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PRAZEPAM METABOLISM *IN VITRO*

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Abstract—^{14}C-prazepam (7-chloro-1-cyclopropylmethyl-5-phenyl-1H-1,4-benzodiazepin-2-one) labeled either in the 5-phenyl ring or in the cyclo propyl sidechain, was incubated with 9000 *g* supernatant fractions prepared from livers of dogs, untreated and phenobarbital-treated rats, and untreated and phenobarbital-treated mice. Ring-labeled prazepam was also incubated with liver supernatants from humans. *N*-dealkylation was the major pathway of prazepam metabolism *in vitro*. The liver enzymes of all species except untreated mice were able to metabolize prazepam to varying amounts of oxazepam. Pretreatment of rats and mice with phenobarbital resulted in an increase of both desalklprazepam and oxazepam formation from prazepam.

A PREVIOUS study has demonstrated that the metabolites *in vivo* of prazepam in man include desalkylprazepam, 3-hydroxyprazepam glucuronide and oxazepam glucuronide.[1] Dogs treated similarly with prazepam also excreted the same metabolites and, in addition, 4-hydroxyprazepam glucuronide.[2,3] It became evident from the study in man that prazepam was hydroxylated more rapidly than it was dealkylated and, furthermore, that hydroxylation appeared to facilitate dealkylation.[4]

The present studies were undertaken to develop comparative information on the biotransformation of prazepam *in vivo* and *in vitro*. It was also of interest to investigate whether species differences would be encountered when prazepam was metabolized by human, dog, rat and mouse liver microsomes.

EXPERIMENTAL

^{14}C-prazepam. Ring-labeled prazepam (7-chloro-1-cyclopropylmethyl-5-phenyl-1H-1,4-benzodiazepin-2-one-5-^{14}C) was synthesized with a specific activity of 1·31 mc/g. Also used was sidechain-labeled prazepam [7-chloro-1-cyclopropyl (methyl-^{14}C)-5-phenyl-1H-1,4-benzodiazepin-2-one] with a specific activity of 0·93 mc/g.

Sidechain-labeled prazepam served a very useful purpose in this study for the following reasons. When prazepam conversion was nearly quantitative, the use of sidechain-labeled drug made it easier to locate residual prazepam by radioscanning, without interference from desalkylprazepam, and to be confident of the absence of 3-hydroxyprazepam.

Enzyme preparations. Human liver tissue was obtained from eight subjects 3–20 hr post-mortem and was packed on ice until arrival at the laboratory. The livers were rinsed with cold 0·1 M Tris (hydroxymethyl) aminomethane hydrochloride, pH 7·4, and homogenized with 4 vol. of this buffer for 20 sec in a Waring Blendor. The homogenates were then centrifuged at 9000 *g* for 20 min at 0° and subsequently assayed for protein content utilizing the method of Lowry *et al.*[5] The enzyme preparations were

stored at −20°. Animal preparations were stable up to 1 month after storage, whereas human preparations were inactive after 2 weeks.

The 9000 *g* supernatant fractions were also prepared from the livers of four dogs (male and female mongrels and male and female beagles) as described above.

Rat liver enzyme preparations were obtained from two groups of three male Carworth Farms rats (New City, N.Y.). One group received three daily i.p. injections of 40 mg phenobarbital/kg and were killed on the fourth day. Another group was given 10 mg/kg prazepam p.o. for 3 days prior to sacrifice on the fourth day. The 9000 *g* supernatant fractions were prepared from the pooled livers of both the control group and each experimental group. The 9000 *g* supernatant fractions from the control group were centrifuged at 105,000 *g* for 1 hr. The pellets were rinsed and resuspended in the original volume of Tris (hydroxymethyl) aminomethane hydrochloride. The 105,000 *g* supernatant fraction was also saved to incubate ring-labeled drug.

Mouse liver enzyme preparations were obtained from two groups of six female Millerton, Ha/ICR mice (Millerton, N.Y.). The experimental group received three daily i.p. injections of 50 mg phenobarbital/kg and were sacrificed on the fourth day. The 9000 *g* supernatant fractions were prepared from the pooled livers of control and experimental groups.

Incubation in vitro. Each incubation mixture consisted of 95 mg of 9000 *g* protein in 6 ml of Tris buffer (equivalent amounts were used of the 105,000 *g* supernatant fraction and of the microsomal pellet prepared from the control rat group), 6 ml of a solution containing 0·2 μmole NADP, 13 μmoles glucose 6-phosphate, 250 μl glucose 6-phosphate dehydrogenase (CalBiochem; specific activity, 200 units/ml), 25 μmoles magnesium chloride and 18 ml of a solution containing 0·36 μmole ^{14}C-prazepam labeled either in the ring or the sidechain. Diazepam at a concentration level of 0·35 μmole was incubated with 9000 *g* enzyme preparations of either control rats or mongrel dogs. The 125-ml flask containing the mixture was placed into a water bath at 37°. Oxygen was delivered to the bottom of the tube at a constant rapid rate to maintain saturation in earlier experiments. Later experiments in the absence of oxygen gave identical results. It was also found that a shallow vessel was superior to a test tube in experiments in which oxygen was not utilized.

The sampling periods were 60 min for the animal enzymes and 3 hr for the human enzyme preparations. Incubation of prazepam in the absence of 9000 *g* liver supernatant fractions for 1-hr and 3-hr periods at 37° showed the drug to be stable. The addition of nicotinamide to the incubation mixtures revealed no qualitative or quantitative differences in the metabolism of ring-labeled or sidechain-labeled prazepam.

Analysis of metabolites. A 10-ml aliquot of each incubation mixture was extracted three times with an equal volume of ethyl acetate. The recoveries of ^{14}C were approximately 95 per cent, except in the experiments involving phenobarbital-treated rats. The latter yielded 85 per cent and 70 per cent of the ^{14}C in two separate trials, and the recoveries were not improved by β-glucuronidase treatment prior to extraction. The extracts of each sample were pooled and evaporated to approximately 1 ml.

Chromatography. Thin-layer chromatography (TLC) was performed on 100-μl aliquots of the concentrated extracts in solvents 307 (chloroform–acetone, 9:1), 306 (chloroform–ethanol–acetone, 8:1:1) and 206 (benzene–ethyl acetate, 5:1). The R_f values of the reference compounds in these solvents as well as the co-chromatographic

technique used to compare the migration of metabolites and reference compounds have been described in a previous study.[3] The radioactive bands were located on chromatograms by using the Packard model 7201 radiochromatogram scanner. The peaks were quantified by measuring their areas with a planimeter. In order to determine the presence of phenolic compounds, plates were sprayed with phenol reagent which was prepared by dissolving 1·0 g ferric chloride and 50 mg potassium ferricyanide in 10 ml water.[6] This test is capable of detecting the presence of phenols in amounts as low as 0·1 μg.

RESULTS

Metabolism by human liver enzymes. The results of incubating ring-labeled prazepam with 9000 *g* supernatant fractions of human liver are shown in Table 1. Preliminary studies on eight samples of human liver obtained at routine post-mortem showed that with only the 9000 *g* supernatant fractions of two of these livers could any measurable metabolism be demonstrated. The main reaction was dealkylation, and 3-hydroxyprazepam was not detected. The most extensive conversion of prazepam to the desalkyl derivative occurred with the liver of a patient who died a sudden death as opposed to all other cases in which the patients had been chronically ill.

TABLE 1. METABOLISM OF PRAZEPAM BY 9000 *g* HUMAN LIVER SUPERNATANT FRACTIONS

Subject	Age	Cause of death	Time elapsed between death and removal of liver samples (hr)	% Radioactivity present after 3-hr incubation			
				Prazepam	3-Hydroxy-prazepam	Desalkyl-prazepam	Oxazepam
Male	37	Aortic insufficiency*	14	16	0	79	5
Male	69	Cancer of prostate	6	>99	0	trace	0
Male	72	Myocardial infarction	8	>99	0	trace	0
Male	92	Perforated jejunum	12	100	0	0	0
Female	68	Cancer of breast	3	93	0	7	0
Female	81	Bronchial pneumonia	20	>99	0	trace	0
Female	75	Congestive heart failure	14	100	0	0	0
Female	53	Cancer of breast	10	100	0	0	0

*Sudden death.

Metabolism by dog liver enzymes. Incubation of ring-labeled prazepam with 9000 *g* mongrel dog liver supernatant fractions resulted in the transformation of prazepam mainly to desalkylprazepam (Table 2). Slight amounts of oxazepam and an additional unidentified metabolite were also formed; the latter gave a negative reaction with the

TABLE 2. METABOLISM OF PRAZEPAM BY 9000 *g* DOG LIVER SUPERNATANT FRACTIONS

Sex	% Radioactivity present after 1-hr incubation				
	Prazepam	3-Hydroxyprazepam	Desalkylprazepam	Oxazepam	Unknown
Male beagle	12	0	71	17	
Female beagle	5	0	88	7	
Male mongrel	17	0	62	5	16*
Female mongrel	8	0	68	10	14*

*Negative phenol test; R_f in solvents 306 and 307 identical to control rat unknown.

phenol reagent. The chromatographic behavior of this unknown metabolite in the various solvents employed was identical to that of an unknown metabolite observed in incubation mixtures containing 9000 *g* liver supernatant fractions of untreated rats. In contrast to mongrel dog liver, beagle dog liver enzymes converted prazepam principally to desalkylprazepam and small amounts of oxazepam. The amounts of oxazepam formed in both cases were comparable, but beagle dog liver enzymes formed more desalkylprazepam than mongrel dog liver enzymes. These differences between the two species may be coincidental, as only four animals were used in this study. Sidechain-labeled prazepam was not oxidized to 3-hydroxyprazepam.

Both 3-hydroxydiazepam and desalkyldiazepam were produced by incubating diazepam with the 9000 *g* supernatant fraction prepared from the liver of a female beagle dog. This finding confirms the observation reported earlier by Schwartz *et al.*[7]

Metabolism by rat liver enzymes. Table 3 lists the results of incubating ring-labeled prazepam with 9000 *g* supernatant fractions of phenobarbital-treated and control rats. The radioactivity was distributed between unchanged prazepam, desalklprazepam, oxazepam and an unidentified metabolite. The unknown metabolite isolated from control rat liver incubation mixtures was chromatographically similar to the unknown metabolite formed by mongrel dog liver enzymes, and it did not give a positive phenol test. Phenobarbital pretreatment led to an increased conversion of desalkyl-

TABLE 3. METABOLISM OF PRAZEPAM BY 9000 *g* LIVER SUPERNATANT FRACTIONS OF UNTREATED AND PHENOBARBITAL-TREATED RATS

Enzyme source	% Radioactivity present after 1-hr incubation				
	Prazepam	3-Hydroxyprazepam	Desalkylprazepam	Oxazepam	Unknown
Untreated rats	3	0	79	10	8*
Phenobarbital-treated rats	10	0	44	36	10†

*Negative phenol test; R_f in solvents 306 and 307 identical to mongrel dog unknown.
†Negative phenol test; different R_f from control rat in solvents 306 and 307.

prazepam to oxazepam and to the production of an unidentified metabolite. However, this unknown metabolite differed in its chromatographic behavior from the unknown metabolite of control animals, and could not be eluted from TLC plates for subsequent purification and spraying with the phenol reagent. Incubation of sidechain-

labeled prazepam with control or phenobarbital-treated rat liver enzymes did not produce any 3-hydroxyprazepam.

Incubation of ring-labeled drug with the microsomal pellet prepared from the livers of control rats revealed an identical profile to that observed with the 9000 *g* supernatant fraction. Incubation of the drug with the 105,000 *g* supernatant fraction demonstrated this preparation to be inactive in the conversion of prazepam to any of the metabolites observed with either the 9000 *g* supernatant fraction or the microsomal pellet. Boiled 9000 *g* supernatant fractions were inactive when incubated with prazepam.

Prazepam administration to rats 3 days prior to sacrifice did not induce the formation of hydroxylating enzymes. The profiles obtained from incubation mixtures containing liver enzymes from these treated rats were identical to those of control rats.

Incubation of diazepam with 9000 *g* supernatant fractions prepared from control rat livers produced 3-hydroxydiazepam and desalkyldiazepam. The metabolites of diazepam were separated by the two-dimensional chromatographic system AB′ described by Schwartz *et al.*[7] The R_f values obtained were 0·24 : 0·35 for diazepam, 0·09 : 0·35 for desalkyldiazepam, and 0·15 : 0·35 for 3-hydroxydiazepam. When prazepam and diazepam were incubated together with control rat liver enzymes, diazepam was metabolized to 3-hydroxydiazepam and desalkyldiazepam.

Metabolism by mouse liver enzymes. It is evident from Table 4 that control mice are unable to metabolize prazepam to the extent that occurs with control rats. Pretreatment with phenobarbital produced more than a 2-fold increase in the amount of prazepam metabolized. Small amounts of oxazepam and an unidentified metabolite were also formed. The unknown compound differed chromatographically from the unknown metabolites formed from prazepam incubated with the liver enzymes of

TABLE 4. METABOLISM OF PRAZEPAM BY 9000 *g* LIVER SUPERNATANT FRACTIONS OF UNTREATED AND PHENOBARBITAL-TREATED MICE

Enzyme source	% Radioactivity present after 1-hr incubation				
	Prazepam	3-Hydroxyprazepam	Desalkylprazepam	Oxazepam	Unknown
Untreated mice	62	0	38	0	
Phenobarbital-treated mice	26	0	60	5	9*

*Negative phenol test.

mongrel dogs and rats (control and phenobarbital-treated). Sidechain-labeled prazepam was not oxidized to 3-hydroxyprazepam with liver enzymes of either control or phenobarbital-treated animals.

DISCUSSION

Previous studies[1–3] have shown that prazepam is metabolized *in vivo* by humans and dogs mainly by two phase 1 reactions,[8] namely by *N*-dealkylation and by hydroxylation at C-3 of the 7-membered ring. The latter pathway in humans is favored to form

3-hydroxyprazepam. This hydroxylated metabolite was not detected *in vitro*, utilizing liver enzymes of humans and animals. Although prazepam has a long half-life, daily administration of the drug to rats did not induce the formation of hydroxylating enzymes. There existed the possibility that polymerized cyclopropylformaldehyde would poison the hydroxylating systems. However, 3-hydroxydiazepam was formed when diazepam was incubated with rat liver enzymes in the presence of prazepam which, presumably, yielded cyclopropylformaldehyde when dealkylated.

The absence of 3-hydroxyprazepam does not imply that this metabolite may not be formed *in vitro*. Human studies *in vivo* show that hydroxylation at the C-3 position facilitates dealkylation.[4] Consequently, this reaction may proceed so rapidly *in vitro* that oxazepam is the metabolite detected through this transformation. It is interesting to note that the incubation of diazepam with liver enzymes of untreated dogs or rats rarely produced oxazepam even in trace quantities.[9,10] On the other hand, prazepam yielded 5–17 per cent oxazepam upon treatment with human, dog and rat liver enzymes. This contrast confirms the observations made in man that the cyclopropylmethyl substituent of prazepam is readily removed after hydroxylation at the C-3 position. In brief, the data suggest that the cyclopropylmethyl group renders prazepam difficult to hydroxylate but easy to dealkylate. This view received further support from the observation that 3-hydroxydiazepam was produced when we incubated diazepam with liver enzymes of beagle dogs or rats. It does not seem unreasonable to assume that a single enzyme hydroxylates both diazepam and prazepam.

Although human, dog, rat and mouse liver enzymes were capable of forming desalkylprazepam and oxazepam, one limitation should be noted. The human enzyme system cannot be compared directly with the animal preparations because only the animal livers were obtained immediately after death. We found that the liver enzymes obtained from a subject who died suddenly were the most efficient in metabolizing prazepam. Studies by Creaven and Williams[11] showed that autopsy samples of human liver obtained 4–28 hr after death were capable of hydroxylating biphenyl to 4-hydroxybiphenyl and coumarin to 7-hydroxycoumarin. Other investigators utilizing 9000 *g* supernatant fractions prepared from liver samples obtained 2 to 5·5 hr post-mortem showed these preparations were capable of metabolizing codeine, hexobarbital and aniline.[12] Furthermore, both studies demonstrated that microsomes obtained from subjects who died suddenly were among the most effective in metabolizing any of the tested drugs. In addition, Darby *et al.*[12] showed that spectral changes occurred in microsomal preparations allowed to stand for varying periods of time.

Microsomes from phenobarbital-treated mice metabolized approximately twice as much prazepam to desalkylprazepam and also yielded small amounts of oxazepam. On the other hand, phenobarbital injections to rats increased the microsomal activity toward desalkylprazepam but tripled the formation of oxazepam. In both rodent species, pretreatment with phenobarbital led to the production of unidentified metabolites. Investigators studying the effects of phenobarbital treatment of rats upon the metabolism of diazepam found an increase in both the dealkylated and hydroxylated metabolites with a concomitant increase in the production of more polar and non-ether-extractable metabolites.[9,13] Phenobarbital treatment in mice, however, resulted in an increase in the formation of 3-hydroxydiazepam.[13]

The results obtained from several studies[9,13,14] of the metabolism of diazepam

in vitro reveal distinct differences when compared to prazepam. The primary route of prazepam metabolism *in vitro* is *N*-dealkylation, although C-3 hydroxylation is necessary for the conversion of prazepam to oxazepam. Diazepam is metabolized to an equivalent extent through *N*-dealkylation and hydroxylation at position C-3. However, in the steady state, mouse liver enzymes converted diazepam mainly to desalkyldiazepam, and rat microsomes produced 3-hydroxydiazepam.[13] Schwartz and Postma[9] showed that diazepam incubated with dog or rat liver enzymes formed equivalent amounts of desalkyldiazepam and 3-hydroxydiazepam after 1 hr. This finding is in contrast to the metabolism *in vivo* of diazepam, which involves mainly *N*-dealkylation. It is of further interest to note that prazepam was metabolized differently from diazepam in humans[7,15] and dogs.[7,16] Prazepam was first attacked in humans almost exclusively by oxidation at C-3, whereas diazepam metabolism proceeded principally through *N*-dealkylation.[15]

It is clear that prazepam biotransformation by liver enzymes *in vitro* proceeds by *N*-dealkylation and hydroxylation at carbon-3. It is not possible to conclude definitely from these studies whether oxazepam arises as a result of hydroxylation of prazepam followed by dealkylation or by dealkylation followed by hydroxylation or by both mechanisms. It is interesting to speculate about the difference between prazepam metabolism *in vivo* and *in vitro*. *In vivo*, 3-hydroxyprazepam is converted to its glucuronide, a reaction which is precluded *in vitro*. Therefore, one may be unable to observe 3-hydroxyprazepam formation *in vitro* because it is not trapped as a conjugate, but is dealkylated readily.

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