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METABOLISM OF ^{14}C-MEDAZEPAM HYDROCHLORIDE IN DOG, RAT AND MAN

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Abstract—Labeled medazepam (7-chloro-2,3-dihydro-1-methyl-5-phenyl-1H-1,4-benzodiazepine-5-^{14}C) and metabolites were identified and measured by two-dimensional thin-layer chromatography. In the dog, 2 mg/kg of medazepam-5-^{14}C HCl given orally or i.v. did not yield measurable blood levels of intact drug. The close agreement of the amount of ^{14}C excreted in the urine after oral and i.v. administration and of the rates of this excretion indicated that the labeled compound was well absorbed. Fractionation of the blood revealed that biotransformation of medazepam was a rapid process. After a higher dose, 20 mg/kg i.v., medazepam disappeared from the blood bi-exponentially with half-lives of 0·16 and 2·7 hr. Pathways of medazepam metabolism, which apparently did not include diazepam as an intermediate, were postulated on the basis of metabolites identified in experiments both *in vivo* and *in vitro*.

In the rat, highest tissue concentrations of ^{14}C were seen 2–4 hr after an oral dose of ^{14}C-medazepam HCl. It was clear from the metabolites identified that the formation of diazepam and phenolic diazepam metabolites represented an important pathway of medazepam metabolism in this species.

The labeled compound was rapidly absorbed by two human subjects who received single 30-mg oral doses. The peak blood levels of medazepam (less than 0·2 μg/ml at 1–2 hr) declined rapidly with a half-life of 1–2 hr. A marked difference between the subjects in rates of elimination of metabolites from the blood and in excretion of urinary metabolites appeared related to body weight and the activity of drug-metabolizing enzymes. Preliminary evidence for diazepam formation in man was also obtained.

MEDAZEPAM hydrochloride (7-chloro-2,3-dihydro-1-methyl-5-phenyl-1H-1,4-benzodiazepine-5-^{14}C HCl) is a member of the 1,4-benzodiazepine class of compounds which includes chlordiazepoxide* and diazepam.† The compound has been shown[1] to possess interesting psychopharmacological activity in animals and is currently marketed‡ as an antianxiety agent. An assay based on extraction of blood at pH 9·0 with ether and gas chromatographic determination of the extracted medazepam was developed by de Silva and Puglisi.§ This method is sensitive to 0·5 μg/ml of blood.

Prior to the synthesis of ^{14}C-medazepam HCl by Kaegi,[2] our studies (unpublished) were concerned with the identification of metabolites by the combination of thin-layer chromatography (TLC) and high-resolution mass spectrometry.|| This technique, used successfully for the identification of metabolites of other benzodiazepines,[3–5]

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* Chlordiazepoxide hydrochloride is the active ingredient in Librium marketed by Hoffmann-La Roche Inc., Nutley, N.J.

† Diazepam is the active ingredient in Valium marketed by Hoffmann-La Roche Inc., Nutley, N.J.

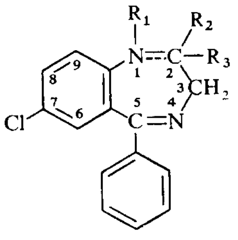
‡ Medazepam is the active ingredient in Nobrium marketed by F. Hoffmann-La Roche and Co., A.G., Basle, Switzerland.

§ J. A. F. de Silva and C. Puglisi, personal communication.

|| The high-resolution mass spectral analysis was performed by Dr. Floie Vane.

TABLE 1. REFERENCE COMPOUNDS USED FOR THE IDENTIFICATION AND ESTIMATION OF METABOLITES BY TWO-DIMENSIONAL TLC

Basic structures



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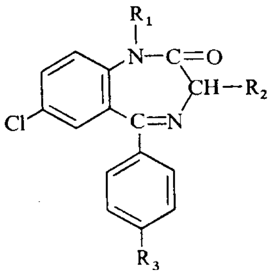

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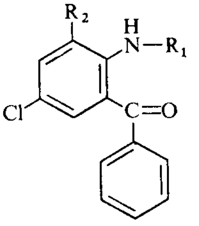

Type C

Compound*	Basic structure type	Substituents		
		R_1	R_2	R_3
Medazepam	A	CH_3	H	H
I	A	H	H	H
II	A	none	H	none
Diazepam	B	CH_3	H	H
III	B	CH_3	OH	H
IV	B	CH_3	H	OH
V	B	CH_3	OH	OH
VI	B	H	H	H
Oxazepam	B	H	OH	H
VII	B	H	H	OH
ACB	C	H	H	
ACHB	C	H	OH	
FACB	C	HC = 0	H	
MACB	C	CH_3	H	

* The chemical name of each reference compound (other than medazepam) follows: I, 7-chloro-2,3-dihydro-5-phenyl-1H-1,4-benzodiazepine; II, 7-chloro-5-phenyl-3H-1,4-benzodiazepine; diazepam, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one; III, 7-chloro-1,3-dihydro-3-hydroxy-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one; IV, 7-chloro-1,3-dihydro-1-methyl-5-(4-hydroxyphenyl)-2H-1,4-benzodiazepin-2-one; V, 7-chloro-1,3dihydro-3-hydroxy-1-methyl-5-(4-hydroxyphenyl)-2H-1,4-benzodiazepin-2-one; VI, 7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one; oxazepam, 7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2H-1,4-benzodiazepin-2-one; VII, 7-chloro-1,3-dihydro-5-(4-hydroxyphenyl)-2H-1,4-benzodiazepin-2-one; ACB, 2-amino-5-chlorobenzophenone; ACHB, 2-amino-3-benzoyl-5-chlorophenol (which is an amino-chloro-hydroxybenzophenone); FACB, 2-formylamino-5-chlorobenzophenone; and MACB, 2-methylamino-5-chlorobenzophenone.

yielded a number of urinary metabolites and one blood metabolite in chronically dosed dogs. Metabolites (structure and chemical name included in Table 1) found were oxazepam, ACB, ACHB, FACB, and MACB in urine and VI in blood. In the present studies with labeled drug, the search for metabolites was continued in the dog and extended to rat and man. In addition, some pharmacokinetic aspects of medazepam metabolism in man were explored.

EXPERIMENTAL

Labeled medazepam hydrochloride. 7-Chloro-2,3-dihydro-1-methyl-5-phenyl-1H-1,4-benzodiazepine-5-^{14}C hydrochloride[2] (structure included in Table 1), with a specific activity of 13·5 μc/mg, was diluted with authentic unlabeled medazepam HCl prior to administration to animals and human subjects. Before administration, the radiochemical purity of labeled doses was confirmed by TLC with System A or System D (described below) or with both, and plate scanning with a model 7200 radio chromatogram scanner (Packard Instrument Co., Downers Grove, Ill.).

Animal experiments. In all the animal experiments, labeled drug with a specific activity of 3·6 μc/mg of medazepam base was used. ^{14}C-medazepam HCl at a dose of 2 mg/kg was given orally in a gelatin capsule to a 9 kg female dog, and 1 month later was injected intravenously into the same dog in 5 ml of physiological saline which contained 2 drops of Tween 80. After both doses, complete collections of urine and feces were made for 1 week and blood (10 ml, heparinized) was drawn by heart puncture at various times. A second female dog (8·2 kg) was given a 20 mg/kg i.v. dose and blood was drawn periodically during the first 24 hr.

A series of male Charles River rats was also given 2 mg/kg oral or i.v. doses of ^{14}C-medazepam HCl. One orally dosed rat and one i.v. injected rat were maintained for a week with complete collections of urine and feces. To study the tissue distribution of ^{14}C, one rat was killed 4 hr after an i.v. dose and others were killed 2, 4, 7 and 24 hr after oral doses; the excised tissues were homogenized in 5–10 vol. of water and counted.

Studies *in vitro* were performed utilizing 100 μg ^{14}C-medazepam HCl as substrate and 9000 *g* supernatant of dog or rat liver as enzyme source. The preparation of the liver supernatants and the composition of the incubation medium, which contained pyridine nucleotides and a NADPH-generating system,[6] deviated from previously described[7] procedures only in the omission of nicotinamide from the incubation mixture. This change was adopted after an initial 60-min incubation experiment demonstrated that nicotinamide had no effect on the extent of ^{14}C-medazepam metabolism or distribution of ^{14}C among metabolites. After incubation at 37° for periods up to 1 hr, the incubation media (3 ml) were extracted twice with 5 ml ethyl acetate. This extraction was shown in preliminary experiments to remove quantitatively the labeled substrate from the incubation medium. The concentrated ethyl acetate extracts were analyzed for labeled substrate and metabolites by 2-dimensional TLC (described below).

Human studies. Two female subjects, H-15 (49-yr old) and H-16 (50-yr old), with apparently normal hepatic and renal function were each given an oral dose of 30 mg ^{14}C-medazepam HCl (sp. act. 1·6 μc/mg of medazepam) in a gelatin capsule. Subject H-15 weighing 100 kg received 0·30 mg/kg of drug, while H-16 (53 kg) received 0·57 mg/kg. Both subjects were given a glass of water every 2 hr during the first day to promote urine production; collection intervals were 0–2, 2–4, 4–6, 6–9, 9–12, 12–24 hr, and daily thereafter until the end of the study. Feces were collected daily throughout the study. Oxalated blood (10 ml) was drawn at 0, 1, 2, 4, 7, 12, 24 hr and at 24-hr intervals thereafter.

Counting techniques. A variety of counting procedures was used in the course these studies. In the dog studies, urine and extracts of urine and blood were counted in Phosphor I[8] using a Packard Instrument Co. model 314-S liquid scintillation spectrometer. Counting efficiency was determined by the use of an internal standard. Blood, plasma and aliquots of 50% ethanol homogenates of feces were counted in a Nuclear-Chicago Corp. D-47 windowless flow-gas counter with correction for selfabsorption. Aliquots of the labeled dose were, of course, counted in both instruments.

In the rat studies, tissue homogenates (0·5 ml) were suspended in Phosphor II[8] and counted in a Nuclear-Chicago Corp. Mark I liquid scintillation spectrometer equipped with a ^{133}Ba external standard; the external standard channels ratio technique was used to determine counting efficiency. The ^{14}C in 0·5-ml samples of whole blood and in aliquots of the 50% alcohol homogenates of feces was determined in the same instrument by the Schöniger combustion, trapping, and counting procedure of Kelly *et al.*[9]

The Mark I liquid scintillation spectrometer was also used for the human studies. Urine (0·2–1·0 ml), urinary extracts and extracts of blood and plasma were counted directly in Phosphor I, while plasma (0·5 ml) was counted as a suspension in Phosphor II. Blood and fecal homogenates were again counted by the Schöniger technique.

Extraction procedures. Aliquots (0·2–5·0 ml) of dog blood and of human blood and plasma were diluted with an equal volume of water and 3 vol. of 1 M borate buffer, pH 9·0, and were extracted twice with 10 vol. of ether. The quantitative extraction of medazepam by this procedure of de Silva and Puglisi* was confirmed by extraction of blood and plasma containing added ^{14}C-medazepam HCl.

Also demonstrated in a recovery experiment was the extraction from urine adjusted to pH 7·0 of over 90 per cent of the ^{14}C of added labeled medazepam HCl. Accordingly, dog and human urine and rat intestinal contents were extracted twice at pH 7·0 with equal volumes of ethyl acetate before and after treatment with Glusulase (Endo Labs., Garden City, N.Y.), a preparation containing a variety of enzymes including β-glucuronidase and sulfatase. Samples were incubated with Glusulase (1%, v/v) for 2–3 hr at 37° and pH 5·5. These incubation conditions were previously found[8] to yield maximum hydrolysis of benzodiazepine conjugates.

Identification and estimation of metabolites by 2-dimensional TLC. The procedure used was essentially the same as that described for the identification and estimation by TLC of the deconjugated urinary metabolites of ^{3}H-diazepam.[8] In brief, identification consisted of demonstrating that a consistent amount of the radioisotope in at least two different 2-dimensional TLC solvent systems exhibited the same migration as a known compound added as an internal standard. From such consistent counting data, quantitation of labeled drug and metabolities was also obtainable. The reference compounds were located by viewing the chromatoplate under shortwave u.v. light, and the silica gel associated with each reference compound was counted in a liquid scintillation spectrometer as a suspension in Phosphor II.

Chromatoplates (20 × 20 cm) containing a 0·3-mm layer of Camag Kieselgel DF-5 (silica gel containing a fluorescent indicator) were employed with the following solvent systems: System A, heptane–chloroform–ethanol (10:10:1); System B′, heptane–chloroform–acetic acid–ethanol (5:5:1:0·3); System B″, heptane–chloroform–acetic acid–ethanol (5:5:1:0·5); System C, isopropanol–conc. ammonia (20:1); System D, heptane–ethyl acetate–ethanol–conc. ammonia (5:5:1:0·3); System F, chloroform–acetone (90:10); and System G, heptane–chloroform–ethanol (5:5:2). The complete 2-dimensional TLC system is designated by the letter of each solvent system given in the order in which it was used [e.g., System AC indicates that development first in heptane–chloroform–ethanol (10:10:1) was followed, after a turn of 90° of the chromatoplate, by development in isopropanol–conc. ammonia (20:1)].

The internal reference compounds added at the origin (usually 4–8 in number) were

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* J. A. F. de Silva and C. Puglisi, personal communication.

chosen from the list of compounds presented together with structures in Table 1. In addition to those metabolites identified in the prior study with unlabeled medazepam, this list includes compounds which were either suspected of being metabolites or were identified as metabolites during the present studies.

RESULTS

Metabolism in the dog

Metabolism in vitro. All of the radioactivity recovered after a 1-hr incubation of ^{14}C medazepam HCl in a medium lacking only dog liver enzymes (9000 *g* supernatant) was still present as intact substrate. The enzymatic conversion of labeled substrate to metabolites on incubation with 9000 *g* supernatant is shown in Table 2. From the consistent data obtained with at least three of the four solvent systems, it is clear that 13 per cent of the substrate was converted to I, 21 per cent to II, 4 per cent to VI, 10 per cent to ACB and 2 per cent to MACB. The deficiencies of this chromatographic assay are also evident: the amount of medazepam remaining was not consistent and could only be estimated as probably between 4 and 9 per cent; the amount of ^{14}C found as diazepam and oxazepam was too small to establish unequivocally their formation; and the roughly 40 per cent of the ^{14}C which did not migrate with any of the internal reference compounds remained unidentified. However, it is clear from the results that not only was medazepam *N*-demethylated to I, which was oxidized to II and VI, but medazepam or some benzodiazepine metabolite was hydrolyzed to the benzophenones, ACB and MACB.

TABLE 2. METABOLITES FORMED ON 1-hr INCUBATION OF ^{14}C-MEDAZEPAM HCl (100 μg) WITH DOG LIVER 9000 *g* SUPERNATANT

Compound	% ^{14}C recovered as each compound*				
	DB′	AD	AB′	AC	Final
Medazepam	4·2	7·4	16·5	9·1	4–9
I	12·8	11·9	25·5	12·9	13
II	26·6	19·8	21·2	20·4	21
VI	3·3	4·2	4·0	3·1	4
ACB	9·0	10·7	10·2	9·9	10
MACB	1·5	2·2	2·1	2·3	2
Diazepam	1·1	1·9			<2
Oxazepam	1·5	1·9			<2

* The 4 solvent systems listed (and described under Experimental) were used for separation and estimation of ^{14}C-substrate and metabolites. The final values for substrate disappearance and metabolite formation were obtained as follows: medazepam, results were not sufficiently consistent to yield more than a range of the lower values; Compounds I and II, the consistent values obtained with 3 of the 4 solvent systems were averaged; Compound VI, ACB and MACB, the values obtained with all four systems were averaged.

Compound II, which has a double bond between the N-1 and C-2 atoms, seemed a likely precursor of ACB and its metabolism was studied in one further experiment. The 9000 *g* supernatant of rat liver was used as the enzyme source and 100 μg of II was incubated for 1 hr with and without the rat liver supernatant. One-dimensional TLC of the ethyl acetate extracts in Systems A and D, followed by inspection of the chromatoplates under shortwave u.v. light, revealed that in the absence of enzymes there was a definite degradation of II to ACB but no detectable formation of VI;

in the complete incubation medium, more II disappeared in 1 hr and both VI and ACB were detectable on the plates. Therefore, II was apparently oxidized enzymatically to VI and also hydrolyzed nonenzymatically to ACB. (These postulated pathways of medazepam metabolism in the dog are included in Fig. 8.)

Metabolism in vivo. The change with time in the levels of plasma ^{14}C, blood ^{14}C, and ether-extractable blood ^{14}C after i.v. injection of 2 mg/kg of ^{14}C-medazepam HCl is shown in Fig. 1. Plasma radioactivity declined for only the first 20 min and then increased to a peak level of 1·2 μg medazepam equiv./ml before exhibiting a biexponential fall-off (half-lives of 3 and 29 hr). The whole blood ^{14}C levels very closely paralleled those of plasma and it was calculated (assuming a hematocrit of 0·45) that during the first 48 hr the whole blood radioactivity was confined almost exclusively to the plasma. Since the ether-extractable blood ^{14}C declined during the time that the whole blood ^{14}C was rising, there appeared to be an extensive biotransformation of medazepam to polar nonextractable metabolites which rapidly entered the plasma. TLC analysis with Systems AB′ and AC resulted in the identification of only one compound in the ether extracts. The levels of this metabolite, ACB, rose to a maximum of 0·07 μg/ml at 2 hr (Fig. 1) and then declined at a rate whose half-life was estimated to be 3 hr. Measurable levels of diazepam and MACB were not found, and the variation in the amounts of ^{14}C migrating as medazepam, I, VI and oxazepam made the conclusive identification and estimation of these compounds impossible.

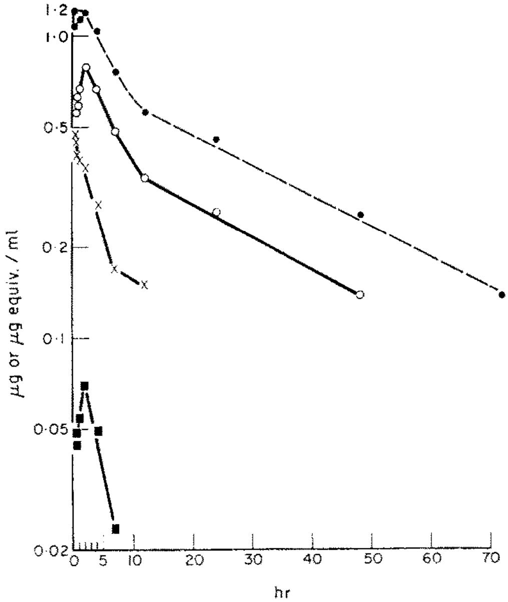

FIG. 1. Radioactivity in the blood of a dog given an i.v. dose of 2 mg/kg of ^{14}C-medazepam HCl. The ^{14}C levels in whole blood (○—○), plasma (●—●) and blood ether extracts (× — ×) in μg medazepam equiv./ml and the levels of ACB (■—■) in μg/ml are plotted logarithmically as a function of time.

TLC analysis of the ether extracts of blood drawn 1, 2, 4, 7 and 12 hr after oral administration of 2 mg/kg of labeled drug to this dog resulted in no medazepam, diazepam, oxazepam, I, ACB or MACB being detected. Compound VI, however, was found to be a blood metabolite and the highest level observed, 0·12 μg/ml at 2 hr, declined with a half-life of 6 hr. The fact that maximum levels of whole blood radioactivity (1·1 μg medazepam equiv./ml) were found at 2 and 4 hr, coupled with the absence of measurable levels of intact drug, indicated that medazepam was readily absorbed and biotransformed into metabolites.

The blood data were not sufficient for a conclusion to be drawn regarding completeness of medazepam absorption. Furthermore, the cumulative excretion data were also not crucial in this respect (Table 3). The excretion of radioactivity was not profoundly influenced by the route of administration of labeled drug and was fairly evenly divided between urine and feces. Since there was a large fecal excretion of radioactivity after i.v. administration, there must have been a large secretion of labeled material into the gastrointestinal (GI) tract. These labeled metabolites, if subsequently absorbed, might have been responsible for the rise in blood ^{14}C seen in Fig. 1.

TABLE 3. EXCRETION OF RADIOACTIVITY BY A DOG AFTER ORAL AND INTRAVENOUS ADMINISTRATION OF 2 mg/kg OF ^{14}C-MEDAZEPAM HYDROCHLORIDE

Route of administration	Excretion of ^{14}C (% administered dose) Urine		Feces	
	1-day	total*	2-day	total*
p.o.	27·9	41·7	36·6	46·9
i.v.	31·2	44·2	27·8	40·9

* The excretion of ^{14}C was measurable for 7 days after oral administration and for 8 days after i.v. administration of labeled medazepam.

The urinary excretion data were of value in the estimation of drug absorption. A semilogarithmic plot of the ^{14}C remaining to be excreted in the urine vs. time[10] revealed very similar rates of excretion of ^{14}C after both routes of administration (Fig. 2). Since the radioactivity excreted in the urine after oral drug administration was similar both in amounts and rates of excretion to that found after i.v. injection of drug, it was concluded that ^{14}C-medazepam HCl was extensively absorbed.

Table 4 summarizes the TLC analysis of urinary ^{14}C extracted at pH 7·0 into ethyl acetate before and after treatment of the urine with Glusulase. Of the directly extractable radioactivity, less than 1 per cent of the urinary ^{14}C was identified as ACB and, after the i.v. dose only, as oxazepam; over 95 per cent of ^{14}C in these fractions (representing 19–20 per cent of the urinary ^{14}C) remained unknown. In contrast, over 50 per cent of the radioactivity excreted as urinary conjugates and extractable only after Glusulase treatment was identified. While oxazepam was the major metabolite in this fraction, significant amounts of ACHB were also found. It is also evident from this table that a total of 78 per cent of the ^{14}C excreted in 2 days after oral administration of labeled medazepam HCl and 69 per cent of the ^{14}C so excreted after i.v. administration was not identified.

In order to obtain information on the rate of elimination of blood medazepam, it

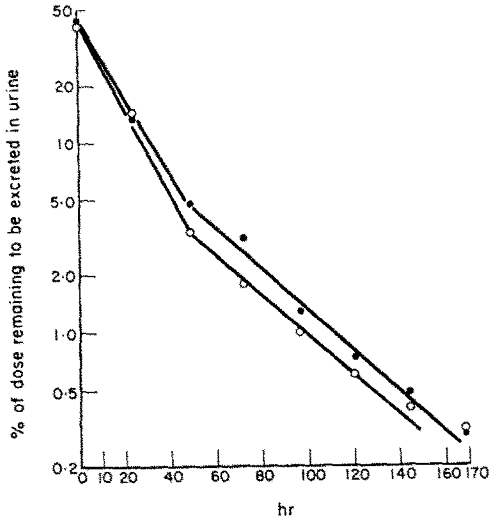

FIG. 2. The urinary excretion of radioactivity by a dog given 2 mg/kg of ^{14}C-medazepam orally and then, a month later, i.v. The ^{14}C remaining to be excreted in the urine after oral (○) and i.v. (●) drug administration is plotted logarithmically as a function of time.

TABLE 4. URINARY METABOLITES EXCRETED BY A DOG DURING THE FIRST 2 DAYS AFTER ORAL AND INTRAVENOUS DOSES OF 2 mg/kg ^{14}C-MEDAZEPAM HCl*

Urinary metabolites	Amount excreted† (% urinary ^{14}C)	
	(p.o. drug)	(i.v. drug)
Directly extractable with ethyl acetate:		
Medazepam	nil‡	nil
ACB	0·8	0·6
Oxazepam	nil	0·2
Unidentified	20	19
Extractable after Glusulase treatment:		
Oxazepam	16	24
ACHB	5	6
Unidentified	9	12
Nonextractable	49	38
Total	100	100

* In 2 days, 39 per cent of the orally administered ^{14}C and 40 per cent of the i.v. injected ^{14}C was excreted in the urine.

† Metabolites identified and estimated by TLC with Systems AB′, AD and GB″.

‡ Nil signifies that less than 0·05 per cent of the urinary ^{14}C was recovered as the designated compound.

was necessary to inject a dog i.v. with 20 mg/kg of ^{14}C-medazepam HCl. Ether extracts of blood were analyzed for medazepam by TLC (Systems AB′, AC and AD) and also by the de Silva and Puglisi gas chromatographic assay. Of the three solvent systems, System AD gave the lowest values for blood medazepam; these presumably were the most accurate because of least contamination with labeled metabolites. Furthermore, as seen in Fig. 3, these values for blood medazepam agreed reasonably well with those obtained by de Silva and Puglisi* using their gas chromatographic assay. With the System AD results accepted as a valid estimate of blood medazepam, it was possible to demonstrate a bi-exponential elimination of intact drug with half-lives of 0·16 and 2·7 hr. The volume of distribution (Vd_{ss}) calculated for a 2-compartmental open system, as described by Riegelman *et al.*,[11] was 42·7 l. or 521 per cent of body weight, which was indicative of extensive tissue uptake of medazepam. It is also seen that during the first 30 min, when medazepam levels declined sharply, the total ether-extractable ^{14}C declined much more slowly and the whole blood ^{14}C scarcely fell at all. Apparently, medazepam was being biotransformed very rapidly into both extractable and nonextractable metabolites which rapidly entered the blood. Two ether-extractable metabolites were identified and estimated by TLC and their levels are shown in Fig. 3. ACB levels rose to 0·9 μg/ml at 30 min and then declined bi-exponentially; VI was still rising at 30 min, after which time a lack of consistent TLC data prevented further estimation. In addition, diazepam, MACB and FACB were not present in detectable amounts, while inconclusive results were obtained with respect to I and oxazepam.

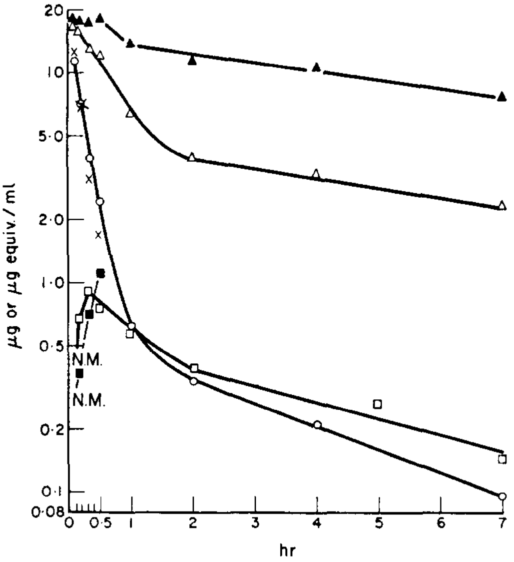

FIG. 3. Blood radioactivity in a dog given an i.v. dose of 20 mg/kg of ^{14}C-medazepam HCl. Whole blood ^{14}C (▲—▲) and ether-extractable blood ^{14}C (△—△) are expressed as μg medazepam equiv./ml. Concentrations in μg/ml of ACB (□—□) and VI (■—■) were determined by TLC (Systems AB′, AC and AD). Medazepam levels were obtained by both TLC with System AD (○—○) and by a gas chromatographic assay (×). N.M. = not measurable.

Metabolism in the rat

Metabolism in vivo. The excretion and tissue distribution of ^{14}C derived from 2 mg/kg i.v. and oral doses of ^{14}C-medazepam HCl are summarized in Table 5. As in the dog, secretion of radioactivity into the GI tract of the rat was evident. In fact, the

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* J. A. F. de Silva and C. Puglisi, personal communication.

total of 66 per cent of the dose in the GI tract 4 hr after the i.v. dose was not markedly less than the 81 per cent of the dose found 4 hr after the oral dose. This suggested that the radioactivity passing through the GI tract after the oral doses (Table 5) was probably in the form of metabolites rather than as intact drug. Support for this idea was obtained on extraction of the intestinal contents from the rat killed 2 hr after oral drug. Extraction at pH 9·0 with ether removed only 8 per cent of the intestinal ^{14}C, which meant that even at this early time at least 92 per cent of the intestinal radioactivity was present as metabolites. The total excretion of fecal metabolites was about the same (58–61 % of the dose) regardless of the route of ^{14}C-medazepam HCl administration. It is also seen that liver and carcass together accounted for almost all of the tissue radioactivity.

TABLE 5. RADIOACTIVITY IN EXCRETA, GASTROINTESTINAL TRACT AND TISSUES OF RATS AFTER 2 mg/kg OF ^{14}C-MEDAZEPAM HCl

Sample	Intravenous dose		Oral dose				
	4 hr (% of dose)	6 days (% of dose)	2 hr (% of dose)	4 hr (% of dose)	7 hr (% of dose)	24 hr (% of dose)	7 days (% of dose)
Urine	8·8	21·8	2·2	4·2	7·2	13·0	16·4
GI tract:							
Stomach + contents	0·3		29·2	6·2	0·3	nil	
Small intest. + contents	41·8		60·5	59·9	11·8	8·0	
Large intest. + contents	23·9		0·6	15·2	55·8	3·7	
Feces	nil	58·2	*	*	6·7	61·0	60·0
Tissues:							
Liver	4·4		6·9	5·1	2·2	0·8	
Carcass	14·0		9·2	3·9	2·7	4·1	
Other	1·1		1·8	1·4	0·6	0·2	
Total recovery	94·3	80·0	110·4	95·9	87·3	90·8	76·4

* No feces excreted.

A more detailed summary of the tissue distribution is given in Table 6. After oral administration, the highest concentrations of ^{14}C were in the liver and kidney. In addition, these organs retained significant levels of ^{14}C for 24 hr, in contrast to lung and perirenal fat, in which the ^{14}C decreased to undetectable levels at 24 hr. The carcass also retained significant levels of ^{14}C for 24 hr and, since no ^{14}C was detected in muscle after 2 hr, it appeared that some other tissue was the source of this radioactivity. The distribution 4 hr after i.v. administration was similar to that after the oral dose, except for the greater uptake of ^{14}C by fat and carcass.

A search for metabolites followed the same procedures which had previously proved successful in identifying conjugated intestinal metabolites of diazepam in the rat.[3] One labeled component liberated by Glusulase from the intestinal contents was purified by TLC and identified by high-resolution mass spectrometry as IV. Since IV had previously been shown to be the major conjugated intestinal metabolite of diazepam in the rat,[3] Compounds III, IV, V and VII, the four metabolites of diazepam in the rat, all had to be considered as possible metabolites of medazepam. Three of these four, IV, V and VII, were found by TLC with Systems DB′ and GB″ to be present as

TABLE 6. TISSUE DISTRIBUTION OF RADIOACTIVITY IN THE RAT AFTER 2 mg/kg OF ^{14}C-MEDAZEPAM HCl

Tissue	Distribution after oral dose								Distribution after i.v. dose	
	2 hr		4 hr		7 hr		24 hr		4 hr	
	(μg-equiv./g)*	tissue/blood	(μg-equiv./g)	tissue/blood	(μg-equiv./g)	tissue/blood	(μg-equiv./g)	tissue/blood	(μg-equiv./g)	tissue/blood
Blood	0·14	1·0	0·081	1·0	0·034	1·0	0·023	1·0	0·054	1·0
Liver	2·72	19·4	2·34	28·9	1·02	30·0	0·39	17·0	1·78	33·0
Kidney	2·04	14·6	2·35	29·0	0·95	27·9	0·34	15·0	1·94	36·0
Heart	0·22	1·6	0·18	2·2	nil	0	nil	0	nil	0
Lung	0·53	3·8	0·39	4·8	0·088	2·6	nil	0	0·16	3·0
Spleen	0·15	1·1	0·20	2·5	nil	0	nil	0	nil	0
Brain	0·19	1·4	0·11	1·4	nil	0	nil	0	nil	0
Testes	0·20	1·4	0·11	1·4	nil	0	nil	0	0·10	1·9
Carcass†	0·22	1·6	0·10	1·2	0·066	1·9	0·088	3·8	0·31	5·7
Muscle	0·11	0·8	nil	0	nil	0	nil	0	nil	0
Fat (perirenal)	1·29	9·2	0·74	9·1	0·41	12·1	nil	0	1·91	35·4

* ^{14}C concentrations presented as μg equivalents medazepam (base)/g of tissue.

† The carcass represents the heterogeneous fraction remaining after removal of samples of blood, muscle and fat, in addition to complete removal of the organs listed above.

conjugated metabolites in small intestinal contents and urine. In the intestinal contents 2 hr after oral ^{14}C-medazepam HCl, 8 per cent of the dose was present as conjugated IV and 3 per cent each as conjugated V and VII. However, there was an additional 47 per cent of the dose present in the intestinal contents as unidentified metabolites. In the 0–24 hr urine of the rat given ^{14}C-medazepam HCl i.v., the same conjugated metabolites were found; 5 per cent of the dose was excreted as VII, 1 per cent each as IV and V, and 10 per cent was unidentified.

A direct demonstration of the metabolism of medazepam to diazepam was obtained by analysis of the liver radioactivity of the rat killed 4 hr after oral ^{14}C-medazepam HCl. Of the 5·1 per cent of the dose in this liver (Table 5), 34 per cent was extracted at pH 9·0; TLC of the ether extract with Systems AB′ and AD revealed that 5·5 and 5·2 per cent of the extracted ^{14}C, respectively (equivalent to 0·09 per cent of the dose), was present as diazepam. This TLC also revealed that compound I was a liver metabolite present to the extent of 0·15 per cent of the dose.

Metabolism in vitro. The formation of diazepam also occurred on incubation of ^{14}C-medazepam HCl with 9000 *g* supernatant of rat liver; 12–14 per cent of substrate ^{14}C was present in the medium as diazepam after 30–60 min (Table 7). This is in sharp contrast to the less than 2 per cent of substrate ^{14}C found as diazepam when dog liver supernatant was used as enzyme source (Table 2). In addition to diazepam, the rat liver enzymes also produced considerable amounts of I and VI. There was, however, no significant accumulation of either II or ACB. It appeared, therefore, that medazepam was metabolized *in vitro* by rat liver enzymes to I (which was not to any significant extent metabolized further to II and hence to VI and ACB) and to diazepam which, as previously shown,[7] was further metabolized to VI. The studies both *in vivo* and *in vitro* clearly demonstrate that the conversion of medazepam to diazepam is a prominent metabolic pathway in the rat.

TABLE 7. METABOLISM OF ^{14}C-MEDAZEPAM HCl (100 μg) BY 9000 *g* SUPERNATANT OF RAT LIVER

Incubation time (min)	% Substrate ^{14}C recovered* as						
	Medazepam	Diazepam	I	II	III	VI	ACB
30	<10	14	14	6	4	16	<2
60	<10	12	10	6	6	22	<2

* Ethyl acetate extracts of the incubation media were analyzed for each compound except II by TLC with AC and DB′; II was analyzed only by TLC with AC so that the value shown represents the maximum amount of II that could be present. The two systems yielded consistent results for the other compounds and the average is presented.

Metabolism in man

Blood medazepam and metabolites. The fall-off of each subject's plasma radioactivity after a 30-mg oral dose of ^{14}C-medazepam HCl is shown in Fig. 4. The curves are markedly different; the plasma ^{14}C levels in subject H-15 dropped rapidly (half-life of roughly 2 hr) from the 1-hr peak, and from the second day exhibited a slow decline (half-life of 15 days), while in H-16 the levels rose to a peak at 2 hr, exhibited a secondary peak at 12 hr (at which time the H-15 levels had declined 3-fold), and from the third day declined with a half-life of 2·5 days. After the third day, plasma radioactivity was eliminated at a 6-fold faster rate by H-16 than by H-15.

Due to low levels, the H-15 whole blood radioactivity was measurable only in the 1-, 2- and 4-hr samples. On the assumption of a hematocrit of 0·45, it was calculated that 84, 76 and 100 per cent of the 1-, 2- and 4-hr whole blood ^{14}C was localized in the plasma, indicating little penetration of drug or metabolites into the red blood cells. This was more clearly evident in subject H-16 whose higher whole blood ^{14}C concentrations could be readily measured. It was calculated that over 92 per cent of the whole blood radioactivity was localized in the plasma of blood drawn at 1, 2, 4, 7, 12, 24, 48, 72, 96 and 120 hr.

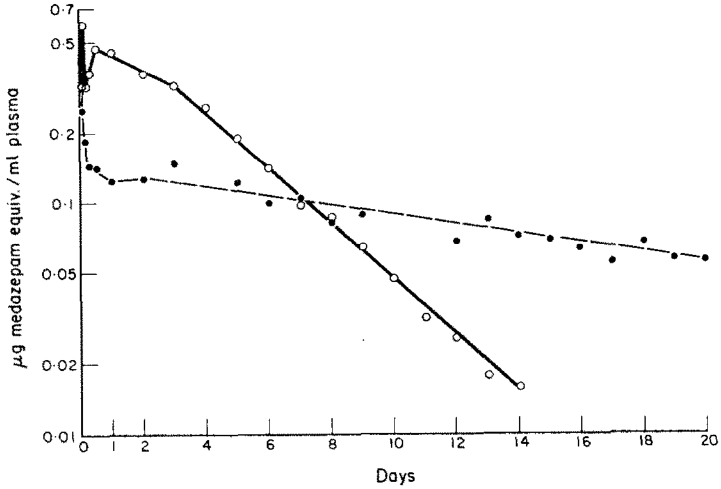

FIG. 4. Plasma ^{14}C concentrations in two subjects, each given 30 mg ^{14}C-medazepam HCl orally, plotted logarithmically as a function of time. Subject H-15 (●—●); subject H-16 (○—○).

Ether extracts of H-15 blood and H-16 plasma were analyzed for intact medazepam by TLC with System AD. In the dog studies presented above (Fig. 3), this system was shown to give a reliable estimate of blood medazepam. Since all the whole blood radioactivity of H-16 was confined to the plasma, the results obtained with plasma extracts could be readily expressed in terms of whole blood for comparison with the H-15 whole blood extraction data. The fall-off of whole blood ^{14}C, ether-extracted blood ^{14}C, and blood medazepam is shown in Fig. 5. In H-15 the medazepam levels, which declined rapidly from 0·10 μg/ml at 1 hr with an apparent half-life of about 2 hr, were no longer measurable at 24 hr. In H-16 the peak medazepam blood level of 0·08 μg/ml at 2 hr dropped to 0·02 μg/ml at 4 hr (apparent half-life of roughly 1 hr) and then declined erratically to nonmeasurable levels at 48 hr. It is also evident that in both subjects the major portion of whole blood radioactivity resided in extractable metabolites of medazepam.

While blood metabolites could not be definitely identified by the use of only one 2-dimensional solvent system (System AD), the ether-extracted ^{14}C from both subjects was found to migrate in this system as I, VI and diazepam, but not as oxazepam, MACB, ACB or II. Furthermore, the marked increase with time of the levels of ^{14}C which migrated as VI raised the possibility that VI was a major blood metabolite of medazepam. However, it must be emphasized that the identification of I, VI and diazepam as blood metabolites in man is very tentative and requires further confirmation.

Excretion of metabolites. Subject H-15 excreted 55·6 per cent of the dose in the urine and 7·8 per cent in the feces in 24 days and was still excreting measurable amounts of urinary ^{14}C (approximately 1 per cent of the dose/day) when the study

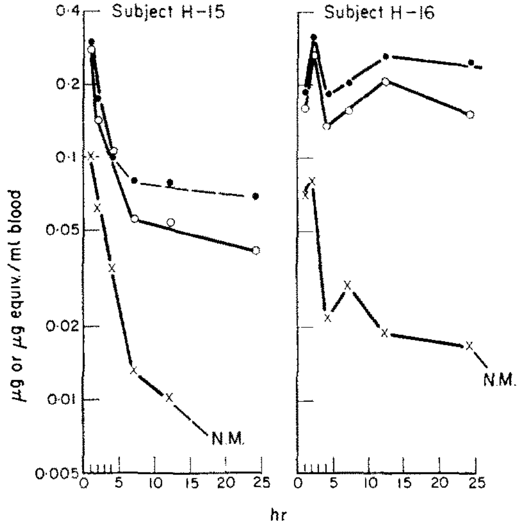

FIG. 5. Fall-off of whole blood ^{14}C (●), ether-extractable blood ^{14}C (○) and blood medazepam (×) in subjects H-15 and H-16. The whole blood ^{14}C levels in H-15 were not measurable after 4 hr but were calculated from the plasma values on the reasonable assumption (see text) that all the ^{14}C was in the plasma. N.M. = less than 0·004 μg medazepam/ml of blood.

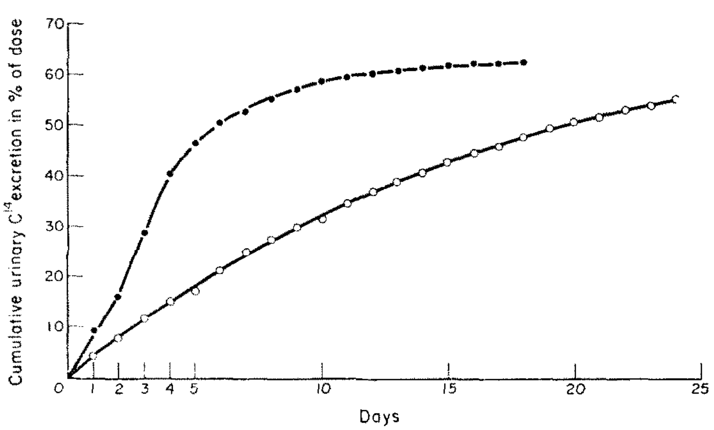

FIG. 6. The cumulative urinary excretion of radioactivity by subject H-15 (○—○) and subject H-16 (●—●) after a 30-mg oral dose of ^{14}C-medazepam HCl.

was terminated. The excretion of ^{14}C by H-16, however, was barely detectable after 18 days, by which time 62·6 per cent of the dose had been excreted in the urine and 22·1 per cent in the feces. In Fig. 6, the cumulative urinary excretion of ^{14}C by subject H-15 is seen to proceed at a slow rate in contrast to that of H-16 which rose sharply and then approached a maximum value.

A more quantitative expression of the rate of excretion of radioactivity by each subject was sought. The semilogarithmic plot of per cent of dose excreted per hour by subject H-15 against time yielded a very erratic fall-off which was not usable for a half-life determination. The "residuals" method suggested by Wagner[10] could not be used directly because the urinary excretion of ^{14}C was not followed until total excretion was obtained, i.e. the cumulative urinary excretion of H-15 did not reach a definite maximum value. It was therefore necessary to assume various values for total excretion and to determine which yielded a linear "residuals" plot. A nearly perfect straight line was obtained (Fig. 7) when the total urinary excretion was placed at 75·0 per cent of the dose. Based on the assumption that H-15 would eventually excrete 75 per cent of the dose in the urine, a half-life of 12 days for the urinary excretion rate was obtained. The determination of the rate of urinary excretion of ^{14}C by H-16 was straightforward; total urinary excretion of ^{14}C was estimated as 63·0 per cent of the dose (asymptote approached in Fig. 6) and the "residuals" plot (Fig. 7) yielded a straight line from which a half-life of 2·5 days was calculated. These rate data indicate that H-16 was excreting radioactive compounds in the urine roughly 5 times faster than was H-15. Also, these urinary excretion rates are seen to be in good agreement with the plasma ^{14}C elimination rates (half-lives of 15 days for H-15 and 2·5 days for H-16).

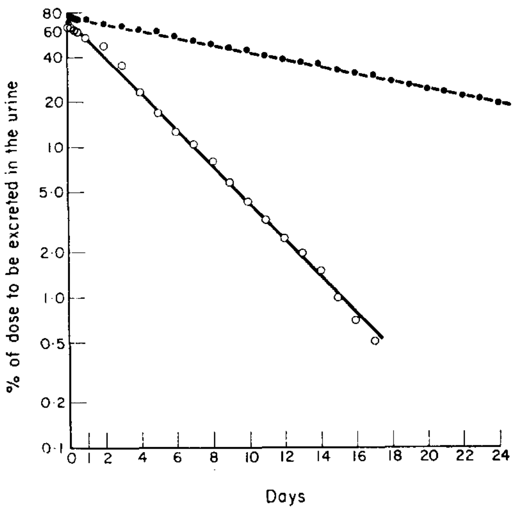

FIG. 7. Semilogarithmic plot (vs. time) of ^{14}C remaining to be excreted in the urine by subjects H-15 (●—●) and H-16 (○—○). The total urinary excretion of ^{14}C by H-15 was estimated as described in the text.

The marked difference between H-15 and H-16 on fractionation of each subject's

TABLE 8. EXTRACTABILITY OF HUMAN URINARY METABOLITES OF MEDAZEPAM

Subject and urine	% Urinary radioactivity		
	Extractable with ethyl acetate*		Nonextractable*
	directly	after Glusulase	
H-5†			
0–7 day pool	30·0	42·8	
0–14 day pool	34·3	45·3	10·8
H-16‡			
0–8 day pool	11·6	74·6	10·0

* The directly extractable ^{14}C was present in basic and neutral metabolites, while the ^{14}C which became extractable after Glusulase treatment was present as glucuronide and/or sulfate conjugates. The nonextractable radioactivity was that found in the urine after the extractions.

† The 0–7 day urine pool represented the excretion of 24·8 per cent of the dose and the 0–14 day pool the excretion of 40·7 per cent of the dose.

‡ The 0–8 day pool represented the excretion of 54·9 per cent of the dose.

TABLE 9. METABOLITES IDENTIFIED BY TLC IN A 0–7 DAY POOL OF H-15 URINE*

Metabolites	Directly extractable† (% of Urinary ^{14}C)	Extractable after Glusulase treatment† (% of Urinary ^{14}C)
I	4·7	2·6
III	nil‡	1·3
VI	0·6	1·1
Oxazepam	nil	7·1
ACHB	nil	2·4
Unidentified	24·7	28·3

* The % of the dose excreted in 7 days by H-15 and the distribution of ^{14}C between directly extractable and conjugated metabolites are included in Table 8.

† The two directly extractable metabolites identified yielded consistent results with Systems AD, DB′, FD and CD; the metabolites extracted after Glusulase treatment were identified with Systems AD, DB′ and FD.

‡ Nil indicates that the metabolite, if present, accounted for less than 0·2 per cent of the urinary ^{14}C.

urine into metabolites directly extractable with ethyl acetate and conjugated metabolites extractable after Glusulase treatment is shown in Table 8. The ratio of conjugated to free metabolites in H-15 urine was 4:3, while this ratio for H-16 urine was almost 7:1. Therefore, H-16 not only excreted metabolites at a much faster rate than did H-15, but also excreted a much greater proportion of conjugated metabolites.

Preliminary results are available on the identity of the urinary metabolites. Analysis by TLC of the metabolites of H-15 urine yielded an identification of only a small portion of the urinary radioactivity (Table 9). I (accounting for 5 per cent of the urinary ^{14}C) and VI (0·6 per cent) were found in the fraction directly extractable with ethyl acetate. Although not included in Table 9, the excretion of intact medazepam was not detectable. After treatment of the urine with Glusulase, oxazepam was the major identified metabolite. Also excreted as urinary conjugates were small amounts of ACHB, I, III and VI. The postulated interrelationships of these metabolites with one another are shown in Fig. 8 and are discussed below.

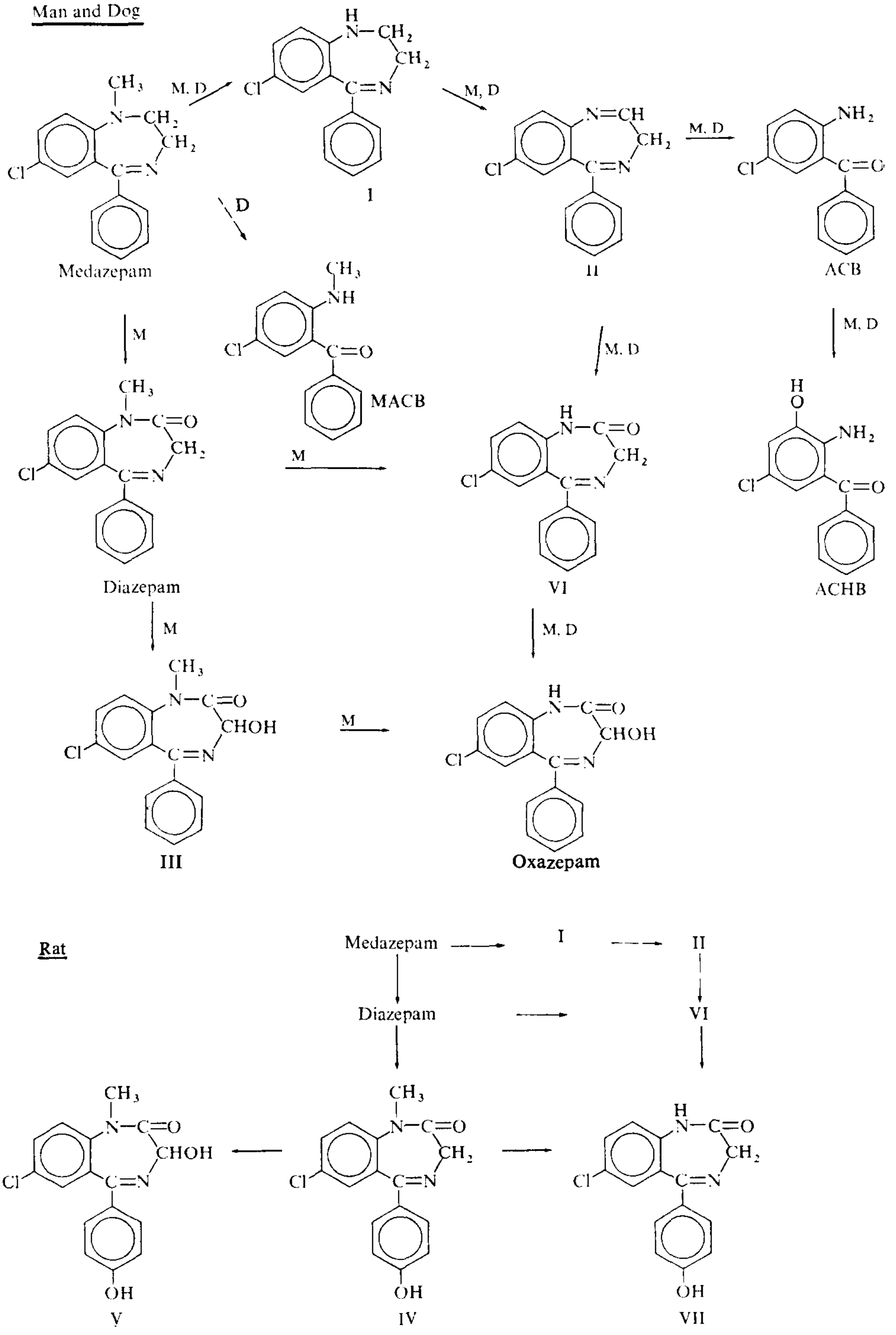

FIG. 8. Postulated pathways of medazepam metabolism in man (M), dog (D) and rat. A broken arrow indicates that the status of the reaction and its product is uncertain.

DISCUSSION

In each of the species studied, at least five metabolites of medazepam were identified. Moreover, since the major portion of excreted radioactivity in each species remained unidentified, there were undoubtedly a considerable number of unidentified metabolites also produced. From those metabolites identified *in vivo* and *in vitro*, however, a partial exposition of the metabolic patterns in each species was possible, and postulated pathways are shown in Fig. 8.

In the dog, except for MACB formed *in vitro*, all identified labeled metabolites no longer retained the *N*-methyl group. This suggests that *N*-demethylation of medazepam to I was a prominent pathway *in vivo*, although I was not detected in blood or urine. The oxidation of I to II and enzymatic conversion of II to VI and nonenzymatic conversion to ACB were discussed with the results of the experiments *in vitro*. The further conversion of VI to oxazepam, the major conjugated urinary metabolite in the dog, was previously demonstrated *in vitro*.[7] ACB was the logical precursor of ACHB (a conjugated urinary metabolite) and, in a separate dog experiment not reported above, the administration of ^{3}H-ACB did result in the urinary excretion of conjugated ACHB. A broken arrow leading to MACB is shown in Fig. 8 to indicate uncertainty as to both its status as a metabolite and pathway of formation. MACB was formed *in vitro* to the extent of only 2 per cent of substrate ^{14}C and in prior experiments was found in the urine of a dog chronically dosed with unlabeled medazepam; however, it was not found *in vivo* in the current work. FACB was also found in the urine of the chronically dosed dog, but was not included in Fig. 8 because it was not detected in the current studies. No evidence was obtained *in vivo* or *in vitro* which indicated that diazepam was a metabolite in the dog.

In man, however, such evidence was obtained and additional pathways proceeding through diazepam are shown for man in Fig. 8. Compound III, previously shown to be a conjugated urinary metabolite of diazepam in man,[8] was definitely detected as a conjugated urinary metabolite in subject H-15, thus strongly suggesting that diazepam was an intermediate in the metabolism of medazepam to III. Furthermore, a small percentage of the ^{14}C extracted from the blood of both subjects did migrate as diazepam in System AD, yielding suggestive but not unequivocal evidence for the presence of diazepam in the blood. The finding of ACHB as another conjugated urinary metabolite in H-15 suggested that the pathway via I, II and ACB found in the dog was also in operation in man. Of these intermediates, only I was identified as a human urinary metabolite.

Diazepam was shown in previous studies *in vitro* with dog[7] and rat and mouse[12] liver preparations to be metabolized to both III and VI, each of which was further metabolized to oxazepam. It is likely, therefore, that oxazepam, the major conjugated urinary metabolite excreted by subject H-15, was formed from both III and VI. Compound VI was excreted in H-15 urine and preliminary, though inconclusive, evidence that VI is a major blood metabolite in man was reported above. In the light of the facts that in man diazepam[8] and medazepam both produced III, VI and oxazepam as urinary metabolites and that VI was a diazepam blood metabolite[8] whose levels rose steadily on chronic diazepam treatment,[13] it appears likely that further work will confirm the status of VI as a major blood metabolite of medazepam.

Also shown in Fig. 8 are the postulated pathways of medazepam metabolism in the rat. While I was formed *in vitro* and was found *in vivo* in the liver, II, ACB and ACHB have not been definitely established as metabolites *in vitro* or *in vivo* in the rat. Therefore, the further metabolism of I to II is in doubt. It is clear, however, that diazepam is an important metabolite of medazepam in this species. Not only was diazepam formed *in vitro* and found *in vivo* in the liver, but the phenolic rat metabolites of diazepam (IV, V and VII)[3] were identified as conjugated intestinal and urinary metabolites of medazepam.

The pharmacodynamic aspects of these studies indicated rapid onset of absorption and rapid biotransformation of oral medazepam in man and dog. The slower rate of urinary excretion of metabolites by subject H-15 (weighing 100 kg) as compared to H-16 (53 kg) appeared related to the less efficient conversion of medazepam and lipid-soluble metabolites to readily excretable conjugates and to the greater body weight of H-15. This additional weight not only provided more adipose tissue for storage of the lipophilic drug and metabolites, but also reduced the dose (mg/kg) so that H-15 received only about half of that given H-16.

Since the metabolites of medazepam in all three species appeared rapidly in blood and excreta, these metabolites are of prime importance in assessing the pharmacological action of medazepam. This is especially true because four metabolites, diazepam, oxazepam, III and VI, have each been shown[14] to possess psychotropic activity in animals. Therefore, further work to establish the relative contribution of medazepam and metabolites to pharmacological activity in animals and man appears warranted.

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