Metabolism of Prazosin in Rat, Dog, and Human Liver Microsomes and Cryopreserved Rat and Human Hepatocytes and Characterization of Metabolites by Liquid Chromatography/Tandem Mass Spectrometry

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ABSTRACT:

Prazosin (2-[4-(2-furanoyl)-piperazin-1-yl]-4-amino-6,7-dimethoxyquinazoline) is an antihypertensive agent that was introduced to the market in 1976. It has since established an excellent safety record. However, in vitro metabolism of prazosin has not been investigated. This study describes the in vitro biotransformation of prazosin in liver microsomes from rats, dogs, and humans, as well as rat and human cryopreserved hepatocytes and characterization of metabolites using liquid chromatography/tandem mass spectrometry. The major in vivo biotransformation pathways reported previously in rats and dogs include demethylation, amide hydrolysis, and O-glucuronidation. These metabolic pathways were also confirmed in our study. In addition, several new metabolites were characterized, including a stable carbinolamine, an iminium species, and an enamine—all formed via oxidation of the piperazine ring. Two ring-opened metabolites generated following oxidative cleavage of the furan ring were also identified. Using semicarbazide hydrochloride as a trapping agent, an intermediate arising from opening of the furan ring was captured as a pyridazine product. In the presence of glutathione, three glutathione conjugates were detected in microsomal incubations, although they were not detected in cryopreserved hepatocytes. These data support ring opening of the furan via a reactive \( \gamma \)-keto-\( \alpha,\beta \)-unsaturated aldehyde intermediate. In the presence of UDP-glucuronic acid, prazosin underwent conjugation to form an N-glucuronide not reported previously. Our in vitro investigations have revealed additional metabolic transformations of prazosin and have shown the potential of prazosin to undergo bioactivation through metabolism of the furan ring to a reactive intermediate.

PRAZOSIN (Fig. 1), a short-acting vasodilator discovered in the mid-1970s, has been widely used in treating hypertension and congestive heart failure (Constantine, 1974; Althuis and Hess, 1977; Stanaszek et al., 1983). It was the first of a new class of direct-acting vasodilators acting by \( \alpha_1 \)-adrenergic receptor blockade. Prazosin was introduced to the U.S. market as MINIPRESS by Pfizer (New York, NY) in 1976. Prazosin is well tolerated, with the most common side effect associated with treatment being postural hypotension. Although no longer a major drug among the antihypertensive agents, based on prazosin’s ability to antagonize centrally located \( \alpha_1 \)-adrenergic receptors, a new indication for treatment of post-traumatic stress disorders (PTSD) encountered during civilian life is being explored in clinical studies (Taylor and Raskind, 2002). In addition, prazosin is also being investigated in treating combat-related nightmares characteristic of PTSD among soldiers recently returned from Operation Iraqi Freedom (Daly et al., 2005).

In vitro metabolism of prazosin in animals or humans has not been reported. Early metabolism studies with \( ^{14} \)C-labeled prazosin (label in quinazoline ring) in rats and dogs revealed that it undergoes extensive hepatic metabolism with biliary excretion as the major route of elimination. In the dog, approximately 50% of the drug is metabolized through first-pass metabolism. Between 74 and 79% of the i.v. dose was recovered in rat and dog feces, respectively, whereas urinary excretion was low (Rubin et al., 1979). In humans, prazosin is highly bound to plasma protein and readily absorbed from the gastrointestinal tract with oral bioavailability ranging from 44 to 77% and a half-life from 2.2 to 3.7 h (Bateman et al., 1979).

In vivo, the primary metabolic route of prazosin consists of 6-\( O \)- and 7-\( O \)-demethylation followed by glucuronidation (Taylor et al., 1977), with 6-hydroxy-prazosin glucuronide the major metabolite. Other routes of metabolism include hydrolysis of the amide linkage to yield 2-(1-piperazinyl)-4-amino-6,7-dimethoxyquinazoline (N-desfuranoyl prazosin) and to a lesser extent, piperazine ring opening and \( N \)-dealkylation to give dimethoxyquinazoline-2,4-diamine (DQ). These metabolites are less potent in lowering blood pressure than prazosin (Althuis and Hess, 1977). The metabolism of prazosin in humans has not been investigated extensively, and only N-desfuranoyl prazosin has been identified as a metabolite in humans (Piotrovskii et al., 1984).

Since the initial investigation of prazosin metabolism reported in
of key product ions. Insert displays the numbering of the quinazoline ring.

In light of these considerations, the purpose of the present study was to investigate the in vitro metabolism of prazosin, including its bioactivation potential, using rat, dog, and human liver microsomes and rat and human cryopreserved hepatocytes combined with state-of-the-art LC/MS techniques.

### Materials and Methods

**Materials.** Prazosin hydrochloride, NADP⁺, NADPH, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, UDP-glucuronic acid (UDPGA) triammonium salt, β-glucuronidase from *Escherichia coli* type VII-A (1000 units/vial), glutathione (GSH), semicarbazide hydrochloride, and magnesium chloride were all purchased from Sigma Chemical Co. (St. Louis, MO). EDTA (0.5 M, pH 8.0) was obtained from GibcoBRL (Grand Island, NY). High-performance liquid chromatography (HPLC)-grade acetonitrile and methanol were obtained from EMD Chemicals (Gibbstown, NJ). Formic acid was obtained from Fisher Scientific (Fair Lawn, NJ). All the other reagents were of analytical grade.

Pooled liver microsomes from 361 male Sprague-Dawley rats and 4 male beagle dogs, pooled mixed gender human liver microsomes from 50 individuals, and pooled liver cytosols from 200 male Sprague-Dawley rats, 4 male beagle dogs, and 10 male human liver cytosols were purchased from Xenotech LLC (Lenexa, KS). Pooled rat cryopreserved hepatocytes (lot no. 42514) and human cryopreserved hepatocytes from a female donor (lot no. 30, donor 51) were purchased from BD-Gentest (Woburn, MA). Hepatocyte incubation media and serum-free and hepatocyte thawing media were purchased from Invitrogen Technologies (Baltimore, MD).

**Microsomal Incubation Conditions.** Prazosin hydrochloride (10 μM) was incubated in a 1.0-ml solution containing magnesium chloride (10 mM), EDTA (2 mM) in potassium phosphate buffer (0.1 M, pH 7.4), rat, dog, or human liver microsomes (1.0 mg/ml), in the presence or absence of an NADPH-generating system (2.62 mM NADP, 7.11 mM glucose 6-phosphate, and 0.8 units/ml glucose-6-phosphate dehydrogenase), and UDPGA at 37°C for 2 h. To assess β-glucuronidase hydrolysis of the aromatic amine N-glucuronide, the incubated mixture of prazosin, UDPGA, and human liver microsomes in phosphate buffer was incubated with β-glucuronidase (250 units) at pH 5.0 and 7.4 for 24 h at 37°C. The incubation mixture without β-glucuronidase was treated as a control. To investigate the stability of metabolites M2, M5, and M8, after performing a human microsomal incubation as described above, an aliquot was evaporated to dryness under N₂, reconstituted in phosphate buffer, and incubated at 37°C for 24 h.

Prazosin was also incubated under the conditions mentioned above with rat, dog, or human liver cytosol (1 mg/ml) and microsomes (1 mg/ml), GSH (2.5 mM), and an NADPH-regenerating system. To trap reactive intermediates of prazosin, semicarbazide hydrochloride (2 mM final concentration) was added to the rat, dog, or human liver microsome incubations together with an NADPH-regenerating system. Incubations of prazosin in buffer and in microsomes without NADPH were run as controls.

**Studies with Cryopreserved Rat and Human Hepatocytes.** The cells were stored in liquid nitrogen until use. Immediately before use, vials of hepatocytes were rapidly thawed in a shaking water bath (37°C, 1.5 min) and then immediately transferred to a 50-ml centrifuge tube that had been precooled on ice. Tubes were kept on ice, and 24 ml of ice-cold thawing media were added drop-wise at a rate of about 4 ml/min with gentle handshaking to prevent the cells from settling. The cell suspension was centrifuged at 50g for 5 min at 4°C; the supernatants were discarded; and the pellets were resuspended in ice-cold incubation media (6 ml/tube). With gentle handshaking, air was introduced into the suspensions using an automatic pipette until the cells were suspended in the media, and the suspensions from the two tubes were pooled. The cell number and viability were determined by the trypan blue exclusion assay (Berry and Friend, 1969) before drug incubations. Prazosin was incubated in 12-well plates at a concentration of 10 μM at 1.0 ml/well containing human hepatocyte suspensions (1.9 million human hepatocytes, viability 37%) or rat hepatocyte suspensions (2.3 million rat hepatocytes, viability 49%) in a gassed (5% CO₂) incubator at 37°C for 4 h. Control samples without hepatocytes were also run.

**Sample Preparation.** At the end of the hepatocyte or microsomal incubations, tubes were transferred to an ice bath. Each sample was extracted with methanol (3 ml) by mixing, sonication, and centrifugation at 1640g for 15 min at 4°C. After each centrifugation, the supernatants were transferred to centri-
fuge tubes and were evaporated to dryness under nitrogen gas at 20°C. The dried extracts were reconstituted in 300 μl of methanol/water (6:4, v/v).

**LC/MS Analysis.** An Agilent model 1100 HPLC system including a binary pump and diode array ultraviolet detector (Agilent Technologies, Palo Alto, CA) was coupled to a mass spectrometer described below. The UV detector was set to monitor absorbance between 190 and 600 nm. Separations were accomplished on a Luna C18(2) column (250 × 2.0 mm, 5 μm) (Phenomenex, Torrance, CA) coupled with a guard cartridge (4 × 2 mm). The sample chamber in the autosampler was maintained at 4°C, whereas the column was at ambient temperature of about 22°C. The mobile phase consisted of 0.2% formic acid in water (A) and acetonitrile with 0.2% formic acid (B) and was delivered at 0.30 ml/min. The gradient started at 0% B and proceeded linearly to 20% B over 40 min, increased to 30% B at 45 min, further increased to 45% B at 50 min, and reached 90% B at 52 min before returning to initial conditions at 54 min. The total run time was 60 min. For hydrogen-deuterium exchange experiments, deuterium oxide was substituted for water in mobile phase A. During LC/MS sample analysis, up to 10 min of the initial flow was diverted away from the mass spectrometer before evaluation of metabolites.

Mass spectral data for the metabolites were obtained with a Micromass quadrupole time-of-flight API-MS mass spectrometer (Waters Corp., Milford, MA). It was equipped with an electrospray ionization source and operated in the positive ionization mode. Full-scan spectra were acquired from 100 to 1500 m/z with a scan time of 0.9 s. The capillary and cone voltages were 3.5 kV and 25 V, respectively. The source block and desolvation gas temperatures were 334°C or 37°C in phosphate buffer, pH 7.4, respectively. The desolvation gas flow was 900 l/h, and the time-of-flight resolution was approximately 8000 (m/Δm). Argon was used for collision-activated dissociation experiments at a pressure setting of 13 psig, and the collision offset for acquiring tandem mass spectra varied between 20 and 35 eV, depending on the metabolite. The quadrupole radiofrequency setting was 0.2.

**Results**

**Metabolism of Prazosin in NADPH- and UDPGA-Fortified Liver Microsomes.** Incubation of prazosin with liver microsomes fortified with NADPH and UDPGA revealed qualitatively similar metabolite profiles among various species (Fig. 2). Several drug-related components were identified in the microsomal incubation mixture, including those that have been identified previously from in vivo studies. A number of new metabolites that have not been identified before were also characterized by LC/MS. Following incubation for 2 h, prazosin remained the largest component in all the species based on UV response (A = 334 nm). LC/MS analysis indicated the presence of MH+ ions at m/z 290 (M1), 370 (M3, M6), 382 (M5, M8), 384 (prazosin), 400 (M2), 404 (M4), and 418 (M7) (see Table 1).

In human liver microsomal incubations containing both NADPH and UDPGA, LC/MS analysis indicated MH+ ions at m/z 546 (M10, M11) and m/z 560 (M13) in addition to those listed above. These three additional metabolites were also observed in rat and dog microsomal incubations and eluted earlier than prazosin, between 25 and 27 min (Fig. 2). Although not apparent in Fig. 2, when comparing extracted ion chromatograms for m/z 546 and m/z 560 individually, species differences were evident (data not shown). In particular, for M10, human > rat > dog; M11, rat > human >> dog; and M13, human >> rat – dog.

**Metabolism of Prazosin in NADPH- and GSH-Fortified Liver Microsomes.** In the presence of both NADPH and GSH, LC/MS analysis indicated additional MH+ ions at m/z 709 (M9, M12, and M14). These three additional metabolites were observed in rat, dog, and human microsomal incubations and eluted earlier than prazosin, between 26 and 28 min (data not shown). Comparing between species, M9 was greatest in humans, whereas M12 was predominant in dogs. In rats, although observed, these metabolites were formed in trace amounts only.

**Metabolism of Prazosin in Cryopreserved Rat and Human Hepatocytes.** In rat and human cryopreserved hepatocytes, LC/MS analysis revealed many of the same metabolites that were observed in the microsomal incubations with the exception of the GSH conjugates and M11. The profiles produced by rat and human hepatocytes were very similar, compared to investigate the stability of these metabolites. After 24 h, M2 had decreased only slightly compared with levels initially present. In contrast, M5, which initially was easily detectable, was absent at 24 h, whereas M8 had more than doubled.

**Identification of Prazosin Metabolites.** Prazosin had an HPLC retention time of about 40.9 min and showed a protonated molecular ion (MH+) at m/z 384. The proposed fragmentation scheme and product ions of m/z 384 for prazosin are shown in Fig. 1. Cleavage of the amide bond generated m/z 288, representing the amino-dimethoxyquinazoline piperazine system, and m/z 95, the acylammonium ion representing the 2-furanoyl moiety, respectively. Cleavage of the piperazine ring with charge retention on the amino-dimethoxyquinazoline or furan side of the molecule yielded product ions at m/z 247 or 138, respectively. Cleavage of the amino-dimethoxyquinazoline portion of prazosin generated the product ions at m/z 205, 204, and 203 following gain of one or two protons or loss of one proton, respectively. LC/MS conducted with D2O revealed an MD+ at m/z 387 consistent with the two exchangeable amino protons.

**M1.** Metabolite M1 had a retention time of about 17.7 min on the HPLC system and showed an MH+ at m/z 290, 94 Da less than for prazosin, suggesting it was a hydrolysis product. LC/MS conducted with D2O produced an MD+ at m/z 294 and indicated three exchangeable protons, one more than prazosin (data not shown). The product ion at m/z 247, identical to prazosin, indicated an unchanged amino-dimethoxyquinazoline group and ethylamine of piperazine. However, the acylammonium ion at m/z 95 representing the 2-furanoyl moiety was absent, which was consistent with loss of the furoyl moiety after hydrolysis of the amide bond. Therefore, M1 was tentatively identified as N-desfuranoyl prazosin.

**M2.** Metabolite M2 had a retention time of about 31.4 min on the HPLC system and showed an MH+ at m/z 400, 16 Da larger than for...
Methyl prazosin, respectively. M3 and M6 were tentatively identified as 6-desmethyl and 7-desmethyl prazosin, respectively. Therefore, M5 was tentatively identified as either 4-(4-amino-6,7-dimethoxy-quinazolin-2-yl)-1-(furan-2-carbonyl)-2,3,4,5-tetrahydro-pyrazin-1-ium or 1-(4-amino-6,7-dimethoxy-quinazolin-2-yl)-4-(furan-2-carbonyl)-2,3,4,5-tetrahydro-pyrazin-1-ium. Hydrogenation of the piperazine ring are consistent with these data. Therefore, M2 was tentatively identified as hydroxyprazosin, indicating it had undergone mono-oxidation. LC/MS conducted with D₂O produced an MD⁺ at m/z 404 and indicated three exchangeable protons, one more than prazosin, consistent with hydroxylation (data not shown). Product ions at m/z 247, 205, and 138, of which only m/z 263 was 16 Da higher than the corresponding ion for prazosin at m/z 247, indicated hydroxylation had occurred on the piperazine ring. Therefore, M2 was tentatively identified as hydroxyprazosin.

M3 and M6. Metabolites M3 and M6 had retention times of about 36.9 and 38.0 min on the HPLC system, respectively, and showed MH⁺ at m/z 370, 14 Da less than prazosin, indicating that demethylation had occurred. LC/MS conducted with D₂O produced an MD⁺ at m/z 374 and indicated three exchangeable protons, one more than prazosin, consistent with demethylation of a methoxy group to reveal a hydroxy function (data not shown). Mass spectral data for M3 and M6 were identical. Product ions at m/z 233 and 190 were 14 Da less than the corresponding ions at m/z 247 and 204 for prazosin, supporting a single demethylation event on the amino-dimethoxyquinazoline group. Previously it was reported that the major demethylated metabolite in vivo was 6-desmethyl prazosin (Taylor et al., 1977), and we observed that the abundance of M3 was larger than M6. Therefore, M3 and M6 were tentatively identified as 6-desmethyl and 7-desmethyl prazosin, respectively.

M4. Metabolite M4 had a retention time of about 37.4 min on the HPLC system and showed an MH⁺ at m/z 404, 20 Da higher than for prazosin. LC/MS conducted with D₂O produced an MD⁺ at m/z 408 and indicated three exchangeable protons, one more than prazosin (data not shown). The proposed fragmentation scheme and product ions of m/z 404 mass spectrum for M4 are shown in Fig. 4. Loss of 18 Da from MH⁺ generated m/z 386 and indicated the presence of an aliphatic hydroxyl group. The product ion at m/z 290, identical to the MH⁺ of M1, indicated an unchanged amino-dimethoxyquinazoline piperazine system. No product ion at m/z 95 was observed as for prazosin, which indicated metabolism of the furan ring had occurred. Ring opening of furan produced a hydroxybutyryl group (HO-CH₂CH₂CH₂-CO-) that generated the product ion at m/z 87. In D₂O, the product ion at m/z 87 shifted to m/z 88. Therefore, M4 was tentatively identified as 5-[4-(4-amino-6,7-dimethoxy-quinazolin-2-yl)-piperazin-1-yl]-4,5-dioxo-pentan-1-ol.

M5. Metabolite M5 had a retention time of about 37.6 min on the HPLC system and showed a protonated MH⁺ at m/z 382, 2 Da less than prazosin, indicating dehydrogenation had occurred. The proposed fragmentation scheme for M5 is shown in Fig. 5. Product ions at m/z 245 and 205, of which m/z 245 was 2 Da less than the corresponding ion at m/z 247 for prazosin, indicated dehydrogenation of the piperazine ring. LC/MS conducted with D₂O revealed an MD⁺ at m/z 384, which indicated two exchangeable protons, identical to prazosin, and also consistent with a metabolite not requiring protonation for acquiring a charge. Moreover, in D₂O the product ion at m/z 245 only shifted 2 Da to m/z 247, whereas in prazosin the corresponding ion at m/z 247 shifted to m/z 250. These observations indicated that M5 possessed a quaternary nitrogen. Two structures in which either one or the other piperazine nitrogen is involved in a carbon-quaternary nitrogen double bond (i.e., iminium ion) following dehydrogenation of the piperazine ring are consistent with these data. Therefore, M5 was tentatively identified as either 4-(4-amino-6,7-dimethoxy-quinazolin-2-yl)-1-(furan-2-carbonyl)-2,3,4,5-tetrahydro-pyrazin-1-i um or 1-(4-amino-6,7-dimethoxy-quinazolin-2-yl)-4-(furan-2-carbonyl)-2,3,4,5-tetrahydro-pyrazin-1-i um.

M7. Metabolite M7 had a retention time of about 39.0 min on the HPLC system and showed a protonated MH⁺ at m/z 418, 34 Da more than for prazosin. LC/MS conducted with D₂O produced an MD⁺ at m/z 422 and indicated three exchangeable protons, one more than prazosin (data not shown). The proposed fragmentation scheme and product ions of m/z 418 mass spectrum for M7 are shown in Fig. 6.

TABLE 1

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Rt (min)</th>
<th>MH⁺ (m/z)</th>
<th>MD⁺ (m/z)</th>
<th>m/z of Select Product Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prazosin</td>
<td>40.9</td>
<td>384</td>
<td>387</td>
<td>288, 247, 231, 204, 164, 138, 120, 95</td>
</tr>
<tr>
<td>M1</td>
<td>17.7</td>
<td>290</td>
<td>294</td>
<td>247, 231, 221, 203</td>
</tr>
<tr>
<td>M2</td>
<td>31.4</td>
<td>400</td>
<td>404</td>
<td>382, 287, 263, 245, 205, 138, 95</td>
</tr>
<tr>
<td>M3</td>
<td>36.9</td>
<td>370</td>
<td>374</td>
<td>352, 233, 218, 190, 164, 138, 120, 95</td>
</tr>
<tr>
<td>M4</td>
<td>37.4</td>
<td>404</td>
<td>408</td>
<td>386, 370, 290, 247, 221, 205, 140, 87</td>
</tr>
<tr>
<td>M5</td>
<td>37.6</td>
<td>382</td>
<td>384</td>
<td>287, 271, 245, 229, 205, 95</td>
</tr>
<tr>
<td>M6</td>
<td>38.0</td>
<td>370</td>
<td>374</td>
<td>352, 233, 218, 190, 164, 138, 120, 95</td>
</tr>
<tr>
<td>M7</td>
<td>39.0</td>
<td>418</td>
<td>422</td>
<td>402, 290, 247, 231, 205, 126, 101</td>
</tr>
<tr>
<td>M8</td>
<td>44.3</td>
<td>382</td>
<td>385</td>
<td>287, 271, 245, 229, 205, 95</td>
</tr>
<tr>
<td>M9</td>
<td>30.8</td>
<td>709</td>
<td>719</td>
<td>691, 562, 436, 418, 386, 290, 247</td>
</tr>
<tr>
<td>M10</td>
<td>31.5</td>
<td>546</td>
<td>553</td>
<td>370, 352, 233, 138</td>
</tr>
<tr>
<td>M11</td>
<td>32.7</td>
<td>546</td>
<td>553</td>
<td>370, 352, 233, 138</td>
</tr>
<tr>
<td>M12</td>
<td>33.4</td>
<td>709</td>
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<tr>
<td>M13</td>
<td>33.2</td>
<td>560</td>
<td>566</td>
<td>456, 426, 384, 247, 233, 138, 95</td>
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<td>M14</td>
<td>34.7</td>
<td>709</td>
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<tr>
<td>M15</td>
<td>32.4</td>
<td>396</td>
<td>399</td>
<td>290, 247, 231, 203, 176, 150, 107</td>
</tr>
</tbody>
</table>

a Trapped as a pyridazine product.

FIG. 3. Extracted ion chromatogram for prazosin and metabolites, including the glucuronides produced in cryopreserved hepatocytes from human (A) and rat (B).
The product ion at \( m/z \) 290, identical to the MH\(^+\) of M1, indicated an intact amino-dimethoxyquinazoline piperazine system. No product ion at \( m/z \) 95 was observed as for prazosin, which indicated metabolism of the furan ring had occurred. Ring opening of furan produced a carboxypropionyl group (HOOC-CH\(_2\)-CH\(_2\)-CO\(-\)) that generated the product ion at \( m/z \) 101. In \( D_2O \), the product ion at \( m/z \) 101 shifted to \( m/z \) 102. Loss of 46 Da (HCOOH) from MH\(^+\) generated \( m/z \) 372 and supported the presence of a carboxylic acid. Therefore, M7 was tentatively identified as 5-[4-(4-amino-6,7-dimethoxy-quinazolin-2-yl)-piperazin-1-yl]-4,5-dioxo-pentanoic acid.

M8. Metabolite M8 had a retention time of about 44.3 min on the HPLC system and showed a protonated MH\(^+\) at \( m/z \) 382, 2 Da less than prazosin, indicating dehydrogenation had occurred. In \( D_2O \), M8 had an identical spectrum to M5, including observation of product ions at \( m/z \) 245 and 205, of which \( m/z \) 245 was 2 Da less than the corresponding ion at \( m/z \) 247 for prazosin, indicating dehydrogenation of the piperazine ring had occurred (see Fig. 5 and insert). However, in contrast to M5, LC/MS conducted with \( D_2O \) revealed an MD\(^+\) at \( m/z \) 385 for M8 rather than \( m/z \) 384 (data not shown). Moreover, in \( D_2O \) the product ion at \( m/z \) 245 shifted 3 Da to \( m/z \) 248 rather than to \( m/z \) 247 as observed for M5. These observations support a carbon-carbon double bond (rather than a carbon-quaternary nitrogen double bond) requiring protonation for MS detection. Therefore, M8 was tentatively identified as 2-[4-(2-furanoyl)-3,4-dihydro-2\(H\)-pyrazin-1-yl]-4-amino-6,7-dimethoxyquinazoline.

M10 and M11. Metabolites M10 and M11 had retention times of about 31.5 and 32.7 min on the HPLC system, respectively, and showed a protonated MH\(^+\) at \( m/z \) 546, 162 Da greater than prazosin, indicating they were conjugates. Furthermore, they were both 176 Da larger than the metabolites with \( m/z \) 370, suggesting that they were glucuronide conjugates of the demethylated metabolites M3 and M6. Mass spectral data for M10 and M11 were similar. LC/MS conducted with \( D_2O \) produced an MD\(^+\) at \( m/z \) 553 and indicated six exchangeable protons (data not shown), four more than prazosin and consistent with the introduction of glucuronic acid. The characteristic neutral loss of 176 Da from MH\(^+\) generated the aglycone at \( m/z \) 370, further supporting the presence of a glucuronide. As described above, M3 was the major O-demethylated metabolite. Because M10 was more abundant than M11, M10 and M11 were tentatively identified as glucuronides of 6-O-desmethyl and 7-O-desmethyl prazosin, respectively.

M13. Metabolite M13 had a retention time of about 33.2 min on the HPLC system and showed a protonated MH\(^+\) at \( m/z \) 560, 176 Da more than for prazosin, indicating it was a conjugate. The characteristic neutral loss of 176 Da from \( m/z \) 560 generated the aglycone at \( m/z \) 384, indicating a glucuronide. LC/MS conducted with \( D_2O \) produced an MD\(^+\) at \( m/z \) 566 and indicated five exchangeable protons (data not shown), three more than prazosin and consistent with the introduction of glucuronic acid on the amino group. The quinazoline amino group was the only possible site on unchanged prazosin capable of undergoing conjugation. Therefore, M13 was identified as prazosin N-glucuronide.

![FIG. 4. Product ions of \( m/z \) 404 mass spectrum of M4 and the proposed origin of key product ions.](image)

![FIG. 5. Product ions of \( m/z \) 382 mass spectrum of M5 (mass spectrum of M8 identical) and the proposed origin of key product ions. For M5, it is unknown which piperazine nitrogen is quaternary. The insert depicts one of two possibilities for the piperazine ring of M5 and the dehydro-piperazine in M8.](image)
M9, M12, and M14. Metabolites M9, M12, and M14 had retention times of about 30.8, 33.4, and 34.7 min, respectively, on the HPLC system and showed protonated MH$^+$ at m/z 709, which was 325 Da more than for prazosin, indicating they were conjugates. These metabolites were also 305 Da higher than M4, suggesting that M9, M12, and M14 were GSH conjugates derived from M4. LC/MS conducted with D$_2$O produced an MD$^+$ at m/z 719 and indicated nine exchangeable protons (data not shown), seven more than prazosin and consistent with the introduction of GSH. The proposed fragmentation scheme and product ions of M9 mass spectrum (similar to spectra for M12 and M14, not shown) are shown in Fig. 7. Loss of 129 Da (pyroglutamic acid moiety) from MH$^+$ with subsequent loss of H$_2$O generated m/z 562, supporting the presence of GSH. The product ion at m/z 290, identical to the MH$^+$ of M1, indicated an intact amino-dimethoxyquinazoline piperazine system. No product ion at m/z 95 was observed for prazosin, which indicated furan had undergone metabolism. The product ion at m/z 418 was a result of addition of GSH to the carbonyl hydroxybutyryl group (HO-CH$_2$CH$_2$CH$_2$-CO-) formed following oxidative cleavage of furan. Therefore, M9, M12, and M14 were tentatively proposed to be GSH conjugates.

**Trapping Reactive Intermediate of Prazosin.** To further investigate the metabolism of the furan ring of prazosin to a reactive metabolite, M15, incubations were conducted in the presence of 2 mM semicarbazide hydrochloride. The semicarbazide-trapped product of M15 had a retention time of about 32.4 min on the HPLC system (Fig. 8) and showed a protonated MH$^+$ at m/z 396, 12 Da higher than for prazosin. LC/MS conducted with D$_2$O produced an MD$^+$ at m/z 399 and indicated two exchangeable protons (data not shown), identical to prazosin. The proposed fragmentation scheme and product ions of the semicarbazide-trapped product of M15 mass spectrum are shown in Fig. 9. The product ion at m/z 290, identical to the MH$^+$ of M1, indicated an unchanged amino-dimethoxyquinazoline piperazine system. No product ion at m/z 95 was observed for prazosin, which indicated furan had undergone metabolism. The observation of an ion at m/z 107 was consistent with a pyridazine acylium ion. Therefore,
the semicarbazide-trapped product of M15 was tentatively identified as 1-[4-amino-6,7-dimethoxy-2-quinazoline]-4[2-pyrazidine]-piperazine.

Discussion

One objective of this work was to use current in vitro methodology and analytical tools to study the metabolism of prazosin and to determine the correlation with in vivo data (Taylor et al., 1977; Piotrovskii et al., 1984). Further understanding prazosin metabolism remains relevant because of renewed interest in prazosin for PTSD. In contrast to the first investigations (Taylor et al., 1977), which used a combination of thin-layer chromatography and gas-LC or solid probe mass spectrometric techniques, we used LC/MS for characterization of metabolites. Microsomes and hepatocytes are now routinely used to investigate metabolism of new chemical entities and are important tools in lead optimization of drug candidates (Ekins et al., 2000). In our studies, the major metabolites detected in rats and dogs were also generated in vitro and characterized by LC/tandem MS. Microsomes and hepatocytes formed 6-O- and 7-O-desmethyl prazosin (M3 and M6) in the expected relative abundance as observed in vivo, i.e., 6-O- > 7-O-desmethyl prazosin. The in vivo metabolite N-desfuranylo prazosin (M1) is expected to result from amidase activity. We identified M1 in microsomes and hepatocytes, although it was more abundant in hepatocytes. Conversion of M1 to DQ was reported to occur in rats and dogs (Taylor et al., 1977). Although we observed DQ in microsomal incubations, as its formation was not NADPH-dependent and it was also seen in our prazosin stock, it was not deemed a metabolite in our studies. In addition, several new metabolites formed by oxidation of the piperazine ring (M2, M5, and M8), furan cleavage (M4 and M7), GSH conjugation (M9, M12, and M14), and N-glucuronidation (M13) were also characterized. These proposed metabolic pathways are shown in Figs. 10 and 11.

In the presence of UDPGA, microsomes produced two O-glucuronides, M10 and M11, derived from M3 and M6, respectively, although in hepatocytes only M10 was detected. O-Glucuronides had been proposed to form in vivo based on experiments in which Glusulase (PerkinElmer, Boston, MA) treatment of urine generated aglycones identical to synthetic 6-O- and 7-O-desmethyl prazosin (Taylor et al., 1977). In humans these glucuronides have not been reported. In addition to O-glucuronides, we also obtained direct evidence for prazosin N-glucuronide (M13) in microsomal incubations supplemented with UDPGA. M13 was not detected in previous animal studies perhaps because of its instability under the extraction and isolation conditions used. Indirect evidence for an N-glucuronide of doxazosin, a close structural analog of prazosin, was obtained based on its colorimetric reaction with naphthoresorcinol and its resistance to β-glucuronidase activity (Kaye et al., 1986). In mouse, it was a major metabolite constituting approximately 17% of the dose, although it was not detected in humans (Kaye et al., 1986). In our studies, M13 was formed in greater amounts than either M10 or M11. With respect to the glucuronide metabolites (M10, M11, and M13), humans showed better concordance with dogs than with rats.

Hydroxylation on the piperazine ring formed M2, a carbinolamine. Carbinolamines are well recognized metabolic products, although acyclic tertiary amines are often unstable and are converted to the corresponding aldehyde (or ketone) and N-dealkylated amine (Rose and Castagnoli, 1983). However, for cyclic tertiary amines, the carbinolamine is reported to remain in equilibrium with the iminium species (Sayre et al., 1997). Our stability results suggest that M2 is stable based on its continued detection for at least 24 h. We cannot assign which carbon of the piperazine group is hydroxylated by the product ion spectrum. However, Prakash and Soliman (1997) characterized two carbinolamines of a drug candidate formed in rat and argued that delocalization of the nitrogen lone pair electrons, made possible by a pyrimidine or succinimide substituent, imparted stability to these carbinolamines. For M2, these considerations could justify hydroxylation on any of the piperazine carbons as each nitrogen substituent (quinazoline or carbonyl) could allow electron delocalization, although the carbon closest to the carbonyl group might be more likely as it is expected to have greater positive character as a result of the electronegativity of oxygen. M2 is expected to be in equilibrium with the iminium species, M5; yet with time, M5 is converted to the more stable enamine, M8. Uncertainty as to the site of hydroxylation for M2 discussed above prevents knowing which of two possible iminium structures for M5 exist (see Fig. 5), and a definitive structure awaits isolation and NMR characterization (in progress).

Evidence for furan bioactivation to a reactive γ-keto-α,β-unsaturated aldehyde, M15, included 1) detection of ring-opened metabolites (M4 and M7), 2) trapping M15 with semicarbazide, and 3) detection of GSH conjugates. The proposed pathway leading to these products is shown in Fig. 11. Kobayashi et al. (1987a) reported that metabolism of TA-1801 converted furan first to a hydroxybutyryl group and then to a carboxypropionic acid. To explain these ring-opened metabolites, they proposed that furan was metabolized by P450 directly to a γ-keto-α,β-unsaturated aldehyde (Kobayashi et al., 1987b), although an epoxide, as suggested by Le Fur and Labaune (1985) based on studies with diclofurne, may also generate the γ-keto-α,β-unsaturated aldehyde. M4 and M7 are also hydroxybutyryl and carboxypropionic acid-containing metabolites, respectively, and are consistent with Kobayashi’s observations. Semicarbazide has been used to trap
an unsaturated γ-keto-α,β-unsaturated aldehyde derived from pulegone (McClanahan et al., 1989) as a pyridazine product, and we also successfully trapped the γ-keto-α,β-unsaturated aldehyde (M15) derived from prazosin with semicarbazide. Finally, semicarbazide greatly reduced the formation of M4 and M7 (Fig. 8) consistent with their formation via M15.

Generation of M4 and M7, the former requiring a net 4-electron reduction, may be rationalized based on consideration of possible enzymes involved in their formation via M15. Many reactive γ-keto-α,β-unsaturated aldehydes are toxic, and the body has several enzymes to detoxify them. Metabolism of the aldehyde to an alcohol (e.g., carbonyl reductase), oxidation to an acid (e.g., aldehyde dehydrogenase or alcohol dehydrogenase), and conjugation with GSH represent detoxification events (Dick et al., 2001). Double bond reduction by hepatic NAD(P)H oxidoreductase also abolishes reactivity of α,β-unsaturated carbonyls (Dick et al., 2001). Carbon-carbon double bond reductive metabolism has been reported for drugs containing this α,β-unsaturated carbonyl structure (Lindstrom and Whittaker, 1984; Taskinen et al., 1991), and it is possible that NAD(P)H oxidoreductase is also involved in the metabolism of prazosin.

M15 has several sites capable of reacting with GSH. 1,4-Addition of GSH across the double bond with reduction of the aldehyde could produce two distinct GSH conjugates. 1,2-Addition of GSH to the aldehyde with reduction of the double bond would generate a GSH conjugate with identical mass. These GSH conjugates, especially that formed through reaction with the aldehyde, may be reversible, although their low abundance precluded a kinetic investigation. It is tempting to assign the three GSH conjugates observed in our incubations to the three possibilities described above. However, we cannot exclude the possibility that two HPLC peaks represent diastereomers. Therefore, until isolated GSH conjugates are further characterized by NMR it is unwise to speculate on the exact structure of these conjugates. In our cryopreserved hepatocyte experiments, although furan ring opening was evident based on the presence of M4 and M7, no GSH conjugates were observed. This observation may reflect decreased GSH concentrations as a result of the cryopreservation pro-
cess, which can be less than 10% of fresh hepatocytes (Sohlenius-Sternbeck and Schmidt, 2005).

Whether our in vitro findings of new metabolites of prazosin correlate with in vivo metabolism of prazosin will require further studies (in progress). Instrumentation available today can enable detection of even minor metabolites in vivo. From a toxicology perspective, however, furan bioactivation may be irrelevant because the toxicity profile of prazosin is established. The primary adverse reaction is postural hypotension and syncope, especially on initiation of medication, a result of its pharmacological activity (Hoffman and Lefkowitz, 1990). No reports of idiosyncratic toxicity to prazosin are in the literature. Idiosyncratic drug toxicity is often explained by electrophilic reactive intermediates that covalently modify protein and initiate a damaging immune response (Uetrecht, 2003). The risk of causing idiosyncratic drug toxicity is often a justification for implementing screening strategies for reactive metabolites (Caldwell and Yan, 2006). We did not determine whether covalent binding to protein occurred and, if so, what levels were reached. Thus, we cannot speculate whether prazosin would have this liability based on guidelines used by some firms (Evans and Baillie, 2005). Kalogutkar and Nguyen (2005) have shown with loperamide that although bioactivation to a potentially neurotoxic pyridinium metabolite was detected in vitro, the absence of such toxicity in humans when taken as prescribed might reflect mitigating events such as P-glycoprotein activity preventing brain accumulation. Our work with prazosin provides another reminder that toxicity is often multifactorial and bioactivation does not always lead to toxicity. Furthermore, Uetrecht (2001) has observed that idiosyncratic toxicity is seldom associated with drugs given at doses of 10 mg or less. The absence of idiosyncratic toxicity associated with prazosin, administered at a maximal daily dose of 5 mg for hypertension (Hoffman and Lefkowitz, 1990) or a mean dose of 9.5 mg/day for PTSD (Raskind et al., 2003), supports this empirical observation.

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