represent 13 and 16% of **1** administered po and iv, respectively, at a dose of 10 mg/kg to a dog. Our finding that the excretion of lorazepam, mostly conjugated, in the urine accounts for 16% of **1** administered to a dog is in agreement with these data. In addition, the finding of a small amount of

[^{14}C]lorazepam (4% of the dose) in the feces raises the possibility that conjugated lorazepam was also secreted in the bile.

The potent central nervous system activity of lorazepam has been reported.[15] The two phenolic metabolites of 1 have not been synthesized, and no direct estimation of their activity is available. However, on the basis of the results of testing other 4′- and 9-hydroxybenzodiazepines,[16] it is probable that they are inactive.

Depending on the structural change, mass spectrometry may be adequate for metabolite identification. However, there are often instances in which mass spectrometry alone will not suffice, and NMR spectroscopy is required for a complete structure. Such an example is provided by the identification of **4** as a metabolite of **1**, but there are examples, e.g., **3**, the 4′-hydroxy metabolite of **1**, in which the usual application of NMR is inadequate. An important element of the present study is the demonstration that substituent parameters for a phenolic hydroxyl group are dependent on its location with respect to other groups on the benzene ring. The application of this principle, as in the present study, significantly increases the power of NMR spectroscopy to solve metabolite structures.

Experimental Section

Labeled 1 Administration and the Measurement of Radioactivity. Compound 1 labeled with ^{14}C in the C-5 position was synthesized by Dr. H. Kaegi and Mr. W. Burger by a published method.[17] This ^{14}C-labeled 1 was diluted with nonlabeled 1 to a specific activity of 0.74 μCi/mg prior to oral administration in a gelatin capsule to a 12.6-kg male beagle. Complete daily collections of urine and feces were made for 6 days. A radiochemical purity check performed using TLC with solvent system 1 (described below), followed by quantitation of the radioactivity on sections of the silica gel layer, revealed that at least 96% of the ^{14}C migrated as cochromatographed authentic 1.

Radioactivity was determined in a liquid scintillation spectrometer (Model 3380; Packard Instrument Co., Downers Grove, Ill.), using the external standard-channels ratio technique for quench correction. A commercial fluor-containing medium (Aquasol, New England Nuclear Corp., Boston, Mass.) was used undiluted (10 mL per vial) to determine the ^{14}C in aqueous or organic solutions and was diluted with sufficient water to bring the aqueous volume to 2.5 mL in order to form a gel for determining the ^{14}C in aliquots of 50% alcohol homogenates of feces and on particles of silica gel.

Isolation Procedures. The excreted radioactivity was fractionated by extracting pools of urine or feces with ethyl acetate. Following the initial extraction of each sample at pH 9.0 and 7.0, the aqueous phase was incubated with a commercial preparation of β-glucuronidase and arylsulfatase (Glusulase, Endo Labs., Garden City, N.Y.), which was added to a final concentration of 1% (v/v). Incubation at pH 5.5 and 37 °C for 3 h, which previously yielded maximum hydrolysis of conjugated benzodiazepine metabolites,[3] was followed by serial extraction at pH 9.0, 7.0, and finally at 2.0. At each pH, two consecutive volumes of ethyl acetate, each equaling the aqueous volume, were shaken with the aqueous phase for approximately 10 min each; all pH adjustments were made by the addition of dilute acid or alkali. The radioactivity in each extract was then determined, and selected extracts were examined by TLC.

Commercial thin-layer plates containing a 0.3-mm layer of silica gel with a fluorescent indicator (Q4F, Quantum Industries, Fairfield, N.J.) were used with the following solvent systems: 1, chloroform–acetone–ethanol (80:5:5); 2, *n*-heptane–ethyl acetate–ethanol–concentrated ammonia (50:50:20:1.5); 3, *n*-heptane–ethyl acetate–ethanol–concentrated ammonia (50:50:20:3); 4, *n*-heptane–chloroform–ethanol–glacial acetic acid (50:50:20:1); 5, *n*-heptane–chloroform–ethanol (10:10:1).

One-dimensional TLC was used for metabolite isolation, and two-dimensional TLC was used for quantitation. Compounds were located by viewing the plates under shortwave UV light.

Metabolite Identification Procedures. Mass spectra were run on a Consolidated Electrodynamics Corp. 21-110 mass spectrometer in which the high-resolution spectrum of a sample of metabolite C was registered with that of a reference compound (perfluorokerosene) on a photographic plate. The distances of the ion traces on the plate were measured with a Gaertner comparator and, after encoding, punched on IBM cards. A computer was used to calculate the masses and the corresponding elemental compositions. For metabolite B, the instrument was used in the low-resolution mode.

Proton NMR spectra were obtained with pulse Fourier-transform techniques on a Varian XL-100 spectrometer or in a continuous-wave mode on a Varian HA-100 spectrometer equipped with a C-1024 time-averaging computer. Each sample (0.1–1 mg) was dissolved in 0.3–0.4 mL of deuterated chloroform ($CDCl_3$), dimethyl sulfoxide (Me_2SO-d_6), or acetone (Me_2CO-d_6), containing 1% (v/v) of tetramethylsilane (Me_4Si) as internal standard. Accumulation of spectra overnight in a continuous-wave mode, using a sweep-width of 50 Hz and a sweep-time of 1 Hz/s, allowed time averaging of up to 1296 scans, representing a 36-fold signal to noise enhancement. Chemical shifts (δ) are expressed in parts per million and spin–spin splittings (J) in Hz. Spectra of larger amounts of model benzodiazepines (1 and **5**–7; 2–5 mg each) as well as the acetophenones (25 mg each) were obtained in the continuous-wave mode, but spectra of **6** and 7 in $CDCl_3$ were obtained in the Fourier-transform mode. The benzodiazepines **1**, **5**, **6**, and 7 were synthesized by the procedure of Sternbach and co-workers.[17] Acetophenone (Matheson, Coleman and Bell) and hydroxyacetophenones (Aldrich Chemical Co., Inc.) used as model compounds were commercially obtained.

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