Hydroxylation of Three Benzodiazepines *In Vitro*

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Abstract □ Three structurally related benzodiazepines were studied as substrates for hydroxylation by liver microsomal enzymes of rats and mice. The V_{max} was comparable for dechlorodesmethyldiazepam, desmethyldiazepam, and 2′-chlorodesmethyldiazepam in the two animal species. The apparent K_m decreased from dechlorodesmethyldiazepam to 2′-chlorodesmethyldiazepam for liver microsomal enzymes from both animal species. The hydroxylation of desmethyldiazepam and 2′-chlorodesmethyldiazepam yielded two pharmacologically active metabolites, oxazepam and lorazepam, respectively.

Keyphrases □ Benzodiazepines, various—hydroxylation by rat and mouse liver microsomal enzymes *in vitro* □ Hydroxylation—three benzodiazepines by rat and mouse liver microsomal enzymes *in vitro* □ Metabolism—three benzodiazepines, hydroxylation by rat and mouse liver microsomal enzymes *in vitro* □ Sedatives—three benzodiazepines, hydroxylation by rat and mouse liver microsomal enzymes *in vitro*

Liver microsomal enzymes metabolize 1,4-benzodiazepines *in vitro* (1). Previously, it was shown that diazepam undergoes a process of *N*-1-demethylation and/or C-3-hydroxylation (2), the relative intensity of these pathways depending, among other factors, on animal species (1, 3), age (4), and induction by other treatments (5). This study was designed to investigate how changes in the chemical structure of a benzodiazepine influence its hydroxylation rate. To this end, desmethyldiazepam (I), dechlorodesmethyldiazepam (II), and 2′-chlorodesmethyldiazepam (III) were selected.

EXPERIMENTAL

Chemicals—Compounds I–III and their corresponding 3-hydroxylated metabolites of pharmaceutical grade purity (99%) were used[1].

Animals—Male Charles River rats, 200 ± 10 g, and male CD_1 mice, 22 ± 3 g, were used.

Preparation of Liver Microsomes—Animals were killed, and the livers were immediately removed and homogenized in ice-cold 1.15% KCl (1:4 w/v) with a polytef–glass homogenizer. The homogenate was centrifuged at 9000×*g* for 20 min, and the supernate was centrifuged again at 105,000×*g* for 1 hr[2].

Incubation *In Vitro*—A microsomal suspension in 1.15% KCl containing 8–10 mg of protein/ml was used. Protein determinations were carried out by the Lowry *et al.* (6) method.

Each incubation mixture consisted of 1.5 ml of microsomal suspension equivalent to 0.4 g of liver, NADP (1.5 μmoles), glucose 6-phosphate dehydrogenase (0.5 unit), magnesium chloride (25 μmoles), niacinamide (50 μmoles), 1.4 ml of 0.2 *M* phosphate buffer (pH 7.4), various concentrations of substrates (I–III) ranging from 10^{-4} to 10^{-3} *M*, and 1.15% KCl to obtain a final volume of 5 ml. The substrate concentrations were selected after preliminary experiments.

The mixtures were incubated in a metabolic shaker[3] at 37° under air for 60 min.

At the end of the incubation period, the mixture was extracted twice with 10 ml of ether. The combined extracts were dried, redissolved in a suitable volume of an acetone solution of internal standard (diazepam), and analyzed by GLC.

GLC Determination—A gas chromatograph[4] and a 1-m long glass column (3 mm i.d.) packed with 3% OV-17 on Gas Chrom Q (100–120 mesh) were used. The oven temperatures were 270 and 260° for I–III and their hydroxylated metabolites; the carrier gas flow (nitrogen) was 30 ml/min.

Recovery studies of tested benzodiazepines and their metabolites from water and from microsomal incubation mixtures without cofactors are reported in Table I.

Table I—Recovery Studies

Benzodiazepines[a]	Water, % ± *SE*	Microsomal Incubation Mixture, % ± *SE*
III	95 ± 3	81 ± 1
Lorazepam	95 ± 2	68 ± 0.5
I	95 ± 2	85 ± 2
Oxazepam	95 ± 3	61 ± 3
II	95 ± 1	88 ± 3
Dechlorooxazepam	95 ± 3	65 ± 2

[a] Added amounts ranged from 10 to 50 μg.

RESULTS AND DISCUSSION

The results summarized in Table II show that the three benzodiazepines can be hydroxylated *in vitro* by liver microsomal enzymes obtained from rats or mice. The maximal velocity of hydroxylation, V_{max}, was comparable for the three benzodiazepines with rat liver microsomes, while it was higher for II when mouse liver microsomes were utilized.

The affinity for liver microsomal enzymes, expressed by the value of the apparent K_m, was II < I < III for both rats and mice.

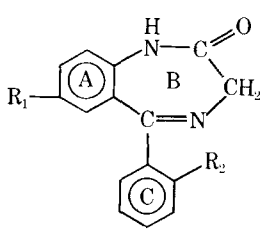
The hydroxylation of 1,4-benzodiazepines may have biological significance, because the hydroxylated products frequently have pharmacological effects[5]. Compound I is, in fact, converted to oxazepam and III is converted to lorazepam, both metabolites being utilized clinically (7–11). It is not known whether the hydroxylated metabolite of II is pharmacologically active, although II was reported (12) to be practically inactive in most experimental tests utilized to screen benzodiazepines.

In mice, lorazepam was more active than oxazepam in antagonizing pentylenetetrazol (13) and the parent compound III was more active than I (12).

The presence of the hydroxylated metabolites in the brain of mice, but not of rats (14), explains the long duration of the effect exerted by I and III in mice but not in rats (12). The facts that both I and III are equally hydroxylated *in vitro* by the liver microsomes of rats and mice but that the hydroxylated metabolites accumulate only in the brain of mice may be due to a rapid metabolism of hydroxylated benzodiazepines in rats (15, 16).

In conclusion, the results indicate that comprehension of the structure–activity relationships of pharmacologically active compounds is tightly bound to studies of drug metabolism and distribution. From a chemical point of view, the presence of a chlorine in positions 7 (ring A) and 2′ (ring C) should influence the energy of the CH bond (ring B) through resonance effects.

The biotransformation of I and III to oxazepam and to chlorooxazepam, respectively, is a stereospecific reaction (17), and the limiting step of this process is the breakdown of the CH bond.



I: R_1 = Cl, R_2 = H
II: R_1 = R_2 = H
III: R_1 = R_2 = Cl

[1] Courtesy of Ravizza, Muggiò, Milan, Italy.
[2] Beckman model L ultracentrifuge with 12.2-m rotor.
[3] Dubnoff.
[4] Model GI, Carlo Erba, Milan, Italy.
[5] S. Garattini, F. Marcucci, and E. Mussini, to be published.

Table II—C-3-Hydroxylation of Benzodiazepines by Liver Microsomal Enzymes of Rats and Mice

Substrates	Rat		Mouse	
	K_m, M	V_{max}, moles/min/mg of Protein	K_m, M	V_{max}, moles/min/mg of Protein
III	4.02×10^{-5} [a]	1.14×10^{-10}	4.52×10^{-4}	6.23×10^{-10}
I	3.30×10^{-4}	1.12×10^{-10}	8.02×10^{-4}	4.58×10^{-10}
II	1.52×10^{-3}	2.35×10^{-10}	1.36×10^{-3}	17.15×10^{-10}

[a] Each figure corresponds to the mean value of four different determinations.

Studies on the distribution of I–III and their metabolites after intravenous administration to rats and mice are in progress. The correlation between *in vitro* and *in vivo* metabolism for both animal species should help explain the species variations in metabolism and pharmacological activity.

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ACKNOWLEDGMENTS AND ADDRESSES

Received September 28, 1976, from the *Istituto di Ricerche Farmacologiche "Mario Negri," Via Eritrea, 62, 20157 Milano, Italy.*

Accepted for publication December 15, 1976.

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Small Animal Model for Myocardial Infarction

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Abstract □ Myocardial infarctions were produced in rats by electrocauterization of the left anterior descending artery, and the extent of myocardial damage was measured by serial serum levels of creatine phosphokinase activity utilizing spectrophotometric analysis. All animals were also evaluated for myocardial damage by electrocardiographic wave alterations. A correlation between myocardial infarct size and serum creatine phosphokinase was demonstrated. Significant arrhythmias and death occurred only in experimental groups where myocardial infarction had been produced. This small animal model offers a quick, inexpensive, and simple method for screening therapeutic agents that alter infarct size.

Keyphrases □ Myocardial infarction—damage correlated with serum creatine phosphokinase levels, rats □ Creatine phosphokinase—serum levels correlated with myocardial damage caused by infarction, rats □ Enzymes—creatine phosphokinase, serum levels correlated with myocardial damage caused by infarction, rats

More than 1,000,000 persons die annually in the United States from cardiovascular disease, the major cause of death in this country. Although a large percentage of deaths resulting from myocardial infarction are associated with cardiac arrhythmias, many patients expire in the coronary care unit due to power failure, especially low output congestive heart failure (1–3). Power failure was suggested to be related to inadequate cardiac output due to necrosis of functional myocardium (4, 5). In power failure, the extent of necrosis, as it is related to the critical mass of functional myocardium, may be used as a determinant of morbidity and mortality in clinical prognosis (6, 7).

BACKGROUND

Several methods have been used to measure the extent of myocardial ischemia, including coronary blood flow, myocardial lactate production, angiography, radioactive scanning, and radioactive potassium tracers (8). Electrocardiographic (ECG) mapping experiments also were used to describe ischemic changes in the myocardium (9, 10). The problem encountered with these methods is that they study the extent of myocardial ischemia without quantitatively ascertaining the infarct size.

The relationship between infarct size and the serum myocardial enzyme levels has been recognized for many years. Myocardial infarct size, measured by excision and weighing of the damaged tissue, correlated closely with the extent of the depletion of myocardial creatine phosphokinase (11). This enzyme offered many advantages over other myocardial enzymes, since it rose first following an infarction and returned to normal levels in approximately 3 days (12). Sequential serum changes of creatine phosphokinase were used to assess damage in the experimentally infarcted dog (13).

A satisfactory quantitative method for the evaluation of myocardial damage would make it possible to determine the effect of therapeutic intervention on limiting the dynamic extension of an infarction. In this study, a simple, inexpensive, small animal model for inducing myocardial infarction, which may prove useful in the preliminary screening of drugs capable of altering infarct size, is presented.