COMPARISON OF HUMAN AND RAT METABOLISM OF MOLINATE IN LIVER MICROSONES AND SLICES

WILLIAM T. JEWELL AND MARION G. MILLER

Miller Laboratory, Department of Environmental Toxicology, University of California, Davis, California

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ABSTRACT:

Molinate undergoes oxidative metabolism forming either ring-hydroxylated metabolites or molinate sulfoxide. Our previous studies strongly implicated the sulfoxidation pathway in molinate-induced testicular toxicity. The present study compares the metabolic capability of rat and human liver microsomes and slices to form either nontoxic ring-hydroxylated metabolites of molinate or the toxic metabolites derived from the sulfoxidation of molinate. $K_m$ and $V_{max}$ values indicate that sulfoxidation would be the preferred high-dose pathway whereas hydroxylation would predominate at low dose levels in both species. Examination of phase II metabolism of molinate in liver slices reveals greater detoxification of molinate sulfoxide by glutathione conjugation in humans with rats forming less conjugate. Oxidative metabolism of molinate in both rats and humans appears to be mediated by cytochrome P-450 and not flavin monooxygenases as indicated by the use of metabolic inhibitors. Overall, the metabolism of molinate would be via the nontoxic hydroxylation pathway in both species at low doses whereas at high doses, where sulfoxidation would predominate, the human is more capable than the rat to detoxify via glutathione conjugation.

In vivo studies in rats have indicated that the major urinary metabolites of molinate are derived from oxidative metabolism via either ring hydroxylation or sulfoxidation (Jewell et al., 1998). Molinate sulfoxide is a more potent testicular toxicant than molinate, suggesting either that the sulfoxide itself or the sulfone metabolite of the sulfoxide is responsible for toxicity (Jewell et al., 1998). Additionally, it is known that the sulfoxide and sulfone metabolites of molinate bind to and inhibit a carboxylesterase, hydrolase A, in both the testes and liver (Jewell and Miller, 1998). Hydrolase A has been shown to possess the ability to hydrolyze cholesterol oleate (Kaphalia et al., 1997), and cholesterol is required for the first step in testosterone biosynthesis. Therefore, it has been proposed that after metabolic activation molinate may induce toxicity via inhibition of the hydrolysis of cholesterol esters with a subsequent decrease in the availability of cholesterol for testosterone biosynthesis (Jewell and Miller, 1998; Ellis et al., 1998).

In vivo studies in rats have indicated that the major urinary metabolites of molinate are derived from oxidative metabolism via either ring hydroxylation or sulfoxidation (Fig. 1; DeBaun et al., 1978). A large proportion of the dose was detected as mercapturic acid, which would have resulted from glutathione conjugation of either molinate sulfoxide or sulfone. Because the sulfone is unstable, it can undergo hydrolysis to liberate the hexamethyleneimine moiety (Casida et al., 1974). Ring-hydroxylated metabolites of molinate are excreted unchanged or undergo phase II conjugation and are excreted as glucuronide conjugates (DeBaun et al., 1978).

Epidemiological studies of workers exposed to molinate did not show any adverse effect on male reproductive capability and thus it was proposed that humans could be less susceptible to molinate toxicity (Cochran et al., 1997). One possible explanation for a species difference in susceptibility to toxicity could be differences in metabolism between the rat and human. Humans may be able to detoxify molinate by utilizing the hydroxylation pathway whereas the rodent could preferentially bioactivate molinate via sulfoxidation. In support of this hypothesis, a human study involving one individual and conducted at very low dose levels found that the mercapturic acid metabolites represented only 1 to 2% of the administered dose and the balance was excreted as hydroxymolinate metabolites (Krieger et al., 1992).

The present study has determined $K_m$ and $V_{max}$ relationships for the formation of nontoxic ring-hydroxylated and toxic sulfoxide metabolites in rat and human liver microsomes and slices. The data indicated that at high doses sulfoxidation would be the preferred pathway but at low doses, ring hydroxylation would predominate. Moreover, as compared to the rat, the human liver slices were also able to substantially detoxify the sulfoxide metabolite via glutathione conjugation.

Materials and Methods

Animals. Male Sprague-Dawley rats (90 days old) weighing 350 to 400 g were purchased from Charles River (Hollister, CA). The animals were housed two per cage in temperature (22 ± 1°C) and humidity (50 ± 10%)–controlled rooms under a 12-h light/dark cycle. Animals were acclimated to housing conditions for at least 1 week before use.

Chemicals. Molinate (>99% purity) was supplied by Chem Service (West Chester, PA). Metabolite standards for molinate were generously provided by Zeneca Ag Products (Richmond, CA). Cytochrome P-450 reductase antibody was purchased from Gentest Corporation (Boston, MA). Except where indicated, all other chemicals were acquired from Sigma Chemical Company (St. Louis, MO).

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Abbreviation used is: FMO, flavin monooxygenase.

Send reprint requests to: Dr. Marion G. Miller, Dept. of Environmental Toxicology, University of California Davis, Davis, CA 95616. E-mail: mgmillersears@ucdavis.edu
Microsome Preparation. Rat liver microsomes were prepared by conventional methods (Lake, 1987). Livers were excised and washed briefly in ice-cold isolation buffer (0.154 M KCl, 50 mM Tris-HCl, pH 7.4). The tissues were homogenized in 4× volume of isolation buffer, then centrifuged (10,000g, 20 min, 4°C). The pellet was discarded and the supernatant was then centrifuged (105,000g, 60 min, 4°C). The microsomal pellet was washed by resuspension in fresh buffer and centrifuged again (105,000g, 60 min, 4°C). Microsomes were resuspended in 2× volume of 0.12 M Tris, pH 7.4, and stored frozen in 1-ml aliquots at −80°C. Human liver microsomes were prepared similarly. They were generously provided by SRI International (Menlo Park, CA).

Microsomal Incubations. Microsomal incubations were carried out at conditions that had been determined previously to give linear metabolism of molinate. Specifically, protein concentration was 3 mg/ml and NADPH concentration was 2 mM. Microsome aliquots were thawed in room temperature water, diluted to 200 µl in 0.12 M Tris, pH 7.4, and preincubated at 37°C for 2 min before addition of substrate (0.25–4 mM). Incubations were carried out for 10 min for kinetic studies and for 1 to 30 min for time course studies. The reactions were terminated by the addition of an equal volume of acetonitrile followed by centrifugation (105,000g, 10 min) and the levels of metabolites in the supernatant were determined by HPLC analysis as described below. All incubations were carried out at 37°C.

Liver Slice Preparation. Liver slices were prepared using a Krumdieck Tissue Slicer (Alabama Research and Development Corp., Munford, AL). Rat liver slice studies were carried out at the University of California (Davis, CA). Human liver slices were prepared and incubated at SRI International (Menlo Park, CA) from three donors through the SRI tissue bank donor program. Tissue cores were taken mechanically with a drill press and corer and trimmed of excess tissue before slicing. Tissues were kept in ice-cold Krebs-Henselheit buffer, pH 7.4, before slicing. Slices were 200 to 250 µM in thickness and transferred to oxygenated (95% O₂, 5% CO₂) ice-cold Krebs-Henselheit buffer, pH 7.4, before incubation.

Liver Slice Incubations. Rat liver slices were prepared and incubated as described previously (Miller et al., 1992). Glass vials were used instead of plastic Petri dishes as molinate binds avidly to plastic. Slices were preincubated in a oxygenated environment (95% O₂, 5% CO₂) in 3 ml of Krebs-Henselheit buffer, pH 7.4, for 1 h before transferring to fresh vials containing the substrate (0.25–4 µM). Incubations were carried out for 2 h. For both human and rat slices, slices were snap-frozen in their vials in an acetone and dry ice bath to terminate metabolism. Media was directly sampled for HPLC analysis as previous determinations with [14C]molinate had shown that the metabolites were not significantly retained in the slices.

HPLC Analysis of Metabolites. HPLC was carried out using a C₁₈ column (Alltech Altima, 250 × 4.6 mm, i.d., 10 µm) on Waters Associates (Milford, MA) equipment as described previously (Tjeerdema and Crosby, 1987). Initial solvent conditions were 25 mM phosphate buffer, pH 6.8, and acetonitrile (80:20) and final conditions (30:70) were reached by linear gradient over 22 min at a flow rate of 2 ml/min. Molinate and metabolites were monitored by UV absorption at 220 nm. Sensitivity was determined to be >0.5 nmol for this system. Liver slice incubation media was treated with type H1 glucuronidase to hydrolyze hydroxymolinate conjugates. The pH of the media was adjusted to 5.0 before the addition of glucuronidase for optimal hydrolysis activity. Liver slice media were treated for 12 h at 37°C. Excess protein was removed by the addition of an equal volume of acetonitrile followed by centrifugation (1000g, 10 min) before HPLC analysis.

Cytochrome P-450 Versus Flavin Monooxygenase (FMO) Microsomal Metabolism of Molinate. Incubations were carried out as described above for the microsomal metabolism experiments. Various inhibitors were used to determine the contributions of cytochrome P-450 and FMO enzymes to molinate metabolism. The contribution of cytochrome P-450 enzymes to molinate metabolism was determined using carbon monoxide and a P-450 reductase antibody to inhibit P-450 activity. Carbon monoxide was bubbled through the microsomal sample for 3 min to inhibit P-450 activity. P-450 reductase activity was determined using carbon monoxide and a P-450 reductase antibody (Gentest Corp.) were used at a concentration of 20 µg serum protein/100 µg microsomal protein with a 30-min preincubation to ensure complete inhibition as recommended (Gentest Corp.). After treatments, microsomal incubations and HPLC analysis were then carried out as described above.

Statistics. Statistically significant differences in the data were determined using a two-tailed Student’s t test as a post hoc test. The level of significance was set at p < .05 unless indicated.

Results

Rat and Human Liver Microsomal Metabolism. In human and rat liver microsomes >95% of all metabolites detected were either molinate sulfoxide or hydroxymolinate. Infrequently, a small peak coeluted with the retention time of the sulfone standard and in some
human microsomes, a small amount of a late eluting metabolite was detected at high substrate concentrations.

Lineweaver-Burke plots clearly demonstrate the difference between the $K_m$ and $V_{max}$ values for molinate sulfoxide and hydroxymolinate formation in rat and human liver microsomes (Fig. 2, A and B). The $K_m$ and $V_{max}$ kinetic constants for hydroxymolinate and molinate sulfoxide production are derived from the $x$- and $y$-intercepts of these graphs and are summarized in Table 1. Values obtained for the sulfoxidation $V_{max}$ were significantly different ($p < .05$) between rat (1426 pmol/min/mg) and human (815 pmol/min/mg) samples. However, the maximal rate of hydroxymolinate production (30–60 pmol/min/mg) was much less than the maximal rate for molinate sulfoxide formation (700–1500 pmol/min/mg) for both species. Species differences in the $V_{max}$ for the hydroxylation of molinate approached statistical significance ($p < .11$), suggesting that the human would have an approximately 1.5-fold greater capacity than the rat to detoxify molinate. The six human samples used in this study were obtained from individuals of differing age, smoking status, drug history, and cause of death, which could account for the variability of the human results.

Comparison of the $K_m$ values indicated that hydroxylation was a higher affinity pathway than sulfoxidation as shown by lower $K_m$.

![Lineweaver-Burke plots for molinate sulfoxide (a) and hydroxymolinate (b) formation in rat (□) and human (●) liver microsomes.](image)

**Fig. 2.** Lineweaver-Burke plots for molinate sulfoxide (a) and hydroxymolinate (b) formation in rat (□) and human (●) liver microsomes.

**Table 1.** Kinetic rate constants for molinate metabolism in liver microsomes

<table>
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<th>$V_{max}$ pmol/min/mg</th>
<th>$K_m$ μM</th>
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<tbody>
<tr>
<td>Sulfoxidation</td>
<td>Rat: 1426 ± 139*</td>
<td>815 ± 140*</td>
</tr>
<tr>
<td></td>
<td>Human: 815 ± 140*</td>
<td>400 ± 15.6**</td>
</tr>
<tr>
<td>Hydroxylation</td>
<td>Rat: 35.4 ± 9.65†</td>
<td>58.6 ± 17.8†</td>
</tr>
<tr>
<td></td>
<td>Human: 58.6 ± 17.8†</td>
<td>138 ± 9.4</td>
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*,$** p < .05, † p < .11; n = 3 for rat microsomes and n = 6 for human microsomes.
TABLE 2

Kinetic rate constants for molinate metabolism in liver slices

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<thead>
<tr>
<th></th>
<th>Rat</th>
<th>Human</th>
<th>Rat</th>
<th>Human</th>
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<tr>
<td></td>
<td>$V_{\text{max}}$</td>
<td>$K_{m}$</td>
<td>$V_{\text{max}}$</td>
<td>$K_{m}$</td>
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<tr>
<td>Sulfoxidation</td>
<td>638 ± 241</td>
<td>510 ± 190</td>
<td>2837 ± 827*</td>
<td>981 ± 411*</td>
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<tr>
<td>and GSH conjugation</td>
<td>(pmol/min/mg)</td>
<td></td>
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<tr>
<td>Hydroxylation</td>
<td>8.33 ± 3.23</td>
<td>42.2 ± 21.3</td>
<td>587 ± 235*</td>
<td>303 ± 143*</td>
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* $p < .05$, $n = 3$.

Discussion

The present study has compared the in vitro capacity of human and rat liver samples to bioactivate and detoxify molinate with the goal of determining the likelihood of molinate adversely affecting human male reproductive capabilities. The formation of molinate sulfoxide and sulfone is a bioactivation step, and there is evidence that either or both of these metabolites generated via sulfoxidation are responsible for molinate’s male reproductive effects whereas the ring hydroxylation of molinate is believed to be a detoxification pathway (Jewell et al., 1998; Ellis et al., 1998).

Liver microsome metabolism experiments showed differences in the metabolic capabilities of human and rats to metabolize molinate. In liver microsomes, $K_{m}$ values for sulfoxidation of molinate were relatively high but similar in rats and humans, indicating comparable substrate affinities. The high $K_{m}$ values indicate that at low dose levels, similar small amounts of sulfoxide would be formed in both species. At higher dose levels, the significantly higher $V_{\text{max}}$ for the rat would indicate that this species could bioactivate more molinate via sulfoxidation.

The microsomal $K_{m}$ value for hydroxylation were also similar in both species. The hydroxylation $K_{m}$ values of 138 µM in the rat and 126 µM in the human were relatively comparable but were lower than the $K_{m}$ values for sulfoxidation in both species (270–400 µM), indicating a higher substrate affinity for this pathway. Thus at low dose levels, ring hydroxylation of molinate would be the predominant metabolic pathway. Overall, ring hydroxylation of molinate was a high-affinity but low-capacity pathway for the metabolism of molinate when compared with the toxic sulfoxidation pathway.

Because molinate is a male reproductive toxicant, only male liver microsomal samples were analyzed. In contrast, in the human liver slice experiments, two male samples and one female sample were analyzed due to the difficulties in procuring fresh liver samples but no significant differences were seen in the molinate metabolism between male and female liver slices. Environmental factors were more important than gender differences in the wide range of human metabolic values obtained.

Liver slice metabolism was included in this study so that the contribution of phase II metabolism toward detoxification of molinate metabolites could be considered. The overall finding from the liver slice experiments confirmed the initial findings in microsomes, namely, that sulfoxidation is a low-affinity but high-capacity pathway whereas molinate hydroxylation represents a higher affinity but lower capacity pathway. Compared with liver microsomes, the slices exhibited a lower rate of metabolism. This difference in metabolism between slices and microsomes has been reported in many other studies using a variety of different substrates (Worboys et al., 1995, 1997; Sidelmann et al., 1996). The lower rate of metabolism in slices as compared with microsomes could be due to the concentrated and soluble nature of enzymes in the microsomal fraction on a per protein milligram basis.

The amount of hydroxymolinate glucuronide conjugate formed in the liver slice system was not significant and was not different between the two species. However, a much higher amount of glutathione conjugate was formed in the human samples than the rat samples. Therefore, the human would be more likely to detoxify the molinate metabolite (sulfoxide or sulfone) that was bioactivated via sulfoxidation.

Liver metabolism of molinate appears to be mediated primarily by cytochrome P-450 in both the rat and human. Using a variety of general P-450 and FMO inhibitors in microsomal incubations, both rat and human incubations had similar inhibition profiles, indicating that...
there are similar metabolizing enzyme systems for molinate in both species. Cytochrome P-450 mediated oxidation of the thiol group of molinate was surprising as FMO has been reported to be capable of oxidizing soft electrophilic atoms like sulfur (Duescher et al., 1994) and could be expected to contribute to the sulfoxidation of molinate. However, the complete inhibition of metabolism after preincubation with P-450 reductase antibodies further indicates the involvement of this enzyme system in molinate metabolism.

Overall, these differences in metabolism could explain why the rodent animal model may be more susceptible to molinate’s male reproductive effects whereas the human has been proposed to be resistant to molinate’s effects. An initial difference in the human metabolism of molinate was noted in a single study (Krieger et al., 1992) and this study has confirmed these findings. Low dose exposures such as in the human administration study reported by Krieger et al. (1992), or those that may occur in an occupational setting, would result in the detoxification of molinate by the formation and excretion of hydroxymolinate and its metabolites. Higher dose exposures, such as those that occur in toxicological studies, would result in the formation of molinate sulfoxide and lead to testicular toxicity. The rat would also appear more susceptible to the male reproductive effects of molinate due to its lower overall hydroxymolinate formation, greater sulfoxidation capacity, and lesser ability to form the glutathione conjugate. Thus a rat exposed to molinate at large doses, as in many of the regulatory studies (Ellis et al., 1998), or in our previous work (Jewell et al., 1998), would be expected to produce more sulfoxide.

**Fig. 3.** Velocity versus substrate bar graphs for formation of molinate sulfoxide (□) and the glutathione conjugate (■) in rat (a) and human (b) liver slices. Values are mean, n = 3.

**Fig. 4.** Molinate metabolism in rat (a) and human (b) liver microsomes. Treatments were control (■), P-450-inhibited (●), and FMO-inhibited (○). Values are mean ± S.E., n = 3.
and show testicular effects. The rat may be more susceptible than the human to molinate’s male reproductive effects and is perhaps not a good animal model for molinate’s potential effects after environmental exposures. However, it may be an excellent model for understanding mechanisms underlying testicular toxicity.

In summary, the metabolism of molinate in both rat and human samples utilizes both the hydroxylation and sulfoxidation pathways and appears to be mediated by P-450. Based on $K_m$ and $V_{max}$ values, low doses of molinate would be metabolized primarily by hydroxylation whereas at higher doses sulfoxidation would be the primary pathway in both species. In addition, the human liver possesses a greater capacity to detoxify molinate sulfoxide via glutathione conjugation. Thus a human exposed to low levels of molinate would be more likely than the rat to detoxify and excrete any sulfoxide intermediate as a glutathione conjugate.

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References


