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Metabolism of Prazepam by Rat Liver Microsomes: Stereoselective Formation and *N*-Dealkylation of 3-Hydroxyprazepam

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SUMMARY

The metabolism of prazepam (7-chloro-1-(cyclopropylmethyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one) (PZ) was studied using liver microsomes prepared from untreated, phenobarbital (PB)-treated, and 3-methylcholanthrene (3MC)-treated male Sprague-Dawley rats. Relative rates of PZ metabolism by liver microsomes prepared from rats were PB-treated > untreated > 3MC-treated. Metabolites of PZ were separated by normal phase high performance liquid chromatography and the relative amounts of major metabolites were found to be *N*-desalkylprazepam (also known as *N*-desmethyldiazepam and nordiazepam) > 3-hydroxy-PZ (3-OH-PZ) > oxazepam. Enantiomers of 3-OH-PZ were resolved by high performance liquid chromatography on an analytical column packed with Pirkle's chiral stationary phase, (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine covalently bonded to spherical particles of γ-aminopropylsilanized silica. 3-OH-PZ formed in the metabolism of PZ by liver microsomes prepared from rats was found to have 3*R*/3*S* enantiomer ratios of 84:16 (untreated), 85:15 (PB-treated), and 84:16 (3MC-treated), respectively. Relative rates of *N*-dealkylation of PZ by three rat liver microsomal preparations were PB-treated > untreated > 3MC-treated. *N*-Dealkylation of 3-OH-PZ by rat liver microsomes was substrate enantioselective; the 3*S*-enantiomer was *N*-dealkylated faster than the 3*R*-enantiomer. The results indicated that both C3-hydroxylation of PZ and *N*-dealkylation of 3-OH-PZ catalyzed by rat liver microsomes were stereoselective, resulting in the formation of a 3-OH-PZ highly enriched in the 3*R*-enantiomer.

The 1,4-benzodiazepines are among the most frequently prescribed drugs and possess anxiolytic, anticonvulsant, sedative, and muscle relaxant properties. 1,4-Benzodiazepines such as DZ, HZ, and PZ (Fig. 1) undergo *N*-dealkylation, C3-hydroxylation, and benzo-ring hydroxylation reactions, which are catalyzed by cytochromes P450 in the microsomal drug-metabolizing enzyme complex (Fig. 2) (1–3). NDZ and OX are common metabolites of DZ, HZ, and PZ and are themselves therapeutically active (see review in Ref. 4).

Enzymatically catalyzed hydroxylation at C3 may be stereoselective, because it is a prochiral site. Microsomal cytochrome P450 isozymes catalyzed hydroxylation reactions at aliphatic carbons of substrates may be highly stereoselective (5, 6). NDZ and DZ may be stereoselectively hydroxylated at the C3 position to form OX and TMZ (3-hydroxy-DZ), respectively, that are enriched in either the 3*R*- or the 3*S*-enantiomer (6). However, enantiomers of OX and of TMZ are known to undergo racemization in aqueous and organic solvents (6–8). Thus, it is difficult, if not impossible, to study the stereoselective/stereospecific hydroxylation reactions by examining the enantiomeric ratios of OX and of TMZ formed in the metabolism of DZ. To circumvent this difficulty, Corbella *et al.* (6) synthesized deuterium-labeled NDZ and DZ with a deuterium atom attached to C3 at either the pro-*S* or the pro-*R* position (Fig. 1), as well as dual-labeled DZ with a ^{14}C and a ^{3}H (either pro-*S* or pro-*R*) at the C3 position. Based on the results of deuterium and tritium retentions of metabolically formed OX and TMZ, Corbella *et al.* (6) concluded that enzymatic hydroxylations at C3 of NDZ and DZ proceed through stereoselective (60–97%) removal of the pro-*S* hydrogen atom.

Because examples are known indicating that stable 3*S*-enantiomers of C3-substituted 1,4-benzodiazepines are more potent

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ABBREVIATIONS: DZ, diazepam; PZ, prazepam; 3-OH-PZ, 3-hydroxyprazepam; HZ, halazepam; OX, oxazepam; NDZ, nordiazepam (*N*-desmethyldiazepam or *N*-desalkylprazepam); LZ, lorazepam; TMZ, temazepam; LMZ, lormetazepam; CMZ, camazepam; CSP, chiral stationary phase; PB, phenobarbital; 3MC, 3-methylcholanthrene; EA7, 7% (v/v) of ethanol/acetonitrile (2:1, v/v) in hexane; D20P5, dichloroethane/2-propanol/hexane (20:5:75, v/v); HPLC, high performance liquid chromatography; (*R*)-DNBPG, (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine.

than the respective antipode in displacing [^{3}H]DZ ([^{3}H]flunitrazepam) binding to benzodiazepine receptors in rat brain (9–12), the results of Corbella *et al.* (6) may be taken as implying that cytochromes P450-catalyzed C3-hydroxylations preferentially produce the therapeutically more effective 3*S*-enantiomer. In this report, we describe the findings on the stability and CSP HPLC separation of 3-OH-PZ enantiomers and on the stereoselective C3-hydroxylation in the metabolism of PZ and the enantioselective *N*-dealkylation of 3-OH-PZ by rat liver microsomes.

Experimental Procedures

Materials

PZ [7-chloro-1-(cyclopropylmethyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one) and 3-OH-PZ were generously provided by Ms. Carol L. Germain of Warner-Lambert Company (Ann Arbor, MI). NDZ (also known as nordiazepam) was provided by Dr. Peter F. Sorter of Hoffmann-La Roche, Inc. (Nutley, NJ). OX and LZ were provided by Dr. Yvon Lefebvre of Wyeth-Ayerst Research (Princeton, NJ). TMZ was provided by Dr. William F. Westlin of Sandoz, Inc. (East Hanover, NJ). Molar extinction coefficients (in cm^{-1} M^{-1}) of these compounds (in methanol) are PZ, ϵ_{229} 30760; 3-OH-PZ, ϵ_{230} 33300; OX, ϵ_{230} 34160 (8); and NDZ, ϵ_{230} 38500. HPLC grade solvents were purchased from Mallinckrodt, Inc. (Paris, KY).

Male Sprague-Dawley rats weighing 80–100 g were treated intraperitoneally once daily for each of 4 consecutive days with either PB (75 mg/kg of body weight, injected in 0.5 ml of water) or with 3MC (25 mg/kg of body weight, injected in 0.5 ml of corn oil). Rats were sacrificed 24 hr after the last treatment. Liver microsomes were prepared as described by Alvares *et al.* (13). Microsomal protein was measured by the method of Lowry *et al.* (14) with bovine serum albumin as the protein standard.

Incubation of PZ and 3-OH-PZ with Rat Liver Microsomes

PZ (8.2 μmol in 3 ml of methanol) was incubated in a 30-ml incubation mixture containing 3 mmol of Tris-HCl (pH 7.5), 0.18 mmol of $MgCl_2$, 60 mg of protein equivalent of rat liver microsomes, 6 NADP units of glucose-6-phosphate dehydrogenase (Type XIV; Sigma Chemical Co., St. Louis, MO), 5.8 mg of $NADP^+$, and 0.12 mmol of glucose-6-phosphate. Before the addition of PZ, the reaction mixture was preincubated at 37° for 2 min in a water shaker bath. After 30 min of incubation following the addition of PZ, the reaction was stopped by the addition of 30 ml of acetone. Unmetabolized PZ and its metabolites were extracted by the addition of 60 ml of ethyl acetate or 30 ml of chloroform. The organic phase was removed by low speed centrifugation and was evaporated to dryness under reduced pressure at ~40°. When most of the organic solvent was evaporated, acetone was added to remove the residual water. It is important to point that the anhydrous $MgSO_4$, commonly used to remove residual water in an organic solvent extract, adsorbs NDZ and should not be used as a drying agent. The dried residue was redissolved in approximately 150 μl of EA7 within 1 min before separation of metabolites by either normal phase or CSP HPLC.

A time-dependent study was performed with a 60-ml incubation mixture, with 2 mg of protein equivalent of liver microsomes from PB-treated rats per ml of incubation mixture and other cofactors as described above. During the incubation, 8-ml aliquots were taken every 10 min into test tubes containing 8 ml of ice-cold acetone, followed by 8 ml of chloroform. Extraction and CSP HPLC analyses were performed as described above.

For the purpose of comparing rates of metabolic formation of OX in the metabolism of 3-OH-PZ and NDZ, incubations were carried out in 3-ml reaction mixtures at 37° for 15 min. Each milliliter of incubation mixture contained 150 nmol of substrate (3-OH-PZ or NDZ, in 50 μl of methanol) and 1 mg of protein equivalent of liver microsomes from PB-treated rats. Acetone (3 ml) was used to stop the reaction and chloroform (3 ml) was subsequently added to extract the unmetabolized 3-OH-PZ (or NDZ) and metabolites, which were analyzed by normal phase HPLC. An internal standard (TMZ, 60 nmol in 80 ml of methanol) for chromatography was added following the addition of acetone.

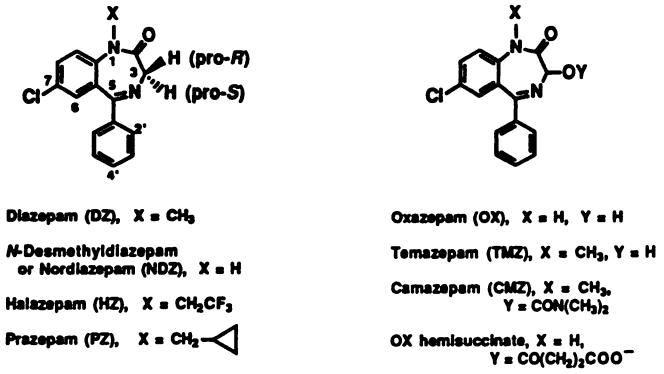

Fig. 1. Structures and abbreviations of some 1,4-benzodiazepines.

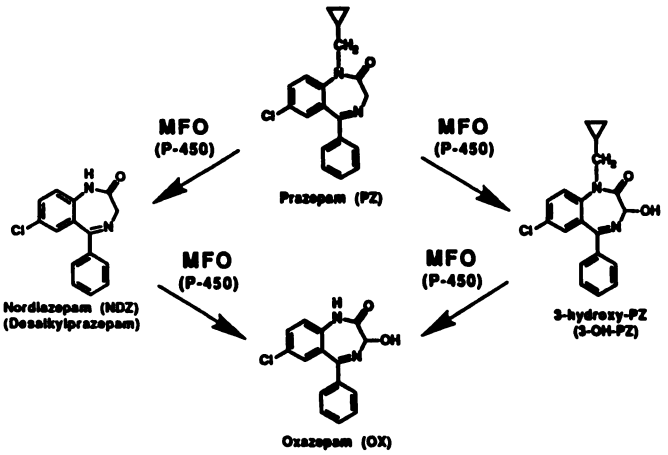

Fig. 2. Major pathways of PZ metabolism by rat liver microsomes (1–3).

To study the substrate-enantioselective *N*-dealkylation of 3-OH-PZ, (0.88 μmol per ml of incubation mixture), 2 mg of protein equivalent of liver microsomes were used and the incubation time was 30 min. Anhydrous $MgSO_4$ was used to remove residual water in the extraction of metabolites by acetone/chloroform. The removal of residual water by using anhydrous $MgSO_4$ reduced the possibility of racemization, allowing a more accurate determination of the enantiomeric ratio of unmetabolized 3-OH-PZ.

HPLC

HPLC was performed using a Water Associates (Milford, MA) liquid chromatograph, consisting of a Model 6000A solvent delivery system, a Model M45 solvent delivery system, a Model 660 solvent programmer, and a Kratos Analytical Instruments (Ramsey, NJ) Model Spectraflow 757 UV-VIS variable wavelength detector. Samples were injected via a Valco Model N60 loop injector (Valco Instruments, Houston, TX). Retention times and areas under chromatographic peaks were determined with a Hewlett-Packard Model 3390A integrator.

Normal phase HPLC. Normal phase HPLC was carried out on a Zorbax SIL column (6.2 mm i.d. × 25 cm; Dupont Co.). Eluent (2 ml/min) used for separation of PZ metabolites was EA7. The solvent of the fraction containing 3-OH-PZ, collected by normal phase HPLC, was evaporated to dryness by a stream of nitrogen in a water bath (~30°). Enantiomeric composition of 3-OH-PZ was determined by CSP HPLC as described below.

CSP HPLC. Enantiomeric pairs of OX, LZ, TMZ, LMZ, and 3-

OH-PZ were separated on a CSP column (4.6 or 10 mm i.d. × 25 cm; Regis Chemical Company, Morton Grove, IL) packed with spherical particles of 5-μm diameter of γ-aminopropylsilanized silica to which (*R*)-DNBPG was bonded covalently. The mobile phase was either EA7 or D20P5 at 2 ml/min. The solvents of the fractions containing 3-OH-PZ enantiomers, collected by CSP HPLC, were evaporated to dryness by a stream of nitrogen in a water bath (~30°). Evaporation in EA7 causes 1–2% of enantiomeric 3-OH-PZ to undergo racemization. No significant racemization was observed when D20P5 was used as the solvent. The retention times of the compounds varied (0.1–0.3 min) from time to time, even when identical mobile phases and columns were used.

A mixture of substrate (either PZ or 3-OH-PZ) and metabolites, obtained by organic solvent (acetone/ethyl acetate or acetone/chloroform) extraction of the *in vitro* incubation mixtures, was also directly analyzed by CSP HPLC. This analytical procedure eliminates the need for isolation of 3-OH-PZ by normal phase HPLC, thereby reducing the possibility of racemization, which can alter the enantiomeric composition of metabolically formed 3-OH-PZ.

Kinetics of Racemization

Changes in ellipticity ($\Delta\Phi$, in millidegrees) of enantiomeric 3-OH-PZ (0.6 to 1.2 A_{229}/ml) were recorded at 255 or 325 nm (at or near the peak of a Cotton effect), as a function of time, in a thermostated quartz cuvette of 1-cm path length (8). Ellipticity at 370 nm was used as the baseline for a completely racemized sample. The half-life of racemization ($t_{1/2}$) was determined by plotting log ($\Delta\Phi$) versus time. Whenever changes in ellipticity can be monitored, racemization of enantiomeric 3-OH-PZ is found to follow first-order kinetics in all solvents studied. The rate constant of racemization (k) can be calculated by $k = 0.693/t_{1/2}$. Each of the 3-OH-PZ enantiomers was dissolved in EA7, D20P5, acetone, ethyl acetate, chloroform, diethyl ether, or 0.1 M Tris-HCl (pH 7.5) for the determination of racemization $t_{1/2}$.

For an enantiomer that undergoes racemization, the percentage difference of two enantiomers between time t_1 and t_2 (assuming $t_2 > t_1$) can be calculated by Eqs. Ia or Ib.

$$P_{R,t_2} - P_{S,t_2} = (P_{R,t_1} - P_{S,t_1})\, e^{-k\,(t_2 - t_1)} = (P_{R,t_1} - P_{S,t_1})\, e^{-0.693\,(t_2 - t_1)/t_{1/2}} \quad \text{(Ia)}$$

or

$$P_{R,t_1} - P_{S,t_1} = (P_{r,t_2} - P_{S,t_2})\, e^{k(t_2 - t_1)} = (P_{R,t_2} - P_{S,t_2})\, e^{0.693\,(t_2 - t_1)/t_{1/2}} \quad \text{(Ib)}$$

where P_{R,t_1} = percentage of *R*-enantiomer at time t_1, P_{S,t_1} = percentage of *S*-enantiomer at time t_1, P_{R,t_2} = percentage of *R*-enantiomer at time t_2, and P_{S,t_2} = percentage of *S*-enantiomer at time t_2.

$$P_{R,t_1} + P_{S,t_1} = 100 \quad \text{(Ic)}$$

and

$$P_{R,t_2} + P_{S,t_2} = 100 \quad \text{(Id)}$$

Percentages of *R*- and *S*-enantiomers at t_1 and t_2 can be calculated by Eqs. Ib and Ic or Ia and Id, respectively.

Two examples are given below for 3-OH-PZ enantiomers in 0.1 M Tris-HCl (pH 7.5) at 37° ($k = 0.0077$ min^{-1} and $t_{1/2} = 90$ min).

Example 1. Based on Eqs. Ia and Id, an optically pure (3*R*)-OH-PZ will result in a 3*R*/3*S* enantiomer ratio of 75:25 in 90 min (one $t_{1/2}$).

Example 2. A 3-OH-PZ of unknown enantiomer ratio results in an enantiomer ratio of 80:20 in 15 min. Based on Eqs. Ib and Ic, the original 3-OH-PZ is calculated to have an enantiomer ratio of 83.7:16.3. Thus, a 3-OH-PZ with a 3*R*/3*S* enantiomer ratio of 83.7:16.3 will change to 80:20 in 15 min in 0.1 M Tris-HCl (pH 7.5) at 37°.

Spectral Analysis

UV-visible absorption spectra of samples were determined using a 1-cm path length quartz cuvette with a Varian Model Cary 118C spectrophotometer. CD spectra of samples in a thermostated quartz cell of 1-cm path length at either room temperature (23°) or 37° were measured using a Jasco Model 500A spectropolarimeter equipped with a Model DP500 data processor. The concentration of the sample is indicated by A_λ ml (absorbance units at wavelength λ_2/ml of solvent). CD spectra are expressed by ellipticity ($\Phi_{\lambda_2}/A_{\lambda_2}$, in millidegrees) for solutions that have an absorbance of A_{λ_2}/ml of solvent at wavelength λ_2 (usually the wavelength of maximal absorption). Under conditions of the measurements indicated above, the molar ellipticity ($[\theta]_{\lambda_1}$, in degree cm^2 dmol^{-1}) and ellipticity ($\Phi_{\lambda_1}/A_{\lambda_2}$, in millidegrees) are related to the molar extinction coefficient (ϵ_{λ_2}, in cm^{-1} M^{-1}) as follows:

$$[\theta]_{\lambda_1} = 0.1\ \epsilon_{\lambda_2}\ (\Phi_{\lambda_1}/A_{\lambda_2})$$

It is apparent from the equation above that molar ellipticity $[\theta]$ of an enantiomer (or diastereomer) must be reported along with its molar extinction coefficient. Unfortunately, molar ellipticities of enantiomers/diastereomers are often reported in the literature in the absence of molar extinction coefficients. Molar extinction coefficients, in a specified solvent, of most chemicals are either not available or difficult to determine accurately. For the purpose of comparing data obtained in different laboratories, we strongly urge investigators to express CD spectral data by ellipticity for a solution of 1.0 absorbance unit at a specified wavelength per ml of solvent, instead of molar ellipticity $[\theta]$.

Results

Effects of Enzyme Inducers on PZ Metabolism. Metabolites of PZ were separated by normal phase HPLC (Fig. 3). The enzymatically formed NDZ, 3-OH-PZ, and OX were identical to the authentic compounds with respect to retention times on both normal phase and CSP HPLC and UV absorption spectra. We chose normal phase HPLC rather than reverse phase HPLC to separate the metabolites because the solvents of collected fractions can be readily evaporated. Due to rela-

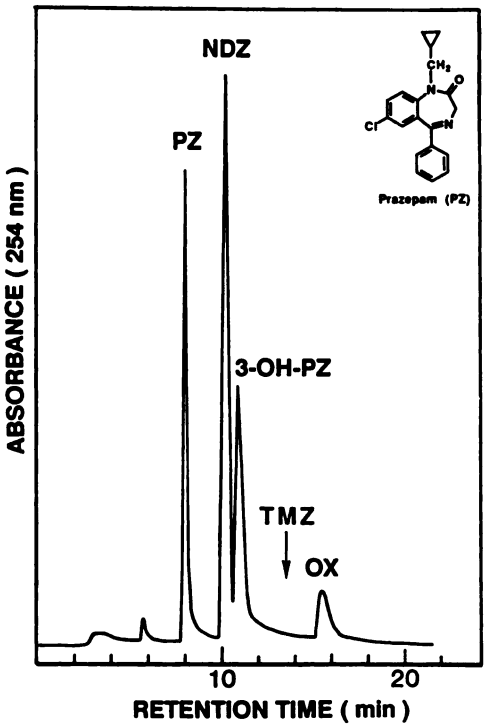

Fig. 3. Normal phase HPLC separation of metabolites formed in the metabolism of PZ by liver microsomes from PB-treated rats. A DuPont Zorbax SIL column (250 mm × 6.2 mm i.d.) was used and the eluent was EA7 at 2 ml/min. Retention time of TMZ is indicated by an *arrow*.

tively fast racemization of enantiomeric 3-OH-PZ (and other 3-hydroxy-1,4-benzodiazepines as well) in aqueous solutions, normal phase HPLC is preferred over reverse phase HPLC in order to preserve the enantiomeric composition of enzymatically formed 3-OH-PZ.

Based on a per milligram of protein basis, the rates of PZ metabolism and distribution of metabolites vary with the different rat liver microsomal preparations (Experiment 1 in Table 1). Liver microsomes from PB-treated rats had the highest activity in catalyzing the metabolism of PZ and liver microsomes from 3MC-treated rats were the least active. The relative amounts of metabolites formed by all three rat liver microsomal preparations were NDZ > 3-OH-PZ ≫ OX. The distribution of PZ metabolites observed in this study is similar to that reported by Matsumoto *et al.* (3), who used a similar substrate concentration of PZ (0.3–0.62 μM) and liver microsomes from untreated rats.

Separation and stability of 3-OH-PZ enantiomers. The enantiomers of 3-OH-PZ (authentic compound) were efficiently separated by CSP HPLC (α = 1.12 and R_s = 1.40) by using eluent EA7 and a covalently bonded (*R*)-DNBPG column (Fig. 4, *left*). Resolved enantiomers have identical UV absorption spectra and their CD spectra are mirror images of each other (Fig. 5). The signs of CD Cotton effects of 3-OH-PZ enantiomers (Fig. 5) are consistent with those of enantiomeric C3-substituted derivatives of NDZ and DZ reported by Corbella *et al.* (6). Both enantiomers of 3-OH-PZ have a racemization $t_{1/2}$ of 570 ± 40 min (three experiments) in EA7 at room temperature. The enantiomers of 3-OH-PZ in EA7 are considerably more stable than those of OX (racemization $t_{1/2}$ = 28 min) (8), LZ, LMZ, and TMZ (Table 2). Enantiomers of 3-OH-PZ were more efficiently separated (α = 1.35 and R_s = 3.0) by using D20P5 as eluent on a covalently bonded (*R*)-DNBPG column. They are fairly stable in D20P5 ($t_{1/2}$ > 5000 min). Because of their relative stability, the 3-OH-PZ enantiomers were isolated on a semipreparative column (25 cm × 10 mm i.d.) with D20P5 as the mobile phase and, after solvent evaporation, were used in the determination of racemization $t_{1/2}$ in other solvents. Racemization $t_{1/2}$ values of 3-OH-PZ enantiomers were determined in solvents that were used either in HPLC or in metabolite extraction (Table 2). The results in Table 2 indicate that enantiomers of 3-OH-PZ are fairly stable in several organic solvents and are relatively less stable in an aqueous medium. However, enantiomers of 3-OH-PZ are much more stable than those of OX, LZ, LMZ, and TMZ in either EA7 or 0.1 M Tris-HCl (pH 7.5) (see $t_{1/2}$ in Table 2).

TABLE 1

Effects of enzyme inducers on the metabolism of PZ, 3-OH-PZ, and NDZ by rat liver microsomes

Substrate (Rat liver microsomes)[a]	Substrate metabolized	NDZ	3-OH-PZ	OX
	%	% of all metabolites formed		
Expt. 1				
PZ (control)	38.6	55.7[b]	39.1[b]	5.2[b]
PZ (PB)	73.8	55.1[b]	34.3[b]	10.6[b]
PZ (3MC)	33.5	53.3[b]	42.5[b]	4.2[b]
Expt. 2				
NDZ (control)	11.7 ± 1.5			0.25 ± 0.1[c]
3-OH-PZ (control)	32.7 ± 0.4			0.84 ± 0.02[c]
NDZ (PB)	48.5 ± 1.5			0.33 ± 0.02[c]
3-OH-PZ (PB)	67.0 ± 2.0			0.65 ± 0.01[c]
NDZ (3MC)	3.5 ± 0.5			0.14 ± 0.01[c]
3-OH-PZ (3MC)	21.2 ± 1.0			0.59 ± 0.06[c]

[a] Metabolites were analyzed as described in Fig. 4. In Experiment 1, each milliliter of incubation mixture contains 2 mg of protein equivalent of liver microsomes and was incubated for 30 min. In Experiment 2, each milliliter of incubation mixture contains 1 mg of protein equivalent of liver microsomes and was incubated for 15 min; TMZ was used as an internal standard.

[b] Percentage of all metabolites formed, measured by area under the curve at 254 nm.

[c] Area ratio of OX and internal standard TMZ.

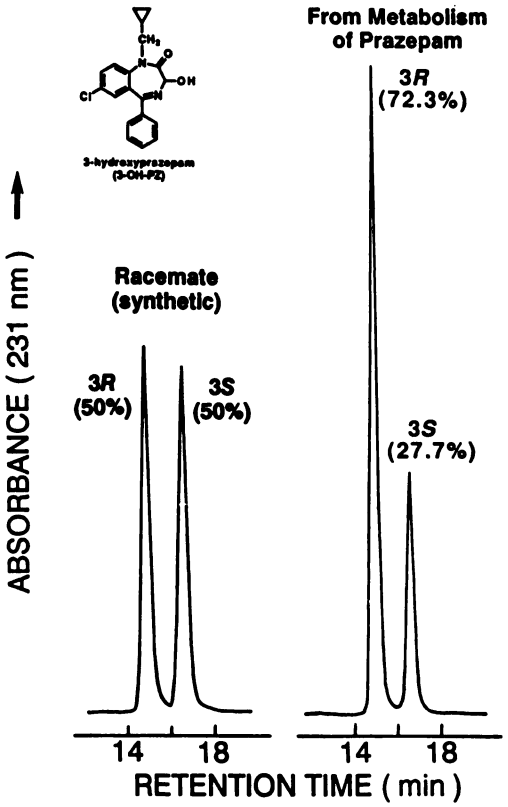

Fig. 4. CSP HPLC separation of 3-OH-PZ enantiomers. *Left*, authentic 3-OH-PZ. *Right*, 3-OH-PZ formed in the metabolism of PZ by liver microsomes from PB-treated rats. A covalently bonded (*R*)-DNBPG column was used and the eluent was EA7 at 2 ml/min.

Stereoselective hydroxylation of PZ. A racemization $t_{1/2}$ of 90 min at 37° in 0.1 M Tris-HCl (pH 7.5) indicates that an optically pure 3*R*- (or 3*S*-) enantiomer will result in a 3*R*/3*S* (or 3*S*/3*R*) enantiomer ratio of 94.55:5.45 in 15 min and 75:25 in 90 min (see Eqs. Ia–Id in Experimental Procedures). Thus, if a 3*R*/3*S* (or 3*S*/3*R*) enantiomer ratio of 3-OH-PZ formed in the metabolism of PZ were determined by CSP HPLC, it would then be possible to calculate/estimate the degree of pro-*R* (or pro-*S*) stereoselective C3-hydroxylation catalyzed by the enzyme preparation.

The enantiomeric composition of 3-OH-PZ formed in the metabolism of PZ by rat liver microsomes from PB-treated rats was determined by CSP HPLC (e.g., Fig. 4, *right*). When acetone and ethyl acetate were used in the extraction procedure, 3-OH-PZ formed in the metabolism of PZ by liver microsomes prepared from rats was found to have 3*R*/3*S* ratios of 70.1:29.9 (untreated), 72.3:27.7 (PB-treated), and 61.3:38.7 (3MC-treated), respectively (Table 3). In these experiments, each milliliter of incubation mixture contained 2 mg of protein equivalent of rat liver microsomes and the incubation was carried out at 37° for 30 min. Metabolites extracted using acetone/ethyl acetate were first isolated by normal phase HPLC (Fig. 3) and the enantiomeric composition of 3-OH-PZ

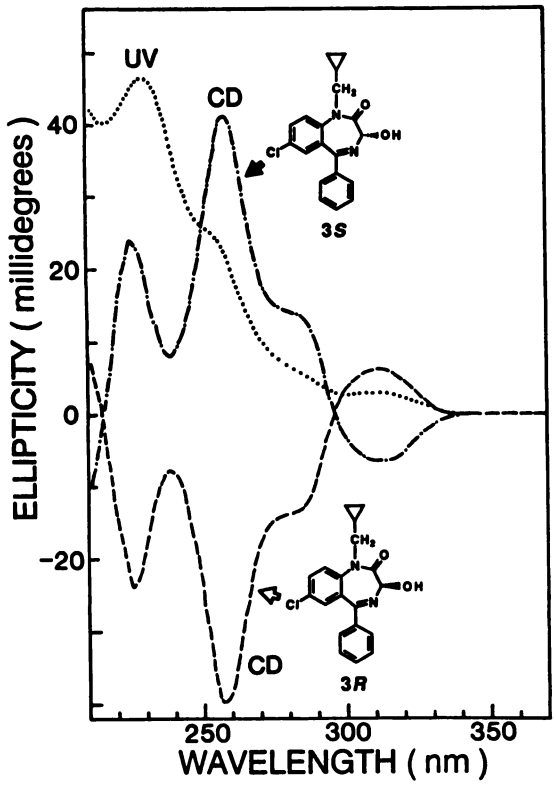

Fig. 5. UV absorption spectrum (· · · · ·) and CD spectra of 3-OH-PZ enantiomers [3*S* (–·–·–), 3*R*/3*S* ≥ 99, concentration, A_{229}/ml = 1.0 in EA7, Φ_{256} = 41.4 millidegree, and 3*R* (——), 3*R*/3*S* ≥ 50, concentration, A_{229}/ml = 1.0 in EA7, Φ_{256} = 40.0 millidegree].

was subsequently determined by CSP HPLC (Fig. 4, *right*). Due to loss of metabolite(s), we have avoided the use of anhydrous $MgSO_4$ to remove the aqueous phase present in the organic solvent extract. Consequently, a small amount of aqueous solution is present when acetone and ethyl acetate are evaporated. This residual aqueous solution was evaporated by the addition of acetone (~0.5 ml at a time for three to five times). The need for removing residual aqueous phase at ~40° has apparently caused racemization of the metabolically formed 3-OH-PZ, whose actual 3*R*/3*S* ratio might be much higher than what was found (Table 3).

Because chloroform is more efficient in metabolite extraction and less hygroscopic than ethyl acetate, the same experiment was repeated using acetone/chloroform in the extraction procedure. Much less acetone was needed to co-evaporate the residual aqueous solution present in the acetone/chloroform extract than when acetone/ethyl acetate was used. Furthermore, the dried metabolite extract was directly analyzed on CSP HPLC (Fig. 6). Under these experimental conditions, the metabolically formed 3-OH-PZ was found to be more highly enriched in the 3*R*-enantiomer (Table 3).

A time-dependent experiment was also performed using liver microsomes from PB-treated rats in the *in vitro* incubations. In this experiment, acetone and chloroform were used in the extraction. Metabolites found were NDZ > 3-OH-PZ ≫ OX (Fig. 7), similar to the results shown in Table 1. The 3*R*/3*S* ratios of 3-OH-PZ formed in PZ metabolism are time dependent (Fig. 7); the percentage of 3*R*-enantiomer is progressively lower as the incubation time increases. Extrapolation of the enantiomeric ratios of 3-OH-PZ to "zero time" gave a 3*R*/3*S* ratio

TABLE 2

Racemization half-lifes of enantiomeric 3-OH-PZ and OX in various solvents

Enantiomer	Solvent	Temperature	$t_{1/2}$[a]	k[b]
			min	$\times 10^3$ *min*$^{-1}$
3*R*-OH-PZ	0.1 M Tris·HCl, pH 7.5	23°	515	1.35
3*R*-OH-PZ	0.1 M Tris·HCl, pH 7.5	37°	90	7.70
3*S*-OH-PZ	0.1 M Tris·HCl, pH 7.5	37°	90	7.70
3*R*-OX	0.1 M Tris·HCl, pH 7.5	37°	~3.0	~231
3*R*-TMZ	0.1 M Tris·HCl, pH 7.5	37°	~3.4	~204
3*R*-LZ	0.1 M Tris·HCl, pH 7.5	37°	~1.3	~533
3*R*-LMZ	0.1 M Tris·HCl, pH 7.5	37°	~0.7	~990
3*R*-OX[c]	EA7	23°	28	2.48
3*R*-LZ	EA7	23°	26	2.67
3*R*-TMZ	EA7	23°	230	0.30
3*R*-LMZ	EA7	23°	457	0.15
3*R*-OH-PZ	EA7	23°	570	1.21
3*R*-OH-PZ	D20P5	23°	>5000[d]	<0.14
3*R*-OH-PZ	Acetone	23°	>5000[d]	<0.14
3*R*-OH-PZ	Ethyl acetate	23°	>5000[d]	<0.14
3*R*-OH-PZ	Chloroform	23°	>5000[d]	<0.14
3*R*-OH-PZ	Diethyl ether	23°	>5000[d]	<0.14
3*R*-OH-PZ	Acetone/diethyl ether (1:2)	23°	>5000[d]	<0.14

[a] Determined as previously described (8).
[b] Rate constant of racemization.
[c] From Ref. 8.
[d] No detectable change in ellipticity for 3–6 hr of monitoring.

TABLE 3

Effects of enzyme inducers and extraction solvents on the enantiomeric compositions of 3-OH-PZ formed in the metabolism of PZ by rat liver microsomes

Rat liver microsomes (time of incubation)	3*R*/3*S* Ratio of 3-OH-PZ determined by CSP HPLC[a]		
	Acetone/ethyl acetate	Acetone/chloroform	Extrapolated value[b]
Control (30 min)	70.1:29.9[c]	80.1:19.9	83.8:16.2
PB (30 min)	72.3:27.7[c]	81.1:18.9	84.9:15.1
PB (10 min)		82.4:17.6	85.0:15.0
3MC (30 min)	61.3:38.7[c]	80.1:19.1	83.8:16.2
PB (10 min)[d]		82.4:17.6	85.0:15.0
PB (20 min)[d]		81.7:18.3	87.0:13.0
PB (30 min)[d]		79.3:20.7	86.9:13.1
PB (40 min)[d]		78.7:21.3	89.0:11.0
PB (50 min)[d]		76.9:23.1	89.5:10.5
PB (60 min)[d]		73.2:26.8	86.8:13.2

[a] Determined as described in Fig. 4. Incubation was carried out at 37° for 30 min with 2 mg of protein equivalent of rat liver microsomes. Volumes of organic solvents used in the extraction of metabolites are described in Experimental Procedures.
[b] Stereoselectivity of rat liver cytochrome P-450 isozymes after extrapolation of the values in the left column to zero time, a measure of stereoheterotopic enzyme-substrate interaction. See Eqs. Ia–Id in Experimental Procedures.
[c] 3-OH-PZ was first isolated by normal phase HPLC (Fig. 3) before its enantiomeric composition was determined by CSP HPLC (Fig. 4).
[d] 3*R*/3*S* enantiomer ratio as a function of incubation time (see also Fig. 7).

of approximately 85:15, which is a measure of stereoheterotopic (selective toward one of two stereochemically different faces) interaction between enzyme and substrate, 85% pro-*R* and 15% pro-*S*.

Rates of OX formation in the metabolisms of 3-OH-PZ and NDZ. A significant amount of OX is formed in the

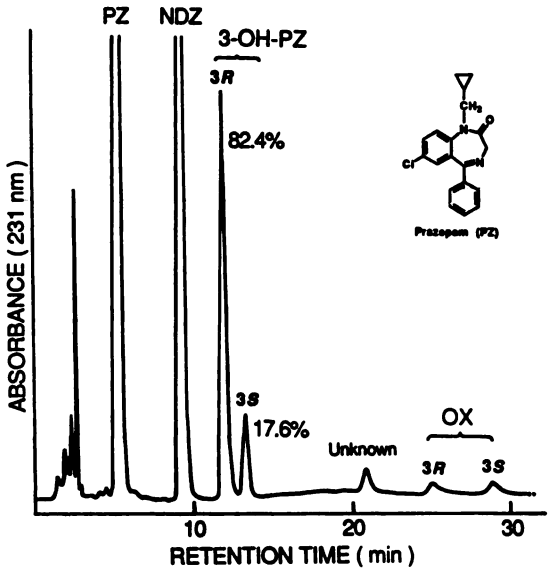

Fig. 6. CSP HPLC analysis of PZ metabolites. A mixture of metabolites, extracted from the incubation mixture after 10 min of incubation with liver microsomes from PB-treated rats, was analyzed on a covalently bonded (*R*)-DNBPG column (see also Fig. 4).

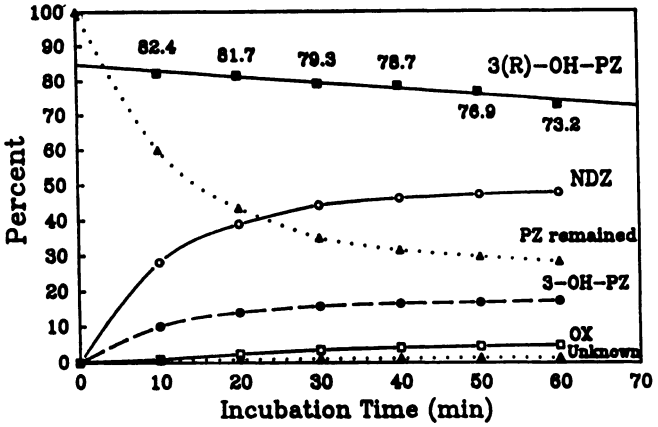

Fig. 7. Time-dependent formation of metabolites (NDZ, 3-OH-PZ, OX, and an unknown) in the metabolism of PZ by liver microsomes from PB-treated rats. Areas under the curves of the metabolites were normalized against that of an internal standard (TMZ) and hence are relative. Metabolites were analyzed as described in Fig. 3. Percentages of the 3*R*-enantiomer of the 3-OH-PZ formed were determined by CSP HPLC (see Fig. 6).

metabolism of PZ (Table 1). OX may be derived either by dealkylation of 3-OH-PZ or by C3-hydroxylation of NDZ (Fig. 2). It is possible that a substrate-enantioselective *N*-dealkylation of 3-OH-PZ also contributes to the 3*R*/3*S* enantiomer ratios of 3-OH-PZ found (Table 3 and Fig. 7). 3*R*/3*S* ratios may be significantly altered if 3-OH-PZ is enantioselectively metabolized to form OX. Thus, it is important to determine the relative contribution of the two metabolic precursors, NDZ and 3-OH-PZ, in the formation of OX.

An experiment was carried out (Table 1) to compare the relative rates for the formation of OX in the metabolism of 3-OH-PZ and NDZ by liver microsomes from untreated and treated rats. Metabolites were analyzed by normal phase HPLC (Fig. 3). In this experiment, TMZ was used as an internal standard in order to assess the relative amount of OX formed and results are shown in Table 1 (Experiment 2). *N*-Dealkylation of 3-OH-PZ by liver microsomes from untreated, PB-treated, and 3MC-treated rats is approximately 3.2-fold, 2-fold, and 4.2-fold faster than the C3-hydroxylation of NDZ, respectively. It is apparent that the majority of OX formed in the metabolism of PZ was derived by *N*-dealkylation of 3-OH-PZ rather than by C3-hydroxylation of NDZ.

Enantioselective *N*-Dealkylation of 3-OH-PZ. The possibility of substrate-enantioselective *N*-dealkylation of any 3-hydroxy-1,4-benzodiazepine has not been studied heretofore. The simple and sensitive CSP HPLC method used in the separation of 3-OH-PZ enantiomers (Figs. 4 and 6) and the relative stability (against racemization) of 3-OH-PZ enantiomers have allowed us to study the *N*-dealkylation reaction of racemic 3-OH-PZ by rat liver microsomes. Under experimental conditions in which greater than 40% of the 3-OH-PZ substrate is *N*-dealkylated, the enantiomeric composition of the remaining substrate (3-OH-PZ) has 3*R*/3*S* enantiomeric ratios of 52:48 (liver microsomes from untreated rats), 55:45 (liver microsomes from PB-treated rats; Fig. 8), and 52:48 (liver microsomes from 3MC-treated rats), respectively. These results indicate that rat liver microsomal cytochrome P450-catalyzed *N*-dealkylation of 3-OH-PZ is substrate enantioselective; the *N*-dealkylation of the 3*S*-enantiomer is slightly favored over that of 3*R*-enantiomer. To date, this is the first example indicating that the *N*-dealkylation reaction of a 3-hydroxybenzodiazepine is substrate enantioselective. Based on the relative amounts of 3-OH-PZ and OX formed in the metabolism of PZ and the relative rates of OX formation in the metabolism of NDZ and 3-OH-PZ (Table 1), it appears that the 3*S*-enantioselective *N*-dealkylation of 3-OH-PZ does not significantly alter the enantiomeric composition of 3-OH-PZ formed in the metabolism of PZ.

Discussion

This study has extended the usefulness of the method reported earlier (8) in the determination of racemization $t_{1/2}$ of enantiomeric 3-hydroxybenzodiazepines that are separated by CSP HPLC. The short racemization half-lives of enantiomeric OX, LZ, TMZ, and LMZ in pH 7.5 solutions (Table 2) indicated

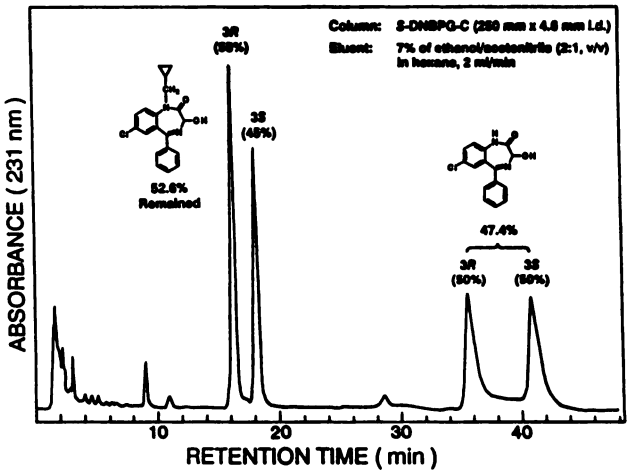

Fig. 8. Substrate-enantioselective *N*-dealkylation of racemic 3-OH-PZ by rat liver microsomes. Products formed in the metabolism of racemic 3-OH-PZ by liver microsomes from PB-treated rats were analyzed by CSP HPLC. Chromatographic conditions are the same as those described in Fig. 6.

that, using CSP HPLC, it was not possible to directly determine the enantiomer ratio of OX, LZ, TMZ, or LMZ formed in the metabolism of the respective parent compound. On the other hand, a $t_{1/4}$ of 90 min for 3-OH-PZ enantiomers in a pH 7.5 solution indicated a sufficient enantiomeric stability to allow the *in vitro* detection of an enzyme-catalyzed stereoselective C3-hydroxylation reaction product of PZ. Other enantiomeric 3-hydroxybenzodiazepines may also be sufficiently stable to allow the study of their stereoselective formations from the respective parent compounds by the methods described in this report.

Cytochrome P450-catalyzed C3-hydroxylation of PZ in rat liver microsomes is highly pro-*R* stereoselective; i.e., the pro-*R* hydrogen at C3 is preferentially removed in oxidative metabolism. Pretreatment of rats with either 3MC or PB alters the metabolizing enzyme activity but has little effect on the stereoselectivity of liver microsomes. It appears that all rat liver cytochrome P450 isozymes have similar stereoselectivity in the C3-hydroxylation of PZ. The pro-*R*-stereoselective C3-hydroxylation of PZ described in this report is in contrast to the pro-*S*-stereoselective C3-hydroxylation of DZ and NDZ reported by Corbella *et al.* (6).

The *N*-dealkylation of 3-OH-PZ is substrate enantioselective; (3*S*)-OH-PZ is dealkylated slightly faster than (3*R*)-OH-PZ. Stereoselective removal of an enantiomeric 3-OH-PZ may also proceed through a stereoselective *O*-glucuronidation reaction, which has been demonstrated in the glucuronidation of OX (15). A substrate-enantioselective *N*-dealkylation reaction was also reported in the metabolism of propranolol; (*R*)-propranolol is *N*-dealkylated faster than (*S*)-propranolol by liver microsomal preparations from dogs and humans (16) and rats (17).

The first enantiomeric pair of a benzodiazepine (Fig. 9) containing an asymmetric carbon atom at the C3 position, shown to differ substantially in their potency in displacing [^{3}H]DZ binding to synaptosomal preparations from rat cerebral cortex, was reported by Möhler and Okada (9). Competition for the receptor by various benzodiazepines closely parallels their pharmacological potency (9). The (+)-enantiomer of Ro 11-6896 [5-(*o*-fluorophenyl)-1,3-dihydro-1,3-dimethyl-7-nitro-2*H*-1,4-benzodiazepin-2-one; Fig. 3] is ~220-fold more potent in displacing [^{3}H]DZ binding to benzodiazepine receptors than the (−)-enantiomer (Ro 11-6893; Fig. 9) in synaptosomal preparations from rat cerebral cortex (9). Ro 11-6896 was found to enhance muscimol-stimulated $^{36}Cl^-$ uptake into rat cerebral cortical synaptoneurosomes, whereas the Ro 11-6893 enantiomer is inactive (10). The (+)-enantiomers of OX hemissuccinate (11) and CMZ (12) were also found to have higher potency in displacing [^{3}H]DZ (or [^{3}H]flunitrazepam) binding to benzodiazepine receptors than the (−)-enantiomers in synaptosomal preparations from rat cerebral cortex. Clinically used chiral benzodiazepines such as OX, LZ, TMZ, LMZ, and CMZ are racemic mixtures. At the present time, enantiomeric 1,4-benzodiazepines have not been used in therapy and their pharmacokinetic properties have not been evaluated.

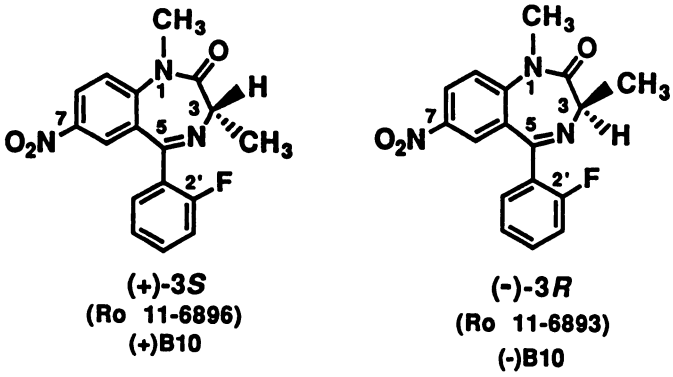

Fig. 9. Structures of (+)-B10 and (−)-B10 used in the study of Möhler and Okada (9).

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