IDENTIFICATION OF A HYDROXYLAMINE GLUCURONIDE METABOLITE OF AN ORAL HYPOGLYCEMIC AGENT

Randall R. Miller, George A. Doss, and Ralph A. Stearns
Department of Drug Metabolism, Merck Research Laboratories, Rahway, New Jersey
(Received April 18, 2003; accepted September 17, 2003)

This article is available online at http://dmd.aspetjournals.org

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-radioactivity in urine from monkeys treated with the aryl piperazine oral hypoglycemic agent 9-[(1S,2R)-2-fluoro-1-methylpropyl]-2-methoxy-6-(1-piperazinyl) purine hydrochloride (1). The mass spectrum of this metabolite indicated that it was both mono- and glucuronidated on the piperazine ring. Possible structures included the N- or O-glucuronic acid conjugates of a carbinalamine, hydroxylamine, or N-oxide. Treatment with β-glucuronidase gave a mono-oxidized derivative of the parent compound. 1H NMR analysis of either the glucuronic acid conjugate or the monooxidated product provided insufficient evidence to unambiguously determine their structures. Incubation of 1 with pig liver microsomes resulted in formation of the same monooxidized derivative derived from β-glucuronidase treatment of the glucuronide metabolite. This in vitro system was used to generate sufficient material for analysis by 13C NMR, and the metabolite was identified as a hydroxylamine derivative. Incubation of the hydroxylamine with monkey liver microsomes and uridine diphospho-5′-glucuronic acid gave the same glucuronic acid conjugate as that observed in monkey urine. 13C NMR analysis of this biosynthetic product led to its unequivocal structure assignment as the O-glucuronic acid conjugate of the hydroxylamine 3.

Materials and Methods

Chemicals. 9-[(1S,2R)-2-fluoro-1-methylpropyl]-2-methoxy-6-(1-piperazinyl) purine hydrochloride 1 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 2 (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).

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 1 (5 Ci/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 nonradiolabeled 1 (5 mg/kg; 2.5 mg/ml in isotonic saline). Oral doses were administered via a nasogastric tube. All animals were

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 O-oxide. Metabolites, typified in the in vivo metabolism of ketoconazole, ciprofloxacin, the inotropic agent OPC-2812 (vesnarinone), and the phenothiazine derivative perazine, include carbinalamine, amide, ring-opened, and N-oxidation products (Breyer, 1972; Gau et al., 1986; Miyamoto et al., 1988; Whitehouse et al., 1994). The hydroxylamine intermediate, formed by oxidation of the secondary amine, if present, is rarely observed in vivo for piperazines or other nitrogen-containing alicyclics. An explanation for this may be found in reports that this intermediate is inherently unstable (Beckett and Salami, 1972; Beckett et al., 1977; Franklin et al., 1977; Ziegler, 1987); however, identifications of stable alicyclic hydroxylamines have been made (Beckett and al-Sarraj, 1972; Beckett et al., 1977; Achari and Beckett, 1983; Rodriguez and Acosta, 1997; Zhang et al., 2000). There are even fewer examples of glucuronic acid conjugation of alicyclic hydroxylamines in the literature (Straub et al., 1988; Delbressine et al., 1992; Schaber et al., 2001), and the structures of these metabolites have been largely deduced from mass spectrometry and in some cases 1H NMR analysis. In the course of investigating the disposition of the oral hypoglycemic agent 1 (Leibowitz et al., 1995) in rhesus monkeys, one major metabolite was observed in urine from monkeys dosed orally. The molecular weight of this metabolite corresponded to addition of an oxygen atom and glucuronic acid to the piperazine ring of the parent drug. Both LC/MS/MS and 1H NMR analysis failed to provide sufficient information to determine the exact structure. This communication describes the unambiguous identification of this metabolite through its biosynthesis, isolation, and structural identification by 13C NMR spectroscopy.

Address correspondence to: Randall R. Miller, Department of Drug Metabolism, Merck Research Laboratories, P.O. Box 2000, RY800B211, Rahway, NJ 07065, E-mail: randy_miller@merck.com

Acknowledgments. The authors wish to acknowledge the technical assistance of Ms. Amy Brondel and Ms. Janet Talley.
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 I (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 methanol followed by 5 ml of water. After loading, the cartridge was washed with 1 ml of water and eluted with 5 ml of methanol. When radiolabeled substrate was used, 50 to 100 μl of the diluted incubation medium, cartridge wash, and methanol eluate was counted by liquid scintillation to account for recovery. Methanol was evaporated at 35°C under nitrogen in a Turbovap LV evaporator (Zymark Corp., Hopkinton, MA). The residue was reconstituted in 100 to 200 μl of mobile phase A (see below), 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 (3.5 mM), magnesium chloride (3.3 mM), and NADP+ (1.3 mM) were added to the suspension along with 3 mg of 13C-labeled I (0.4 μCi/mg). The flask was placed in a reciprocating water bath at 37°C and preincubated for 5 min. Metabolism was initiated by addition of 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 10 ml of the diluted incubation medium, suspension that was separated by centrifugation. The organic solvent was transferred by pipette to a 16 ml of the diluted incubation medium, residue was reconstituted in 10 ml of MeOH to give a mobile phase consisting of 15% CH3CN/30% CH3OH/100 mM NH4OAc/1% acetic acid. Fractions with absorbance at 280 nm were collected. To obtain enough hydroxylamine metabolite for further investigation, this procedure was repeated on an 8-times scale. The final yield of hydroxylamine metabolite was 7 mg from 24 mg of starting material I.

**Glucuronidation.** Liver microsomes from male monkeys (final concentration, 3.39 mg protein/ml) were added to 100 ml of 50 mM potassium phosphate mobile phase of methanol followed by 5 ml of water. After loading, the cartridge was washed with 3 ml of water and eluted with 5 ml of methanol. When radiolabeled substrate was used, 50 to 100 μl of the diluted incubation medium, cartridge wash, and methanol eluate was counted by liquid scintillation to account for recovery. Methanol was evaporated at 35°C under nitrogen in a Turbovap LV evaporator (Zymark Corp., Hopkinton, MA). The residue was reconstituted in 100 to 200 μl of mobile phase A (see below), 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 (3.5 mM), magnesium chloride (3.3 mM), and NADP+ (1.3 mM) were added to the suspension along with 3 mg of 13C-labeled I (0.4 μCi/mg). The flask was placed in a reciprocating water bath at 37°C and preincubated for 5 min. Metabolism was initiated by addition of 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 10 ml of the diluted incubation medium, suspension that was separated by centrifugation. The organic solvent was transferred by pipette to a 16 ml of the diluted incubation medium, residue was reconstituted in 10 ml of MeOH to give a mobile phase consisting of 15% CH3CN/30% CH3OH/100 mM NH4OAc/1% acetic acid. Fractions with absorbance at 280 nm were collected. To obtain enough hydroxylamine metabolite for further investigation, this procedure was repeated on an 8-times scale. The final yield of hydroxylamine metabolite was 7 mg from 24 mg of starting material I.

**Glucuronidation.** Liver microsomes from male monkeys (final concentration, 3.39 mg protein/ml) were added to 100 ml of 50 mM potassium phosphate mobile phase of methanol followed by 5 ml of water. After loading, the cartridge was washed with 3 ml of water and eluted with 5 ml of methanol. When radiolabeled substrate was used, 50 to 100 μl of the diluted incubation medium, cartridge wash, and methanol eluate was counted by liquid scintillation to account for recovery. Methanol was evaporated at 35°C under nitrogen in a Turbovap LV evaporator (Zymark Corp., Hopkinton, MA). The residue was reconstituted in 100 to 200 μl of mobile phase A (see below), 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 (3.5 mM), magnesium chloride (3.3 mM), and NADP+ (1.3 mM) were added to the suspension along with 3 mg of 13C-labeled I (0.4 μCi/mg). The flask was placed in a reciprocating water bath at 37°C and preincubated for 5 min. Metabolism was initiated by addition of 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 10 ml of the diluted incubation medium, suspension that was separated by centrifugation. The organic solvent was transferred by pipette to a 16 ml of the diluted incubation medium, residue was reconstituted in 10 ml of MeOH to give a mobile phase consisting of 15% CH3CN/30% CH3OH/100 mM NH4OAc/1% acetic acid. Fractions with absorbance at 280 nm were collected. To obtain enough hydroxylamine metabolite for further investigation, this procedure was repeated on an 8-times scale. The final yield of hydroxylamine metabolite was 7 mg from 24 mg of starting material I.

**Glucuronidation.** Liver microsomes from male monkeys (final concentration, 3.39 mg protein/ml) were added to 100 ml of 50 mM potassium phosphate mobile phase of methanol followed by 5 ml of water. After loading, the cartridge was washed with 3 ml of water and eluted with 5 ml of methanol. When radiolabeled substrate was used, 50 to 100 μl of the diluted incubation medium, cartridge wash, and methanol eluate was counted by liquid scintillation to account for recovery. Methanol was evaporated at 35°C under nitrogen in a Turbovap LV evaporator (Zymark Corp., Hopkinton, MA). The residue was reconstituted in 100 to 200 μl of mobile phase A (see below), 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 (3.5 mM), magnesium chloride (3.3 mM), and NADP+ (1.3 mM) were added to the suspension along with 3 mg of 13C-labeled I (0.4 μCi/mg). The flask was placed in a reciprocating water bath at 37°C and preincubated for 5 min. Metabolism was initiated by addition of 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 10 ml of the diluted incubation medium, suspension that was separated by centrifugation. The organic solvent was transferred by pipette to a 16 ml of the diluted incubation medium, residue was reconstituted in 10 ml of MeOH to give a mobile phase consisting of 15% CH3CN/30% CH3OH/100 mM NH4OAc/1% acetic acid. Fractions with absorbance at 280 nm were collected. To obtain enough hydroxylamine metabolite for further investigation, this procedure was repeated on an 8-times scale. The final yield of hydroxylamine metabolite was 7 mg from 24 mg of starting material I.

**Glucuronidation.** Liver microsomes from male monkeys (final concentration, 3.39 mg protein/ml) were added to 100 ml of 50 mM potassium phosphate mobile phase of methanol followed by 5 ml of water. After loading, the cartridge was washed with 3 ml of water and eluted with 5 ml of methanol. When radiolabeled substrate was used, 50 to 100 μl of the diluted incubation medium, cartridge wash, and methanol eluate was counted by liquid scintillation to account for recovery. Methanol was evaporated at 35°C under nitrogen in a Turbovap LV evaporator (Zymark Corp., Hopkinton, MA). The residue was reconstituted in 100 to 200 μl of mobile phase A (see below), and 25 to 50 μl was analyzed by HPLC.
buffer, pH 7.7, containing 10 mM MgCl₂, 1 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 Zorbax 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

---

**Fig. 2.** HPLC radiohistogram (A) and LC/MS reconstructed ion current chromatograms for m/z 309 (B) and m/z 501 (C) of 0- to 24-h monkey urine.
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 $\sim 25$ and $\sim 50\%$, 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 $m/z$ of 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 $m/z$ of 325 was

![Fig. 3. MS/MS spectrum obtained from collision-induced dissociation of the MH$^+$ ion at $m/z$ 509 of the hydroxylamine glucuronide metabolite 3 isolated from monkey urine.](image)

(Delbressine et al., 1992; Schaber et al., 2001) but was not conclusive. The $^1$H NMR spectrum of this metabolite revealed the presence of signals characteristic of a $\beta$-glucuronic acid moiety (anomeric proton, doublet at 4.68 ppm, $J = 8.4$ Hz). The absence of perturbation of the NMR signals of the fluorinated side chain or aromatic protons supported the characterization that the site of metabolism was on the piperazine group. The 1 H NMR spectrum of this metabolite revealed the presence of signals characteristic of a $\beta$-glucuronic acid moiety (anomeric proton, doublet at 4.68 ppm, $J = 8.4$ Hz). The absence of perturbation of the NMR signals of the fluorinated side chain or aromatic protons supported the characterization that the site of metabolism was on the piperazine group.
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
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 1H NMR characteristics to that of the aglycone were isolated for 13C NMR analysis. Two signals were attributed to the piperazine carbons in the 13C NMR spectrum of the aglycone. This corroborated the observation from 1H 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 β-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 1H 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 13C NMR data were consistent with the O-glucuronide structure 3 where the C3/5 methylenes experienced an (upfield) γ-effect and the C2/6 methylenes experience a (negligible) δ-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 β-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.** 1H 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.


