IDENTIFICATION OF A HYDROXYLAMINE GLUCURONIDE METABOLITE OF AN ORAL HYPOGLYCEMIC AGENT

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ABSTRACT:

Glucuronides of piperazine hydroxylamines are rarely reported in the literature, and even more rarely are their structures unambiguously identified. One major metabolite was detected by liquid chromatography/mass spectrometry, ammonium acetate; TFA, high-performance liquid chromatography; NH₂OAc, ammonium acetate; TFA, trifluoroacetic acid; MeOH, methanol; UDPGA, uridine diphospho-5'-glucuronic acid.

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The oxidative metabolism of N-aryl or N-alkyl piperazines is typically characterized by carbon oxidation or oxidation of the tertiary amine to give the N-oxide. Metabolites, typified in the in vivo metabolism of ketoconazole, ciprofloxacin, the inotropic agent OPC-24361, and the structures conjugation of alicyclic hydroxylamines in the literature (Straub et al., 1972; Achari and Beckett, 1983; Rodriguez and Acosta, 1997; Zhang et al., 1998; Delbressine et al., 1992; Schaber et al., 2001), and the structures exhibited by the Labeled Compound Synthesis Group (Merck Research Laboratories). The purity of the labeled and unlabeled compounds was >98.5%. HPLC grade solvents were from Fisher Scientific Co. (Fair Lawn, NJ). Glucose 6-phosphate, glucose-6-phosphate dehydrogenase (type V from baker’s yeast), β-glucuronidase (type HA-4 from Helix aspersa), glycerol, polyoxyethylene 9 lauryl ether (Labrol PX), and NADP⁺ were purchased from Sigma-Aldrich (St. Louis, MO). EDTA, ammonium acetate, and TFA were purchased from Aldrich Chemical Co. (Milwaukee, WI). Dibasic potassium phosphate, monobasic sodium phosphate, potassium chloride, and magnesium chloride were obtained from Mallinckrodt (St. Louis, MO).

Animal Studies. Animal studies were conducted with approval of the Institutional Animal Care and Use Committee. One male rhesus monkey (3 kg) was dosed orally with 14C-labeled I (5 μCi/mg; 5 mg/kg at 1.5 mg/ml) dissolved in isotonic saline solution. Two additional monkeys (7.6 and 8 kg) were dosed orally with radiolabeled I (5 mg/kg; 2.5 mg/ml in isotonic saline). Oral doses were administered via a nasogastric tube. All animals were

Materials and Methods

Chemicals. 9-[(1S,2R)-2-fluoro-1-methylpropyl]-2-methoxy-6-(1-piperazinyl) purine hydrochloride I was obtained from Merck Research Laboratories (West Point, PA). 9-[(1S,2R)-2-fluoro-1-methylpropyl]-2-methoxy-6-(1-piperazinyl)-2-[14C]purine hydrochloride I (28.54 μCi/mg) was synthesized by the Labeled Compound Synthesis Group (Merck Research Laboratories). The purity of the labeled and unlabeled compounds was >98.5%. HPLC grade solvents were from Fisher Scientific Co. (Fair Lawn, NJ). Glucose 6-phosphate, glucose-6-phosphate dehydrogenase (type V from baker’s yeast), β-glucuronidase (type HA-4 from Helix aspersa), glycerol, polyoxyethylene 9 lauryl ether (Labrol PX), and NADP⁺ were purchased from Sigma-Aldrich (St. Louis, MO). EDTA, ammonium acetate, and TFA were purchased from Aldrich Chemical Co. (Milwaukee, WI). Dibasic potassium phosphate, monobasic sodium phosphate, potassium chloride, and magnesium chloride were obtained from Mallinckrodt (St. Louis, MO).
fasted for 16 h before dosing. Urine and feces were collected for 48 h post dose.

**Biological Preparations.** Pig liver microsomes were prepared as described previously (Ziegler and Poulsen, 1978). Microsomes were prepared as described previously (Levin et al., 1972) from monkey livers that were removed within 30 min of death, frozen in liquid nitrogen, and stored at −80°C. Protein concentrations were determined by using the Lowry method (Lowry et al., 1951). The hydroxylamine glucuronide of 1 (100 µg, 40 µM) was incubated for 18 h at 37°C with 25,000 units of β-glucuronidase in 5 ml of 20 mM sodium acetate buffer, pH 5.

**Metabolite Extraction.** Typically, aliquots of the incubation medium (0.5–1 ml) were passed through a 3-ml Analytichem BondElut C18 solid-phase extraction cartridge (Varian, Inc., Palo Alto, CA) that was prewashed with 5 ml of 5 ml of water and eluted with 70 ml of MeOH. Methanol was evaporated in a 250-ml round-bottom flask on a water bath at 37°C and then reconstituted in 100 to 200 μl of mobile phase A (see above), and 25 to 50 μl was analyzed by HPLC.

**Preparative Scale Biosynthesis of Hydroxylamine Glucuronide (3). Oxidation.** Pig liver microsomes (final concentration 2.6 mg protein/ml; 0.042 nmol cytochrome P450/ml) were suspended in 50 ml of 0.1 M glycine buffer, pH 8.5, containing 1 mM EDTA in a 250-ml Erlenmeyer flask. Glucose 6-phosphate (3.3 mM), magnesium chloride (3.3 mM), and NADP+(1.3 mM) were added to the suspension along with 3 mg of [14C]-labeled glucose 6-phosphate dehydrogenase (1 unit/ml). After 1-h incubation, the medium was passed through a 60-ml C18 BondElut cartridge that had been pre-equilibrated by washing with 50 ml of water, and retained metabolites were eluted with 70 ml of MeOH. Methanol was evaporated at 35°C under nitrogen in a 250-ml round-bottom flask on a water bath at 37°C and then reconstituted in 100 to 200 μl of mobile phase A (see above), and 25 to 50 μl was analyzed by HPLC.

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**TABLE 1**

<table>
<thead>
<tr>
<th>1H NMR chemical shifts for 1, hydroxylamine 2, and hydroxylamine glucuronide 3 (in CD3OD at 400 MHz)</th>
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<tr>
<td>1 Aryl piperazine</td>
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<tr>
<td>7.97 (d, 1H, J1, 1H = 11, H-8), 4.95 (ddq, 1H, J1, 1H = 48.2; JHM, 1H = 42, 6.3 Hz, FCH2CH3), 4.68 (ddq, 1H, JHM, 1H = 21.1; JHM, 1H = 4.2, 7.1 Hz, NCH2CH3), 4.48 (br m, 4H, piperazine), 3.95 (s, 3H, OMe), 3.29 (br m, 4H, piperazine, 1.62 (dd, 3H, JHM, 1H = 0.9; JHM, 1H = 7.1 Hz, NCH2CH3), 1.34 (d, 3H, JHM, 1H = 24.0; JHM, 1H = 6.3, FCH2CH3)</td>
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<tr>
<td>2 Hydroxylamine</td>
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<tr>
<td>7.97 (d, 1H, J1, 1H = 11, H-8), 5.23 (br m, 2H, piperazine eq), 4.93 (ddq, 1H, J1, 1H = 48.7; JHM, 1H = 4.2, 6.3 Hz, FCH2CH3), 4.65 (ddq, 1H, J1, 1H = 21.0; JHM, 1H = 4.4, 7.3 Hz, NCH2CH3), 3.93 (s, 3H, OMe), 3.40 (br m, 2H, piperazine ax), 3.27 (br m, 2H, piperazine eq), 2.63 (br m, 2H, piperazine ax), 1.61 (dd, 3H, JHM, 1H = 1.0; JHM, 1H = 7.1 Hz, NCH2CH3), 1.33 (dd, 3H, JHM, 1H = 24.0; JHM, 1H = 6.3, FCH2CH3)</td>
</tr>
<tr>
<td>3 Glucuronide</td>
</tr>
<tr>
<td>7.97 (d, 1H, J1, 1H = 11, H-8), 5.23 (br m, 2H, piperazine eq), 4.93 (ddq, 1H, J1, 1H = 48.7; JHM, 1H = 4.2, 6.3 Hz, FCH2CH3), 4.68 (d, 1H, J = 8.4 Hz, anameric proton) 4.65 (ddq, 1H, J1, 1H = 21.0; JHM, 1H = 4.4, 7.3 Hz, NCH2CH3), 3.93 (s, 3H, OMe), 3.40 (br m, 2H, piperazine ax), 3.33, 3.39, 3.41, 3.61 (m, 4H, sugar protons), 3.27 (br m, 2H, piperazine eq), 2.63 (br m, 2H, piperazine ax), 1.61 (dd, 3H, JHM, 1H = 1.0; JHM, 1H = 7.1 Hz, NCH2CH3), 1.33 (dd, 3H, JHM, 1H = 24.0; JHM, 1H = 6.3, FCH2CH3)</td>
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**TABLE 2**

<table>
<thead>
<tr>
<th>13C NMR chemical shifts for 1, hydroxylamine 2, and hydroxylamine glucuronide 3 (in CD3OD at 125 MHz)</th>
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<tr>
<td>1 Aryl piperazine</td>
</tr>
<tr>
<td>162.9 (C-2), 155.5 and 154.3 (C-4 and C-6), 139.0 (C-8), 117.1 (C-5), 92.5 (d, JCP, 2H = 172.9 Hz, CHF), 55.3 (d, JCP, 2H = 24.0 Hz, 55.2 (OMe), 44.8 (piperazine, C-3’-5’), 43.9 (br, piperazine, C-2’-6’), 17.7 (d, JCP, 2H = 22.2 Hz, FCH2CH3), 14.1 (d, JCP, 2H = 4.6 Hz, FCH2CH3)</td>
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<tr>
<td>2 Hydroxylamine</td>
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<tr>
<td>163.0 (C-2), 155.6 and 154.0 (C-4 and C-6), 138.5 (C-8), 117.0 (C-5), 92.3 (d, JCP, 2H = 177 Hz, CHF), 58.8 (piperazine, C-3’-5’), 55.2 (JCP, 2H = 23 Hz, 55.1 (OMe), 44.8 (piperazine, C-2’-6’), 17.8 (d, JCP, 26 Hz, FCH2CH3), 14.1 (d, JCP, 2H = 4.7 Hz, FCH2CH3)</td>
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<tr>
<td>3 Glucuronide</td>
</tr>
<tr>
<td>176.7 (Glu, CO2H), 162.9 (C-2), 155.4 and 153.9 (C-4 and C-6), 138.3 (C-8), 116.9 (C-5), 106.6 (Glu, anomic carbon), 92.3 (d, JCP, 2H = 172.9 Hz, CHF), 78.3, 76.4, 73.7, 73.4 (4 carbons, Glu), 58.3 and 56.8 (piperazine, C-3’-5’), 55.2 (JCP, 2H = 23 Hz, NCH2CH3), 55.1 (OMe), 44.9 (br, piperazine, C-2’-6’), 17.8 (d, JCP, 22 Hz, FCH2CH3), 14.1 (d, JCP, 4 Hz, FCH2CH3)</td>
</tr>
</tbody>
</table>
buffer, pH 7.7, containing 10 mM MgCl₂, 3 mM UDPGA, 2% glycerol, 0.02% Lubrol PX, and 7 mg of hydroxylamine 2 (Huskey et al., 1993). This suspension was incubated for 1 h at 37°C, and the reaction was stopped by freezing. The hydroxylamine glucuronide metabolite 3 (4.6 mg) was purified as described above for the purification of the hydroxylamine (retention time ~5 min). Before NMR analysis, this metabolite was repurified by HPLC on a 4.6 × 250-mm Zorbax RX C8 column eluted at 1 ml/min with a mobile phase consisting of 10% CH₃CN/15% CH₃OH/100 mM NH₄OAc/1% acetic acid. Under these conditions, the glucuronic acid conjugate eluted at approximately 20 min.

HPLC Analysis. The HPLC system consisted of two Shimadzu LC-600 pumps (Kyoto, Japan), a Shimadzu static-bed mixer, and a Rhodyne 7125 injector (Cotati, CA). Chromatography was performed on a DuPont Zorbx RX C8 column (4.6 × 250 mm) eluted at 1 ml/min with a linear gradient from (A), 20% CH₃CN/10 mM NH₄OAc/1% TFA to (B), 40% CH₃CN/10 mM NH₄OAc/1% TFA in 30 min and held at 100% (B) for 10 min. UV detection was performed using a Spectra Physics SpectraFocus scanning UV detector (Spectra Physics, San Jose, CA). Fractions were collected using an Amersham Biosciences fraction collector (Amersham Biosciences, Uppsala, Sweden).

NMR Spectroscopy. NMR spectra were obtained on a Varian Unity 400 or 500 MHz spectrometer. Chemical shifts are given in ppm downfield from tetramethylsilane. Residual solvent signals were used as an internal reference (CD₃OD, 3.30 ppm for proton; 49.0 ppm for ¹³C). Coupling constants (J) are given in hertz.

Mass Spectrometry. Mass spectral analysis was performed by LC/MS on an MDS Sciex (Concord, ON, Canada) API III tandem mass spectrometer using the ionspray interface. Spectra were acquired using positive ion detection, scanning from 150 to 600 Da with a 5- to 10-ms dwell time (orifice potential = 65 V). Argon was used as the collision gas for LC/MS/MS experiments.

Radioactivity Measurement. Radioactivity in incubation extracts or HPLC fractions was determined by counting 0.1- to 1-ml aliquots in glass vials containing 6 ml of liquid scintillation cocktail (Insta-Gel XF; PerkinElmer Life and Analytical Sciences, Boston, MA). Measurement of radioactivity was
performed by liquid scintillation counting using a Beckman LS5000TD liquid scintillation spectrometer (Beckman Coulter, Fullerton, CA).

Results

In a rhesus monkey dosed orally with $^{14}$C-labeled 1 (Fig. 1), approximately 70% of the administered radioactivity was excreted in the urine within 24 h of oral dosing. The HPLC radiohistogram and LC/MS-reconstructed ion current chromatograms ($m/z$ 309 and 501) of an extract of urine collected from 0 to 24 h postdose are shown in Fig. 2. Parent drug and a metabolite of similar polarity (retention time = 29–30 s accounted for ~25 and ~50%, respectively, of the total radioactivity recovered in urine. Both parent drug and metabolite were observed in urine from two other monkeys dosed with unlabeled drug, and the metabolite ratio, determined by comparison of the parent drug ion current to that of the metabolite, was similar in all three monkeys. Based on its mass spectrum, the metabolite gave an $\text{M}^+ m/z$ 501, an addition of 192 Da to the mass of the parent drug. This is equivalent to the addition of an oxygen atom and conjugation with glucuronic acid. Collisional activation of this ion gave the MS/MS spectrum shown in Fig. 3. The product ions at 325, 307, 266, 253, and 240 Da were consistent with monooxidation of the piperazine group. The $^1$H NMR spectrum of this material was consistent with this characterization, since the eight piperazine protons were present as four broad resonances at 2.63, 3.27, 3.40, and 5.23 ppm.

In vitro biosynthesis of these metabolites was pursued to obtain sufficient material for further characterization. Compound 1 was incubated with pig liver microsomes, and the HPLC-UV chromatogram (280 nm) and associated LC/MS-reconstructed ion current chromatogram of the extracts of this incubation are shown in Fig. 5. The metabolite that eluted at 18 min with an $\text{M}^+ m/z$ 325 was
isolated. It coeluted by HPLC and had $^1$H NMR (Fig. 6) and MS/MS spectra identical to those of the product derived from $\beta$-glucuronidase treatment of the major metabolite from monkey urine. From a large-scale incubation of 1 with pig liver microsomes, approximately 7 mg of this metabolite was isolated and purified for $^{13}$C NMR analysis (Fig. 7). Similar to the parent compound, two $^{13}$C signals for the piperazine ring were observed in the spectrum of this metabolite. However, in the metabolite, there was a significant downfield shift of the C3/5 methylene signals of the piperazine ring from 44.8 to 58.8 ppm. The C2/6 methylene signals were shifted only marginally from 43.9 to 44.8 ppm.

The biosynthetic monooxygenated piperazine metabolite was then incubated for 1 h with monkey liver microsomes that contained Lubrol PX and UDPGA to generate glucuronides for $^{13}$C NMR analysis. The reaction was virtually quantitative to one product, and approximately 4.6 mg of this was isolated preparatively. This material coeluted by HPLC and had $^1$H NMR (Fig. 8) and MS/MS spectra identical to those of the major metabolite identified in the urine from monkeys treated with 1. Comparison of the chemical shifts in the $^{13}$C NMR spectrum of the glucuronide (Fig. 9) with those of the related hydroxylamine metabolite revealed a slight upfield shift in the piperazine C3/5 carbons, which were now split into two peaks at 58.3 and 56.8 ppm. There was virtually no change in the C2/6 signals.

**Discussion**

The major metabolite of the aryl piperazine hypoglycemic agent 1 in rhesus monkeys was identified by LC/MS/MS and $^1$H NMR as being both oxygenated and glucuronidated on the piperazine functional group. Assignment of an exact structure, however, was not trivial due in part to extensive line broadening of the proton signals of the piperazine ring observed in its $^1$H NMR spectrum. This broadening was not resolved by changes in either solvent or temperature, rendering them relatively uninformative. This broadening is commonly seen with piperazine rings and is attributed to flipping of the piperazine ring in the time frame of NMR analysis (Straub et al., 1988). To unambiguously determine the glucuronide metabolite structure, sufficient material was needed for analysis by $^{13}$C NMR.

Also of interest and crucial to the structural identification of the glucuronide was the structure of the aglycone. Treatment of the glucuronide metabolite with $\beta$-glucuronidase liberated a derivative that could be partially identified by LC/MS/MS and $^1$H NMR as monooxygenated on the piperazine, although, as with the parent compound, the $^1$H NMR spectrum of the aglycone suffered from broad signals arising from the piperazine protons, precluding its exact structure determination by this technique. However, due to the symmetry of the observed signals, it was clear that the additional oxygen atom was attached to one of the two piperazine nitrogen atoms to give either an N-oxide or a hydroxylamine. This metabolite was stable under the conditions used to isolate as well as characterize it. To assign the structure of this metabolite unequivocally, it was again necessary to obtain a sufficient quantity for $^{13}$C NMR analysis.

To produce sufficient amounts of both the glucuronide and the aglycone and obtain them with adequate purity, methods of in vitro biosynthesis were investigated. The metabolism of 1 was evaluated in

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**Fig. 4.** MS/MS spectrum obtained from collision-induced dissociation of the MH$^+$ ion at m/z 325 of the hydroxylamine 2 resulting from B-glucuronidase treatment of the glucuronide metabolite isolated from monkey urine.
pig liver microsomes because of the expected catalytic capacity of this in vitro system to provide a biosynthetic route to the monooxygenated piperazine compound. Using this in vitro system, sufficient quantities of a metabolite with identical HPLC, MS/MS, and $^1$H NMR characteristics to that of the aglycone were isolated for $^{13}$C NMR analysis. Two signals were attributed to the piperazine carbons in the $^{13}$C NMR spectrum of the aglycone. This corroborated the observation from $^1$H NMR analysis that the symmetry of the piperazine was still intact and limited the site of oxidation to one of the two nitrogens. Additionally, there was a large (14 ppm) downfield shift of only the C3/5 methylene signals of the piperazine, which can be attributed to the $\beta$-effect of the N-OH group, although there was only a marginal shift of the C2/6 methylene signals (0.9 ppm). The significant shift of the C3/5 methylene indicated that the site of oxygen attachment must therefore be on the secondary nitrogen. Using this rationale, the exact structure of the aglycone was established as the hydroxylamine derivative of 1 and not the N-oxide of the tertiary piperazine nitrogen.

Incubation of this biosynthetic hydroxylamine with monkey liver microsomes and UDPGA produced a glucuronide that had HPLC, MS/MS, and $^1$H NMR characteristics identical to those of the major metabolite of 1 isolated from monkey urine. With the structure of the hydroxylamine definitively established, these observations allowed for the otherwise nontrivial distinction between the two possible sites of glucuronide attachment, which were either at the oxygen 3 or the nitrogen 4. These $^{13}$C NMR data were consistent with the O-glucuronide structure 3 where the C3/5 methylenes experienced an (upfield) $\gamma$-effect and the C2/6 methylenes experience a (negligible) $\delta$-effect by the glucuronic acid moiety. Also, the C3/5 methylenes displayed larger splitting, more so than C2/6, due to close proximity to the chiral glucuronic acid moiety. In contrast, if the structure had been the N-glucuronide 4, the C3/5 methylenes would have exhibited a significant downfield shift due to the $\beta$-effect of the glucuronic acid moiety, and this was not observed. The culmination of all of these results led to the unambiguous structural determination of the major metabolite found in the urine of monkeys treated with the hypoglycemic agent 1 as the O-glucuronide of the hydroxylamine 3.

Fig. 5. HPLC-UV chromatogram (A) and LC/MS reconstructed ion current chromatograms of m/z 309 (B) and m/z 325 (C) of an incubation of 1 with pig liver microsomes.

Fig. 6. $^1$H NMR spectrum of the hydroxylamine metabolite 2.
Fig. 7. $^{13}$C NMR spectrum of the hydroxylamine metabolite 2.

Fig. 8. $^1$H NMR spectrum of the glucuronide metabolite 3.
References


