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SHORT COMMUNICATIONS

Comparative metabolism of clinically important precursors of *N*-desmethyldiazepam using phenobarbitone-pretreated rat liver microsomes

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Abstract—Phenobarbitone-pretreated male Sprague–Dawley rat liver microsomes were used to examine C_3-hydroxylation and N-dealkylation of four clinically important benzodiazepines: diazepam (DZP), prazepam (PZP), pinazepam (PIN) and halazepam (HZP). These substrates differ only in the nature of the N-substituent of the B ring and *N*-desmethyldiazepam (DMD) is the N-dealkylation product in each case. C_3-Hydroxylation was accordingly also studied with DMD as substrate. All monooxygenations were studied with substrates at a concentration of 10 μM, in the absence of solubilizing agents, and under conditions where the production of secondary metabolites was minimized. A 20-fold variation in the rate of C_3-hydroxylation was recorded across the five substrates with HZP showing the highest rate and DMD showing the lowest rate. An almost equally large range of variation was shown for the N-dealkylation reaction, with PZP undergoing this biotransformation more than 17 times faster than DZP. Log *P* values (a measure of lipophilicity) for the five substrates were determined using an HPLC method and a remarkable lack of correspondence between this substrate parameter and either of the monooxygenations was noted. This suggests that multiple substrate determinants govern the relative rates of these monooxygenations. It was, however, notable that the additive rate of metabolism of these substrates by both monooxygenase routes did show an excellent correlation with substrate lipophilicity.

At least eight of the 1,4-benzodiazepines which are, or have been, in clinical use yield a common metabolite, *N*-desmethyldiazepam (DMD*). Four of these (diazepam, DZP; halazepam, HZP; pinazepam, PIN; and prazepam, PZP) differ only in the nature of the substituent at N_1 (see Table 1); all are oxidized metabolically at C_3 in addition to being N-dealkylated. Despite their long and widespread clinical use and their extensive study, many aspects of the metabolic disposition of these compounds remain poorly understood. The metabolism of DZP has been recently reviewed [1]. Our review of the literature has shown a fragmentary and confused picture. Some of the conclusions have been based on *in vivo* studies in humans or animals, but authors have failed to appreciate that, for example, the finding of a higher plasma AUC for DMD than for DZP following a dose of the latter does not imply that DMD is a quantitatively major excretion product of DZP. Few *in vivo* studies have quantified urinary metabolites. Other workers have used *in vitro* methods, in which methodological shortcomings abound, and attempts at correlating their results with the findings of *in vivo* studies have been inadequate, largely as a consequence of the confused picture from the latter. Although the metabolic dispositions of HZP [2], PIN [3] and PZP [4] have each been compared to that of DZP, and a comparative study of the metabolism of HZP and PZP was recently reported [5], no detailed, internally consistent study of the comparative metabolism of all members of this interesting series of compounds has appeared.

We have recently studied aspects of the *in vitro* metabolism of these benzodiazepines, using human [6] or rat liver microsomes [7, 8]. We have addressed a number of methodological problems attaching to such studies, foremost among which are the low water solubilities of the substrates, and the non-classical kinetics shown by both the N-demethylation and the C_3-hydroxylation reactions, at least for DZP [6]. We have shown that C_3-hydroxylation, but not N-demethylation of DZP is catalysed by enzymes of the cytochrome P450IIIA sub-family in rats [7]. The present communication reports the results of comparative metabolic studies on DZP, HZP, PZP and PIN, using microsomes prepared from the livers of phenobarbitone-pretreated adult male Sprague–Dawley rats, which we had shown previously to have relatively high activities for both C_3-hydroxylation and N_1-demethylation of DZP [7].

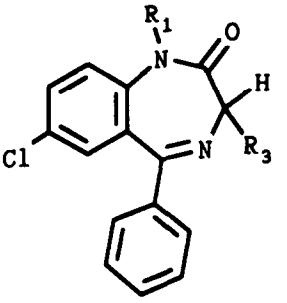
Materials and Methods

Chemicals, drugs and metabolites. D-Glucose-6-phosphate (disodium salt) and D-glucose-6-phosphate dehydrogenase (G6PDH) (yeast enzyme, grade 1) were obtained from Boehringer Mannheim Pty Ltd (Sydney, Australia). NADPH was purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Phenobarbitone sodium was obtained from Queensland Ethicals (Brisbane, Australia). All other chemicals and solvents were of analytical reagent grade. The benzodiazepines and their metabolites (except 3-OH-PIN) were all gifts from pharmaceutical companies as follows: camazepam (CZP), Boehringer Ingelheim Pty Ltd (Sydney, Australia); DZP, DMD and temazepam (TZP), Roche Products Pty Ltd (Sydney, Australia); HZP and 3-OH-HZP, Essex Laboratories Pty Ltd (Sydney, Australia); PIN, Zambeletti s.p.a. (Milan, Italy); PZP and 3-OH-PZP, Parke Davis Pty Ltd (Sydney, Australia). 3-OH-PIN was synthesized by the reaction of oxazepam (OZP) with propargyl bromide in dimethylformamide in the presence of sodium hydride [9, 10]; the identity of the product was confirmed by mass spectrometry and by proton magnetic resonance spectroscopy.

Microsomes and incubation conditions. Twelve random outbred male Sprague–Dawley rats weighing 150–175 g were obtained from the University of Queensland Medical School Animal House. They were fed a standard laboratory rodent diet *ad lib.* and maintained in a temperature

* Abbreviations: AUC, area under plasma concentration–time curve; CZP, camazepam; DMD, *N*-desmethyldiazepam; DZP, diazepam; G6PDH, D-glucose-6-phosphate dehydrogenase; HZP, halazepam; 3-OH-HZP, 3-hydroxy-halazepam; 3-OH-PIN, 3-hydroxy-pinazepam; 3-OH-PZP, 3-hydroxy-prazepam; OZP, oxazepam; PIN, pinazepam; PZP, prazepam; TZP, temazepam (3-hydroxy-diazepam).

Table 1. Chemical structures of diazepam, its major metabolites, the congeners studied, and camazepam, the internal standard for the HPLC assay



Compound	R_1	R_3
Diazepam	CH_3	H
Metabolites		
Temazepam	CH_3	OH
N-Desmethyldiazepam	H	H
Oxazepam	H	OH
Congeners		
Camazepam	CH_3	$OCON(CH_3)_2$
Halazepam	CF_3CH_2	H
Pinazepam	Propargyl	H
Prazepam	Cyclopropylmethyl	H

controlled room (20–25°) under a 12 hr light–dark cycle. They were administered phenobarbitone, dissolved in drinking water at a concentration of 1 mg/mL for 7 days, and this was replaced with tap water and food withdrawn, 16 hr prior to decapitation and preparation of pooled liver microsomes by the procedure of Guengerich [11]. Microsomal protein and cytochrome P450 concentrations were assayed [12, 13], and monooxygenase activities with benzodiazepines as substrates were determined at 37° in 200 μL reaction mixtures containing 100 mM Tris–HCl, pH 7.9, 2.5 mM glucose-6-phosphate, 1 IU G6PDH and 1.0 mM $NADP^+$ as described previously [7, 8]. Owing to the extreme insolubility in aqueous media of the benzodiazepine substrates of interest, and the requirement to avoid the use of solubilizing agents to increase substrate concentrations [6], monooxygenase activities in the present study were determined for each benzodiazepine solubilized directly in incubation buffer by sonication [6], at a concentration of 10 μM. This figure was dictated by the maximum attainable concentrations of PZP and HZP, the two least soluble of the substrates under study. The use of this very low substrate concentration for all of the substrates was validated by the apparent lack of saturability (apparent first-order kinetics) of both N-dealkylation and C_3-hydroxylation with DZP as substrate over the range 10–200 μM [7, 8], which suggested that at this concentration we would obtain a measure of substrate oxidation that reflected the formation of the enzyme–substrate complex. This, we reasoned, would serve to compare the metabolism of the different benzodiazepines, given our inability to determine K_m and V_{max} for any of these substrates. Indeed HZP, PIN and PZP were not soluble enough, given our lower limit of detection of product formation, even to determine a range of concentration over which an apparent first-order relationship between substrate and product concentrations may have held. Further support for adopting this method was, however, provided by the results of preliminary studies which were conducted to determine the assay durations and cytochrome P450 concentrations for each of the substrates that yielded linear formation of the C_3-hydroxy and *N*-desalkyl products. OZP production resulting secondarily from dealkylation of TZP or from C_3-hydroxylation of DMD, with DZP as substrate, was not significant at this relatively low concentration, but secondary production of OZP was notable with the other substrates. Because of this, assay conditions with respect to both duration and cytochrome P450 concentration had to be adjusted for each of the substrates so that the formation of the primary products was optimal with undetectable secondary OZP production. The incubation conditions that satisfied these requirements were as follows: DZP: 10 min, 0.5 μM P450; PIN: 10 min, 0.1 μM P450; HZP: 5 min, 0.25 μM P450; PZP: 5 min, 0.1 μM P450. OZP was the only monooxygenase product of incubations conducted with DMD as substrate at 10 μM and the assays with this compound as substrate were conducted for 10 min at 0.5 μM P450. Microsomal reactions were terminated by the addition of tetrahydrofuran (50 μL) containing CZP (10 μg/mL), the internal standard for the HPLC assay of the benzodiazepines in the incubation mixtures. Full details of the analytical method have been reported recently [7]. Replicate analyses (N = 3) were carried out, and turnover numbers were calculated by normalization on the basis of the cytochrome P450 concentrations in the incubates.

Determination of log P *values.* The octanol–water partition coefficients (log *P* values) of the benzodiazepines were determined by a HPLC method [14]. The chromatographic capacity factors (log *k'*) of the various compounds were calculated as follows: $\log k' = \log (t_r - t_0)$ where t_r is the retention time of the compound of interest, and t_0 is the retention time of an unretained solute (sodium nitrate). A calibration relationship between known log *P* values of standards and experimentally determined log *k'* values was established, and was subsequently used to estimate the log *P* values of the benzodiazepines from their measured log *k'* values. The experiments were all conducted using one mobile phase which gave convenient retention times (65% methanol in phosphate buffer (0.1 M, pH 7.4)); this procedure has been validated for the determination of log *P* values of an extensive series of benzodiazepines [15]. The reported values are averages of three separate determinations.

Table 2. Rates of metabolism by N-dealkylation and C_3-hydroxylation, and log P values of the benzodiazepines

Substrate	Turnover number (μM/min/μM P450) C_3-Hydroxylation	N-Dealkylation	Log P
DMD	0.192 ± 0.003	NA	2.55 ± 0.006
DZP	0.316 ± 0.006	0.269 ± 0.007	2.72 ± 0.001
PIN	0.401 ± 0.028	2.045 ± 0.049	2.72 ± 0.006
HZP	3.970 ± 0.064	0.409 ± 0.059	3.02 ± 0.006
PZP	2.926 ± 0.169	4.655 ± 0.074	3.34 ± 0.010

Turnover numbers (N = 3) and log P values (N = 3) are given as mean ± SD. NA, not applicable.

Results and Discussion

Table 2 shows that there was substantial variation among the substrates in the rates of both C_3-hydroxylation and N-dealkylation. A 20-fold variation in the rate of C_3-hydroxylation was recorded across the five substrates, with HZP showing the highest and DMD the lowest rate. An almost equally large range of variation was shown for the N-dealkylation reaction, with PZP undergoing this biotransformation more than 17 times faster than DZP. Furthermore, the relative rates of metabolism by these two pathways varied according to substrate. Thus, C_3-hydroxylation was favoured for HZP and DZP, though only marginally for the latter. On the other hand N-dealkylation was the dominant pathway for PIN and PZP. Previous authors have provided some data with which ours can be compared. Hence, our findings for HZP and PZP agree well with the recent report of Lu *et al.* [5], who also found predominance of C_3-hydroxylation for HZP and of N-dealkylation for PZP; those authors additionally examined the product enantioselectivity in C_3-hydroxylation, an important issue which our study was not designed to address. We note that N-dealkylation was recently shown to be the principal route for metabolism of HZP in dogs [16], which probably reflects the substantial inter-species variability characteristic of benzodiazepine metabolism. Our results for PIN and DZP are also consistent with earlier reports [3, 17] which showed that PIN was mainly dealkylated while C_3-hydroxylation was favoured for DZP. It seems likely on the basis of previous work with DZP [7, 18] that the P450 isoenzyme(s) which catalyse the N-dealkylation of these substrates (probably of the IIB sub-family [19]) differ from those (in the IIIA sub-family [7]) which catalyse the C_3-hydroxylation. This conclusion is consistent with the differing rank-ordering of the substrates in terms of the rate of metabolism by the competing pathways (Table 2). Presumably many attributes of the substrates, as well as those of the metabolizing tissue, determine their metabolic fates. The one attribute which we were able to examine was lipophilicity, which has been shown to be important in the disposition of many classes of drugs. The apparent lack of a relationship between log P values and rate of metabolism by either pathway separately (Table 2) suggests that lipophilicity is not a critical parameter for these drugs, except insofar as their highly lipophilic character renders them all subject to extensive metabolism. The relatively small range of log P values among these substrates would, in any case, make such relationships difficult to determine. Nevertheless, it was striking that the sum of the metabolic rates by the two pathways showed an excellent correlation with lipophilicity (Table 2).

Two aspects of the possible relevance of these studies to the metabolism of the compounds in man warrant comment. Firstly, the limitations imposed by the sensitivity of analytical methods for the quantification of products have meant that most *in vitro* metabolism studies have been conducted with substrate concentrations which exceed the likely *in vivo* concentrations by one or more orders of magnitude. We have worked with substrate concentrations which are low in comparison with most published work, but which probably still exceed the levels attained in hepatocytes during clinical use of these drugs. The study of clinically relevant enzyme kinetics of these benzodiazepines must await the development of more sensitive analytical methods. Secondly, as noted above, it is hazardous to attempt to predict quantitative aspects of the elimination of drugs *in vivo* on the basis of their relative metabolic rates *in vitro*. Thus, the finding of a faster formation of TZP than of DMD from DZP *in vitro* might suggest that the former would be the quantitatively more important metabolite. However, several workers have estimated that the fractional conversion of DZP to DMD *in vivo* is 50% or more [1], and the most important urinary metabolite following DZP administration in man is OZP [20], most of which is presumably derived from DMD. Comparative *in vivo* disposition data for the compounds studied here *in vitro* are scant.

This study, the first report of comparative metabolism of the congeneric benzodiazepines DZP, HZP, PIN and PZP, has highlighted the gross differences in their relative rates of N-dealkylation and C_3-hydroxylation. The data suggest that lipophilicity is not an important determinant of their metabolic routes, but those factors which do determine the observed differences remain to be elucidated.

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Effects of deoxyspergualin on dipeptidyl peptidase-II and -IV in the spleen of BXSB mice and MRL/*lpr* mice during the development of the lupus erythematosus-like syndrome

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Several murine strains such as (NZB × NZW)F_1 mice, MRL/Mp-*lpr*/*lpr* (MRL/*lpr*) mice or male BXSB mice are known as animal models of systemic lupus erythematosus (SLE*) [1, 2]. The etiopathogenesis of SLE in these strains of mice has been a subject of extensive study. Significant changes in some peptidases in tissues or serum have been demonstrated in these animal models of SLE. In our previous studies [3, 4], we found increased activity of DPP II (EC 3.4.14.2) and decreased activity of DPP IV (EC 3.4.14.5) in the spleen of NZB mice, male BXSB mice and male MRL/*lpr* mice with lupus erythematosus-like syndrome as compared with the activities of male MRL/++ mice, female BXSB mice and female BALB/c mice, as controls without SLE. As a result, an increase in the ratio of DPP II/DPP IV activities in the spleen of these lupus mice was noted as compared to the activities of control mice [3, 4]. As in lupus mice, serum DPP II activity in patients with rheumatoid arthritis or SLE was increased

* Abbreviations: SLE, systemic lupus erythematous; DPP, dipeptidyl peptidase; DSP, 15-deoxyspergualin (1-amino-19-guanidino -11- hydroxy-4,9,12-triazanadecane -10,13 - dione); Lys - Ala - MCA, 7 - (Lys - Ala) - 4 - methylcoumarinamide; Gly-Pro-MCA, 7-(Gly-Pro) -4-methylcoumarinamide.