BIOTRANSFORMATION OF A GABA<sub>A</sub> RECEPTOR PARTIAL AGONIST IN SPRAGUE-DAWLEY RATS AND CYNOMOLGUS MONKEYS: IDENTIFICATION OF TWO UNIQUE N-CARBAMOYL METABOLITES

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Received March 7, 2005; accepted August 3, 2005

ABSTRACT:
The absorption, metabolism, and excretion of N-[3-fluoro-4-[2-(propylamino)ethoxy]phenyl]-4,5,6,7-tetrahydro-4-oxo-1H-indole-3-carboxamide monomethanesulfonate (1), a GABA<sub>A</sub> receptor partial agonist potentially useful in treating generalized anxiety disorder, have been evaluated in both Sprague-Dawley rats and cynomolgus monkeys using <sup>14</sup>C]1. In both species, mass balance was achieved within 48 h postdose, with the majority of drug-related material excreted within the feces; the clearance of 1 in each species had both metabolic and renal components. In addition to the metabolites produced by aliphatic hydroxylation and/or N-dealkylation of 1, two unique metabolites were detected: a putative carboxylic acid (M7) in rat plasma and monkey bile, and an N-carbamoyl glucuronide (M8) in both rat and monkey bile. Metabolite M8 was structurally deciphered by liquid chromatography-tandem mass spectrometry and NMR, and was readily generated in vitro upon incubation of [<sup>14</sup>C]1 with rat liver microsomes fortified with uridine 5’-diphosphoglucuronic acid trisodium salt and alanethicin under a CO<sub>2</sub> atmosphere. Treatment of M8 with β-glucuronidase afforded 1 directly. The presence of M8 in bile and its notable absence from other matrices suggests the enterohepatic cycling of 1 via M8. Although the structure of M7 was not elucidated unequivocally due to its inability to be formed in vitro and its minimal absolute quantities in limited biological matrices, data herein clearly support its structural rationalization. Furthermore, since M7 is the precursor of M8, detection of M8 is indirect evidence of its existence. It is proposed that M7 arises from an equilibrium between 1 and dissolved CO<sub>2</sub>-equivalents both in vivo and in vitro, similar to carboxamido bonds observed in hemoglobin and certain amino acids, respectively.

Anxiety disorders, the most common of which are generalized anxiety disorder, phobias, and panic, are some of the most prevalent psychiatric sufferings (Kessler et al., 1994). Although benzodiazepines are often effective for the treatment of generalized anxiety disorder, they exhibit several undesirable side effects such as sedation, cognitive impairment, withdrawal, and abuse potential. More recently, serotonergic anxiolytics, such as buspirone, have been introduced as safer alternatives to benzodiazepines (Lader, 1988). However, such serotonergics often lack the rapid and robust efficacy of the benzodiazepines, resulting in their less frequent prescription. Thus, a medical need exists for an anxiolytic agent demonstrating benzodiazepine-like efficacy while concurrently providing a better safety profile for the general and chronic treatment of anxiety.

Benzodiazepines exert their efficacy via allosteric modulation of the GABA<sub>A</sub> receptor complex (Olsen and Tobin, 1990), which enhances GABA-mediated neuronal inhibition (Sieghart, 1992). Unlike full agonists, partial agonists of the GABA<sub>A</sub> receptor may afford the desired anxiolytic effects of a full agonist while eliminating its undesired properties. With this in mind, 1 (Fig. 1) is a potent subtype-selective partial agonist at the GABA<sub>A</sub> receptor complex that has demonstrated in vivo anxiolytic activity in rat models without evidence of benzodiazepine-related adverse effects. Reported within this paper are the results of the radiolabeled mass balance studies conducted in adult male and female Sprague-Dawley (SD) rats and cynomolgus monkeys. In addition to the identification of all major metabolites in excreta, bile, and plasma, two unique metabolites were detected: a putative carboxylic acid

ABBREVIATIONS: GABA<sub>A</sub>, γ-aminobutyric acid type-A receptor; 1, N-[3-fluoro-4-[2-(propylamino)ethoxy]phenyl]-4,5,6,7-tetrahydro-4-oxo-1H-indole-3-carboxamide monomethanesulfonate; 2, 2-fluoro-4-[4-oxo-4,5,6,7-tetrahydro-1H-indole-3-carbonyl]-amino]-phenoxy acetic acid; 3, 4-oxo-4,5,6,7-tetrahydro-1H-indole-3-carboxylic acid [3-fluoro-4-(2-hydroxy-ethoxy)-phenyl]-amide; [<sup>14</sup>C]1, N-[3-fluoro-4-[2-(propylamino)ethoxy]phenyl]-4,5,6,7-tetrahydro-4-oxo-1H-[<sup>14</sup>C]indole-3-carboxamide monomethanesulfonate; SD, Sprague-Dawley; PGRD, Pfizer Global Research and Development; RLM, rat liver microsome; MLM, monkey liver microsome; HPLC, high-performance liquid chromatography; MeCN, acetonitrile; rcf, relative centrifugal force; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MRM, multiple-reaction monitoring; AUC, area under the plasma concentration-time curve; k<sub>e</sub>, elimination rate constant; NADPH, reduced β-nicotinamide adenine dinucleotide phosphate; LC, liquid chromatography; CID, collision-induced dissociation; UGT, uridine 5’-diphosphoglucuronic acid transferase; amu, atomic mass unit(s).
at XenoBiotic Laboratories, Inc. (Plainsboro, NJ) in accordance with the Guide for the Care and Use of Laboratory Animals. A value of 0 was used when a measured in-life value was below its lower limit of quantification.

In Vivo Studies in Cynomolgus Monkeys. The in-life portion of the study was conducted at Charles River Laboratories, Inc. (Wilmington, MA) in accordance with the Guide for the Care and Use of Laboratory Animals. A single dose containing ca. 30 µCi of $[^{14}C]$ in 0.5% aqueous methyl cellulose (15 mg/ml) was administered orally at a target dose level of 60 mg/kg to each rat. Individual animal doses were calculated based on respective pretreatment body weights and a dose volume of ca. 4 ml/kg. The actual amount of dose solution administered to each animal was determined by weighing the loaded dosing syringe before and after it was dispensed. The study included three groups of rats. Group 1 (two per sex): From intact animals, urine was collected predose and from 0 to 8 and 24 h during day 1, and in 24-h intervals from 24 to 168 h postdose. From the same animals, feces and cage rinse were collected predose and in 24-h intervals from 0 to 168 h postdose. Group 2 (two per sex): From bile duct-cannulated animals, which received an infusion of a bile salts replacement solution (44 mmol of cholic acid and 13 mmol of NaHCO₃, per liter of 0.9% NaCl, pH 7.0–7.4) during the collection period, bile was collected predose and from 0 to 24 and 24 to 48 h postdose. Group 3 (two per sex per time point): Blood samples from intact animals euthanized at 2, 4, 8, 12, and 24 h postdose were collected in heparinized tubes by cardiac puncture and processed to obtain plasma. Control plasma was harvested from blood collected from untreated animals that were not sacrificed.

In Vivo Studies in Cynomolgus Monkeys. The in-life portion of the study was conducted at Charles River Laboratories, Inc. (Wilmington, MA) in accordance with the Guide for the Care and Use of Laboratory Animals. A single dose containing ca. 150 µCi of $[^{14}C]$ in 0.5% aqueous methyl cellulose (1.5 mg/ml) was administered orally at a target dose level of 6 mg/kg to each monkey. Individual animal doses were calculated based on respective pretreatment body weights and a dose volume of ca. 4 ml/kg. The actual amount of dose solution administered to each animal was determined as previously described. The study included two groups of monkeys. Group 1 (two per sex): From intact animals, excreta and cage debris/rinse were collected as described above for rats. Blood samples (ca. 3 ml) were collected into heparinized tubes from a vascular access port or peripheral vessel by venipuncture predose and at 0.5, 1, 2, 4, 8, 12, 24, 48, 72, 96, 120, 144, and 168 h postdose, and processed to obtain plasma. Group 2 (two per sex): From bile duct-cannulated animals, which received an infusion of a bile salts replacement solution (described above) during the collection period, bile and excreta were collected predose and from 0 to 24 and 24 to 48 h postdose.

Determination of Radioactivity within Biological Matrices. Tripletic gravimetric aliquots of each sample of urine (0.2–0.5 g), bile (0.05–0.1 g), and plasma (0.05–0.1 g) were mixed with liquid scintillation cocktail (15 ml) and counted for 2 min in a model LS 6000 or LS 6500 liquid scintillation counter (Beckman Coulter, Fullerton, CA). Fecal samples were homogenized with Mili-Q H₂O (20% w/w, feces/H₂O) using a probe-type homogenizer. Cage debris/rinse samples (collected in 50% reagent alcohol in H₂O) were homogenized directly with a probe-type homogenizer. Tripletic gravimetric aliquots (0.2–0.5 g for either fecal homogenate or cage debris/rinse homogenate) were transferred into tared cones and pads, weighed, and combusted prior to radio-analysis. Sample combustion was performed using a PerkinElmer model 307 or 387 sample oxidizer (PerkinElmer Life and Analytical Sciences, Boston, MA). Combustion efficiency using a 14C-labeled standard was determined daily prior to the combustion of study samples, and the measured radioactivity content in feces and cage debris/rinse was adjusted using daily combustion efficiency values. Liberated $[^{14}C]$CO₂ was trapped in Carbo-Sorb (PerkinElmer Life and Analytical Sciences) and mixed with Perma-Fluor scintillation fluid (PerkinElmer Life and Analytical Sciences), and the samples were analyzed for total radioactivity in a model LS 6000 or LS 6500 liquid scintillation counter (Beckman Coulter) for 2 min. Scintillation counter data were automatically corrected for counting efficiency using an external standardization technique and an instrument-stored quench curve generated from a series of sealed quench standards.

Quantitative Analysis of 1 in Plasma. Plasma concentrations of 1 were quantified using an LC-MS/MS method. To each plasma sample (100 µl) contained in a DW 96-well plate was added MeCN (200 µl) containing an internal standard, and the samples were vortex-mixed and centrifuged (832 rcf for 10 min) to sediment precipitated proteins. The respective supernatants (280 µl) were transferred to a second DW 96-well plate, concentrated under N₂ (200 µl) containing an internal standard, and the samples were vortex-mixed and centrifuged (832 rcf for 10 min) to sediment precipitated proteins. The respective supernatants (280 µl) were automatically corrected for counting efficiency using an external standardization technique and an instrument-stored quench curve generated from a series of sealed quench standard.

Pharmacokinetic Calculations. Pharmacokinetic parameters were calculated for each animal by noncompartmental analyses using WinNonlin Version 3.2 (Pharsight, Mountain View, CA). Values used to determine the pharmacokinetic parameters of total radioactivity were calculated by converting the raw data generated by liquid scintillation counting to concentrations (ng-Eq/ml) using the specific activity of administered $[^{14}C]$I. The AUC$_{0-tau}$ was calculated using the linear trapezoidal method, $k_d$ was determined by linear regression of the log concentration versus time data during the last observable elimination phase, and $t_{1/2}$ was calculated as 0.693/$k_d$. Both maximal plasma concentration (C$_{max}$) and its time of occurrence (T$_{max}$) were taken directly from the concentration versus time data. Means and standard deviations were calculated when half or greater of the values exceeded the lower limit of quantification for 1 (2 ng/ml) or total radioactivity (90 and 43 ng-Eq/ml in rat and monkey plasma, respectively). A value of 0 was used when a measured value was below its lower limit of quantification.

Sample Preparation for Metabolite Profiling and Identification. At each step during the sample preparation of all biological matrices, total radioactivity levels were determined by liquid scintillation counting for recovery calculations. Following preparation, all samples were analyzed as described below by LC-MS/MS with radiometric detection. Predose and blank samples served as controls for determining background radioactivity and endogenous, non-drug...
related ions observed within respective matrices or their extracts by LC-MS/MS.

**Urine.** Urine samples from each animal collected from 0 to 24 h postdose representing ≥95% of total urine radioactivity were pooled proportionally to the amount of urine in each sampling period to afford the analytical sample.

**Bile.** Bile collected from 0 to 24 h postdose representing ≥95% of total biliary radioactivity was centrifuged (9300 rcf for 5 min) to yield the analytical supernatant.

**Bile.** Fecal homogenates from each animal collected from 0 to 24 or 48 h postdose representing ≥88% of total fecal radioactivity were pooled proportionally to the amount of feces in each sampling period. Pooled homogenates were diluted with H2O (1 ml of H2O/g of homogenate) and shaken at 37°C for 16 h in a water bath, subsequently diluted with MeCN (2 ml/g homogenate), vortex-mixed and centrifuged (1500 rcf for 10 min), and the resulting supernatants were isolated. If necessary, the remaining fecal pellets were extracted further with 33% H2O in MeCN (9 ml) until ≥90% of the radioactivity from each pooled sample was extracted. The supernatants were concentrated to dryness and reconstituted in solvent A (600 µl) for analysis.

**Rat Plasma.** Plasma from blood samples collected at 2, 4, and 8 h postdose were used for circulatory metabolite profiling and identification since >80% of the total radioactivity AUC0–12 was captured by its AUC0–8. Plasma aliquots (0.5 ml from each rat, two per sex per time point) were pooled between genders for each time point. The pooled samples (2 ml) were diluted with 30% H2O in MeCN (7 ml), vortex-mixed for 30 min and centrifuged (2465 rcf for 10 min), and the resulting supernatants were isolated. The remaining plasma protein pellets were extracted an additional time with MeCN (4 ml) to ensure that >90% of the radioactivity injected from each pooled plasma sample was extracted. The combined supernatants were concentrated to near dryness and reconstituted in solvent A (300 µl) for analysis.

**Monkey Plasma.** Plasma from blood samples collected at 2 h postdose (the approximate 14C T1/2) was used for circulatory metabolite profiling and identification. The samples were pooled within genders, and each pooled sample (ca. 4.8 ml) was diluted with 33% H2O in MeCN (15 ml) and processed as described for rat plasma. Each analytical sample contained >90% of the radioactivity originally contained in its respective pooled plasma sample.

**Metabolite Profiling and Identification.** Samples were analyzed by an LC-MS/MS (described previously) equipped with a Column Engineering Monitor C18 analytical column (5 µ, 4.6 × 150 mm) in series with a β-RAM radiometric detector (IN/US Systems, Inc., Tampa, FL) containing a liquid scintillant cell (250 µl). Analytes within sample aliquots (20–100 µl) were eluted at 1 ml/min with 1% iPrOH in 2 mM ammonium acetate, pH 3.5 (solvent B) and MeCN (solvent C). The following two-step gradient was used: 0 to 45 min, 5% to 60% solvent C in solvent B; 45 to 50 min, 60% to 80% C in B. Following the elution of I and its metabolites, the column was returned over 3 min to 5% C in B where it remained for 7 min before the next injection. For the 1H-NMR matrix, >95% of the radioactivity injected onto the column eluted during the first 40 min of the gradient program. HPLC effluent was split 1/9 between the mass spectrometer and the radiometric flow detector; liquid scintillation cocktail flowed at 3 ml/min to the radiometric detector. Mass spectral data were collected using positive or negative ionization in full, precursor ion, neutral loss, product ion, and MRM scanning modes. Instrument settings and potentials were adjusted to provide optimal data in each mode. Masschrom version 1.1.1 (PerkinElmer Life and Analytical Sciences) and Winflow version 1.4 (IN/US Systems, Inc.) software were used for the acquisition and processing of mass spectral and radiochromatographic data, respectively.

**TiCl3 Reaction (Kulanthaivel et al., 2004).** To determine whether a monoxygenated metabolite contained an N-oxide functionality, a fekal extract aliquot (100 µl) was combined with 20% tinous chloride solution (twice, at 20 µl), vortext-mixed, and left at room temperature for 1 h. The sample was then centrifuged, and an aliquot (75 µl) of the supernatant was analyzed by LC-MS/MS. Quantitative formation of N-methylmorpholine from its N-oxide served as a positive control for TiCl3 effectiveness.

**Micromosal Incubations with 2 and 3.** Incubations (1 ml) were performed in duplicate with and without NADPH (1.3 µmol) in round-bottom glass test tubes open to air at 37°C in a shaking water bath. Each incubation contained RLMs or MLMs (1 mg of protein/ml of 0.1 M KH2PO4 buffer, pH 7.4), MgCl2 (3 µmol), and 2 or 3 (10 nmol). Sample aliquots (0.4 ml) were removed by micropipette at 0 and 60 min after NADPH (or buffer) addition, quenched with MeCN (1 ml) and centrifuged (1500 rcf for 5 min), and the resulting supernatant was concentrated and reconstituted in solvent A (0.4 ml) for LC-MS/MS analysis.

**In Vitro Generation of N-Carbamoyl Glucuronide M8 (Schaefer, 1992).** Incubations (5 ml) were performed in a 25-ml Erlenmeyer flask under a CO2-saturated atmosphere at 37°C in a shaking water bath. Each incubation contained male RLMs (2 mg of protein/ml of 0.1 M NaHCO3 buffer, pH 7.5), MgCl2 (50 µmol), [14C] CH 3-glucuronide acid trisodium salt (25 µmol), alamethicin (50 µg), and β-saccharolactone (23 µmol). After 2 h, the incubation was quenched with MeCN (5 ml) and centrifuged (1500 rcf for 5 min), and the resulting supernatant was concentrated and reconstituted in solvent A (1 ml) for LC-MS/MS analysis.

**Isolation of M8.** From an RLM incubation conducted to generate it, [14C]M8 was isolated for β-glucuronidase treatment and NMR analysis by preparative HPLC using an HPLC-MS/MS system composed of a Thermo Electron LC-Q (liquid chromatography-quadrupole ion trap) mass spectrometer with an electrospray ionization source (Thermo Electron Corporation, Waltham, MA), a Gilson model 322 pump (Gilson Medical Electronics, Middleton, WI), and a Gilson 215 Liquid Handler. The system was equipped with a Kromasil C18 column (5 µ, 150 × 10 mm, 100 Å) in series with a β-RAM radiometric detector containing a solid scintillator cell (100 µl). Effluent containing M8 was isolated as it exited the β-RAM-detector. Analytes within sample aliquots (500 µl) were eluted at 4 ml/min using the two-step gradient described previously but using 10 mM ammonium formate, pH 3.4, as solvent B. All fractions containing M8 were combined to afford 10 ml, which was divided equally among four glass Kimmle centrifuge tubes (2.5 ml/tube), and each was concentrated under N2 at 37°C to afford ca. 7.3 µg (12 nmol, 7.6 nCi/nmol) [14C]M8 per tube.

**Treatment of M8 with β-Glucuronidase.** Purified [14C]M8 (12 nmol, 7.6 nCi/nmol) was dissolved in KH2PO4 (0.1 M, pH 7.4) buffer (0.6 ml), and equal aliquots (275 µl) were transferred to two separate vials. To the first vial was added more buffer (275 µl), and to the second vial was added β-glucuronidase [2750 units in 275 µl of KH2PO4 (0.1 M, pH 7.4) buffer]. Each solution was incubated in a shaking water bath at 37°C for 2 h. Aliquots (250 µl) were removed from each vial after 0 and 2 h of incubation, quenched with MeCN (250 µl), vortex-mixed and centrifuged (1500 rcf for 5 min), and the resulting supernatant was concentrated and reconstituted in solvent A (100 µl) for LC-MS/MS analysis.

**LC-NMR Analysis of M8.** Prior to each of two NMR analyses, isolated [14C]M8 (12 nmol) within each glass Kimmle centrifuge tube was reconstituted in solvent A (110 µl). Samples were analyzed by an LC-NMR system consisting of an Agilent 1100 binary pump and auto injector (Agilent Technologies, Palo Alto, CA), a Bruker BioSpin BSFU-0 column oven (Bruker BioSpin Corporation, Billerica, MA), a Bruker BioSpin photodiode array detector, a Bruker Biospin BNMIF interface unit using a 100:1 split, a Bruker Daltonics Esquire 3000 ion trap MS (Bruker Daltonics Inc., Billerica, MA) equipped with an electrospray source, a Bruker BioSpin BPSU-36 peak storage unit, and a Bruker BioSpin 600 MHz Avance DRX Spectrometer equipped with a 4 mm 1H, 13C inverse z-gradient LC flow probe. Analytes within sample aliquots (60 µl) were eluted at 0.5 ml/min over a Phenomenex Luna C12(2) (5 µ, 3 × 150 mm; Phenomenex, Torrance, CA) at room temperature with MeCN-d3 (solvent D) and 0.1% TFA-d in D2O (solvent E) using the following linear gradient: 0 to 60 min, 10% to 40% D in E. One percent of the column effluent was split post-column and diluted with 10% D2O in MeCN-d3 (containing 0.2% acetic acid-d4) at a flow rate of 20 µl/min before entering the mass spectrometer; the remaining 99% of the effluent passed through the diode array detector. Peaks of interest within the diode array effluent were stored in the BPSU-36 unit using the loop storage technique and subsequently introduced into the NMR spectrometer. 1H, correlation spectroscopy, and total correlation spectroscopy NMR spectra were obtained at 298 K on the LC peak of interest using double presaturation of solvent NMR resonances. Proton chemical shifts are reported in ppm (δ) relative to tetramethylsilane as referenced from the shift of residual protons in MeCN-d3 (1.93 ppm). To obtain 1H NMR spectra of M8 at multiple temperatures (298 K, 318 K, and 338 K), a second aliquot (100 µl) of a similar but less concentrated sample was analyzed using the same LC-NMR conditions as above except for a slightly modified linear gradient: 0 to 40 min, 20%
to 40% D in E. For the LC-NMR analysis of \(1\) under equivalent conditions, an aqueous aliquot (35 μL) containing \(1\) (189 nmol) was analyzed as described above using the following linear gradient: 0 to 50 min, 20% to 70% D in E. \(^1H\) and correlation spectroscopy NMR spectra of \(1\) were obtained at 298 K using double presaturation of solvent NMR resonances.

### Results

#### Excretion of Radioactivity in Rats

In males, the mean overall recovery of drug-related material within excreta and cage rinse was 94.3% ± 2.8%. The cumulative amount of radioactive dose detected in male urine and feces was 10.0% ± 1.1% and 83.1% ± 4.1%, respectively. In females, the mean overall recovery of drug-related material within excreta and cage rinse was 93.8% ± 1.2%. The cumulative amount of radioactive dose detected in female urine and feces was 10.5% ± 2.7% and 80.4% ± 3.8%, respectively. The excretion of total drug-related material was rapid in both genders; on average, >94% of the administered radioactivity was excreted within the first 24 h. Due to the lack of readily apparent gender-related differences in overall excretory routes or mass recoveries, averages of combined male and female recovery values are listed in Table 1. On average, in male and female bile duct-cannulated animals, 35.4% ± 12.0% of the dose was detected in bile from 0 to 48 h postdose, with the 0- to 24-h bile sample comprising >96% of the 0- to 48-h biliary radioactivity (Table 1).

#### Quantitative Profile of [\(^{14}C\)]1 and Its Metabolites in Rat Excreta

**Urine.** In addition to \(1\), three metabolites were observed in both male and female urine (Fig. 2). Two (M1 and M2) metabolites were tentatively identified as monohydroxylated regioisomers of \(1\), whereas the other metabolite was \(2\) (Table 1). On average, in urine, 6.6% ± 1.6% of the administered dose was unchanged \(1\), which equated to a renal clearance of 1.9× glomerular filtration rate (Davies and Morris, 1993).

**Feces.** In addition to \(1\), the three urinary metabolites (M1, M2, and \(2\)) were also observed in both male and female feces (Fig. 3). On average, 45.0% ± 3.9% of the administered dose was unchanged \(1\) (Table 1).

**Bile.** In addition to \(1\), three monohydroxylated regioisomers of \(1\) (M1, M2, and M3), and an \(N\)-carbamoyl glucuronide (M8) were detected in bile (Fig. 4). M8 comprised nearly 50% of total biliary radioactivity (Table 1).

#### Quantitative Profile of [\(^{14}C\)]1 and Its Metabolites in Rat Plasma

In addition to \(1\), five radioactive peaks were identified in both male and female plasma (Fig. 5): regioisomers M1, M2, and M3, 2, and a carbamic acid of \(1\) (M7). On average, in plasma, \(1\) comprised 78.5% of total circulatory radioactivity, whereas the two most significant metabolites, M1 and M7, accounted for 5.2% and 5.6%, respectively (Table 2).

### TABLE 1

**Cross-species comparison of excretory routes and metabolite profiles**

<table>
<thead>
<tr>
<th></th>
<th>Rat (^a)</th>
<th>Monkey (^a)</th>
<th>Rat Bile (^b)</th>
<th>Monkey Bile (^b)</th>
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<tr>
<td></td>
<td>Urine</td>
<td>Feces</td>
<td>Urine</td>
<td>Feces</td>
</tr>
<tr>
<td><strong>Recovery (% of dose)</strong></td>
<td>94.1</td>
<td>91.9</td>
<td>35.4(^c)</td>
<td>31.8(^d)</td>
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<td>% of dose excreted</td>
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<td>81.8</td>
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<td>% of (^{14}C) in excreta profiled</td>
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<td>94.6</td>
<td>89.5</td>
<td>96.1</td>
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<tr>
<td><strong>Metabolite Profile (% of dose)</strong></td>
<td></td>
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<td>95.8</td>
<td>92.1</td>
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<td>Compound 1</td>
<td></td>
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<tr>
<td>M1 (hydroxylated 1)</td>
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<td>45.0</td>
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<td>M8 (N-carbamoyl glucuronide)</td>
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<td>4.4</td>
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</table>

\(^a\) Intact animals (group 1).

\(^b\) Bile duct-cannulated animals (group 2).

\(^c\) Percentage of the administered dose detected in bile from 0 to 48 h postdose; excreta were not collected from bile duct-cannulated rats.

\(^d\) Percentage of the administered dose detected in bile from 0 to 48 h postdose; 10.3% and 41.7% of the administered dose was excreted in the urine and feces, respectively, of bile duct-cannulated monkeys.

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FIG. 2. Radiochromatograms of rat (top) and monkey (bottom) urine.
Pharmacokinetics of 1 and Total Drug-Related Material in Rats

Due to the lack of readily apparent gender-related differences in systemic exposure to 1 or total radioactivity, all pharmacokinetic parameters are an average of all study animals. For 1, its average plasma concentration versus time is plotted in Fig. 6, and its pharmacokinetic parameters are listed in Table 3. On average, in males and females, plasma concentrations of 1 rose to a mean \( C_{\text{max}} \) of 7100 ± 1400 ng/ml at a mean \( T_{\text{max}} \) of 3.0 ± 1.2 h postdose, with a mean \( t_{1/2} \) of 1.6 ± 0.4 h.

For total radioactivity of 1, the average plasma concentration versus time is plotted in Fig. 6, and the pharmacokinetic parameters are listed in Table 3. On average, in males and females, the mean \( C_{\text{max}} \) and \( T_{\text{max}} \) values of total radioactivity were 8740 ± 1710 ng-Eq/ml and 3.5 ± 1.0 h, respectively, whereas the mean \( t_{1/2} \) was 3.0 ± 1.1 h. On average, in both genders, the AUC\(_{0-24}\) for 1 represented 76.9% of the AUC\(_{0-24}\) for total drug-related material.

Excretion of Drug-Related Material in Monkeys

In males, the mean overall recovery of drug-related material within excreta and cage rinse was 92.7%. The cumulative amount of radioactive dose detected in male urine and feces was 19.9% and 67.9%, respectively. In females, the mean overall recovery of drug-related material within excreta and cage rinse was 91.2%. The cumulative amount of radioactive dose detected in female urine and feces was 24.0% and 61.0%, respectively. The excretion of total drug-related material was rapid in both males and females; on average, >90% of the administered radioactivity excreted within the first 48 h postdose. Due to the lack of readily apparent gender-related differences in overall excretory routes or mass recoveries, averages of combined male and female recovery values are listed in Table 1. On average, in male and female bile duct-cannulated monkeys, 31.8% of the dose was detected in bile from 0 to 48 h postdose, with 10.3% and 41.7% of the administered dose detected in the urine and feces, respectively; the 0- to 24-h bile sample comprised 97% of the 0- to 48-h biliary radioactivity (Table 1).

Quantitative Profile of [\(^{14}\text{C}\)]1 and Its Metabolites in Monkey Excreta

**Urine.** In addition to 1, five radioactive peaks were observed in both male and female urine (Fig. 2). Four of the metabolites were tentatively identified as monohydroxylated 1 regiosomers (M1 and M3), monohydroxylated 2 (M4), and monooxygenated 3 (M5); the fifth metabolite was unequivocally identified as 2. In both male and female urine, 5.7% of the administered dose was excreted as unchanged 1, which equated to active renal secretion at 5.9× glomerular filtration rate (Davies and Morris, 1993).

**Feces.** In addition to 1, six radioactive peaks were observed in both male and female feces (Fig. 3). The metabolites were identified as M1, M3, M4, M5 and its regioisomer M6, and 2. In male and female feces, 9.9% and 9.1% of the administered dose, respectively, was detected as unchanged 1.

**Bile.** In addition to 1, seven metabolites were detected in the bile (Fig. 4), five of which were also detected in excreta. However, M7 and M8, which displayed LC and MS properties identical to those of the M7 and M8 observed in rats, were unique biliary metabolites comprising 0.3% and 4.4% of the dose, respectively (Table 1).

Quantitative Profile of [\(^{14}\text{C}\)]1 and Its Metabolites in Monkey Plasma

In addition to 1, four radioactive peaks were structurally identified in the plasma of both males and females at \( T_{\text{max}} \) (Fig. 5). In each
gender, two of the metabolites were tentatively identified as M1 and M3, whereas two of the metabolites were unequivocally identified as 2 and 3. On average, in males and females, 1 comprised 37.4% of $T_{\text{max}}$ total radioactivity. The percentages of total plasma radioactivity contained within each peak as an average of both genders are listed in Table 2.

**Pharmacokinetics of 1 and Total Drug-Related Material in Monkeys**

Due to the lack of readily apparent gender-related differences in systemic exposures to 1 or total radioactivity, all reported pharmacokinetic parameters are an average of all study animals. For 1, its average plasma concentration versus time is plotted in Fig. 6, and its pharmacokinetic parameters are listed in Table 3. On average, plasma concentrations of 1 rose to a mean $C_{\text{max}}$ of 365 ± 65 ng/ml at a mean $T_{\text{max}}$ of 1.3 ± 0.5 h postdose, with a mean $t_{1/2}$ of 3.9 ± 0.3 h.

For total radioactivity of 1, its average plasma concentration versus time is plotted in Fig. 6, and its pharmacokinetic parameters are listed in Table 3. On average, the mean $C_{\text{max}}$ and $T_{\text{max}}$ values of total radioactivity were 1050 ± 145 ng-Eq/ml and 1.3 ± 0.5 h, respectively, whereas the mean $t_{1/2}$ was 6.4 ± 1.3 h. The $T_{1/2}$ for 1 represented 28.4% of the $C_{\text{max}}$ for total drug-related material.

**Identification of Hepatic Microsomal Metabolites of 2 and 3**

Incubation of 2 with RLMs and MLMs generated M4 exclusively in an NADPH-dependent manner. Incubation of 3 with RLMs and MLMs produced solely M5 and M6, which required NADPH. Based purely on response factors in MRM chromatograms, M5 was produced in greater amounts than M6 in both species.

### Structural Rationalization of 1 and Its Metabolites

**Compound 1.** Compound 1 had a protonated molecular ion of $m/z$ 374 and an LC retention time ($t_{\text{R}}$) of ca. 19.8 min. The CID product ion spectrum of $m/z$ 374 contained fragment ions with $m/z$ 271, 213, 193, 180, and 86. Fragment ion $m/z$ 271 resulted from loss of the 2-amino-propylhydroxide moiety. Fragment ion $m/z$ 213 corresponded to the positively charged 3-fluoro-4-(2-amino-propylhydroxy)aniline formed by cleavage of the parent amide bond; loss of HF from this fragment afforded $m/z$ 193. Fragment ion $m/z$ 162, hydration of which generates $m/z$ 180, was a positively charged 4-oxotetrahydroindole-3-ketene formed upon lysis of the parent amide bond. Fragment ion $m/z$ 86 was protonated N-propyl-N-vinylamine. A complete $^1$H NMR characterization of 1 is summarized in Table 4. Based upon the
fragmentation pattern of 1, precursor ion scans of m/z 162 and 213 were used for metabolite structure elucidation since each fragment reported on molecular modification to either the left or right portion of its amide bond, respectively.

$M_1$. $M_1$ had a protonated molecular ion of m/z 390, 16 amu greater than 1, and an LC $t_R$ of ca. 14.7 min. $M_1$ was unaffected by treatment with $\text{TiCl}_3$, suggesting that it was not an N-oxide. The CID product ion spectrum of $M_1$ contained fragment ions with m/z 287, 213, 196, 178, and 86. Fragment ions m/z 287, 196, and 178 were 16 amu greater than the three corresponding fragments observed for 1, suggesting that hydroxylation had occurred on the 4-oxotetrahydroindole region of 1. Fragment ions m/z 213 and 86 were the same as those observed in the parent CID spectrum, suggesting that the 2-amino propylether moiety was unchanged. Based on these data, $M_1$ was tentatively identified as monohydroxy-1.

$M_2$. $M_2$ had a protonated molecular ion of m/z 390, 16 amu greater than 1, and an LC $t_R$ of ca. 15.8 min. $M_2$ was inert to $\text{TiCl}_3$. The CID product ion spectrum of $M_2$ contained fragment ions with m/z 287, 213, 196, 178, and 86. All fragment ions, except for m/z 160, were identical to those fragments observed for $M_1$, suggesting that the 4-oxotetrahydroindole of $M_2$ was oxygenated. Not only was $M_2$ distinct from $M_1$ based on LC $t_R$, but the m/z 178 fragment of $M_2$ underwent a unique loss of H$_2$O to afford fragment ion m/z 160. Formation of fragment ion m/z 160 by $M_2$ but not by $M_1$ suggested that $M_2$ was a distinct regiosomer of $M_1$. More specifically, $M_2$ is believed to be hydroxylated at either C-6 or C-7 of the tetrahydroindole structure, and its m/z 160 cation is an a,b-unsaturated ketone resulting from dehydration of fragment ion m/z 178. If this is correct rationalization, then logic suggests that $M_1$ does not generate m/z 160 because it is hydroxylated at C-5, which would allow for intramolecular hydrogen bonding between the carbonyl oxygen and the hydroxyl proton within the 4-oxotetrahydroindole, hence precluding dehydration of m/z 178. Based on these data, $M_2$ was tentatively identified as monohydroxy-1, a regiosomer of $M_1$.

$M_3$. $M_3$ had a protonated molecular ion of m/z 390, 16 amu greater than 1, an LC $t_R$ of ca. 13.1 min, and was unchanged by $\text{TiCl}_3$. The CID product ion spectrum of $M_3$ was identical to that of $M_1$. Based on these data, $M_3$ was tentatively identified as monohydroxy-1, a regiosomer of $M_1$ and $M_2$.

Compound 2. Metabolite 2 had a protonated molecular ion of m/z 347, 27 amu less than 1, and an LC $t_R$ of ca. 16.8 min. The CID product ion spectrum of 2 contained fragment ions with m/z 329, 180, and 162. In identical matrices, the CID product ion spectrum and LC $t_R$ of this metabolite were indistinguishable from those of synthetic standard 2. Fragment ion m/z 329 (M + $\text{H}^+$ – 18) resulted from facile dehydration of 2 at its protonated carboxylic acid to afford an acylum species. Fragment ions m/z 180 and 162 confirmed the presence of the unmodified 4-oxotetrahydroindole moiety, whereas the absence of fragment ion m/z 86 verified the lack of a 2-aminopropyl group in 2 relative to 1.

$M_4$. $M_4$ had a protonated molecular ion of m/z 363, 16 amu greater than 2, and an LC $t_R$ of ca. 12.6 min. $M_4$ was unaltered by $\text{TiCl}_3$. The CID product ion spectrum of $M_4$ contained fragment ions with m/z 345, 196, 178, 160, and 132. Fragment ion m/z 345, 18 amu less than the parent ion, suggested that dehydration of the parent ion had occurred. Fragment ions m/z 196 and 178, each 16 amu less than the corresponding ions m/z 180 and 162 in the CID spectrum of 2, implied that the 4-oxotetrahydroindole of $M_4$ was monoxygenated. Moreover, the observation of fragment ion m/z 160, which is only observed in $M_2$ and is proposed to be formed by dehydration of fragment ion m/z 178, suggested that 4-oxotetrahydroindole monoxygenation within $M_4$ occurred at the same carbon as in $M_2$. Loss of CO from m/z 160 yielded m/z 132. The absence of fragment ion m/z 86 suggested that a chemical modification had occurred at the 2-aminopropyloxy portion of 1. Thus, $M_4$ was tentatively identified as monohydroxy-2.

Compound 3. Metabolite 3 had a protonated molecular ion of m/z 333, 41 amu less than 1, and an LC $t_R$ of ca. 21.6 min. The CID product ion spectrum of 3 contained fragment ions with m/z 315, 271, 180, and 162. The CID product ion spectrum and LC $t_R$ of 3 were indistinguishable from those of authentic standard 3 in identical matrices. Fragment ion m/z 315 (M + $\text{H}^+$ – 18) resulted from dehydration of 3 at its protonated alcohol to a carbocation. Fragment ions m/z 271, 180, and 162 confirmed the presence of unmodified 4-oxotetrahydroindole and fluorooanilide regions, whereas the absence of fragment ion m/z 86 verified the lack of the 2-aminopropyl group in 3 relative to 1.

$M_5$. $M_5$ had a protonated molecular ion of m/z 349, 16 amu greater than 3, an LC $t_R$ of ca. 17.2 min, and was impervious to $\text{TiCl}_3$. The CID product ion spectrum of $M_5$ contained fragment ions with m/z 331, 313, 296, 287, 196, 178, 160, and 132. Fragment ion m/z 331 was a result of dehydration of $M_5$ at its protonated alcohol to form a carbonium, a fragmentation mechanism common to 3. Fragment ion m/z 178, which is 16 amu greater than the corresponding m/z 162 ion in the CID spectrum of 3, implied that an oxygen atom had been incorporated into the 4-oxotetrahydroindole moiety of $M_5$. Furthermore, the observation of fragment ion m/z 160, common to $M_2$, $M_4$, and $M_6$, suggested that 4-oxotetrahydroindole monoxygenation within $M_5$ occurred at the same carbon as in these three metabolites. Hence, $M_5$ was tentatively identified as monoxydihydroxy-3.

$M_6$. $M_6$ had a protonated molecular ion of m/z 349, 16 amu greater than 3, and an LC $t_R$ of ca. 17.6 min. $M_6$ was unaltered by $\text{TiCl}_3$. The CID product ion spectrum of $M_6$ contained the same fragment ions as that of $M_5$, but its base peak was m/z 296 versus m/z 178 for $M_5$. Based on nearly identical CID spectra, $M_6$ was tentatively identified as monoxydihydroxy-3, a regiosomer of $M_5$.

$M_7$. $M_7$ had a protonated molecular ion of m/z 418, 44 amu greater than 1, and an LC $t_R$ of ca. 23.7 min. The CID product ion spectrum of $M_7$, whose molecular ion corresponded to 1 + $\text{CO}_2$, contained fragment ions with m/z 400, 374, 271, 239, 213, 180, 162, and 86 (Fig. 7). The presence of fragment ions m/z 374, 271, 213, 180, 162, and 86, all of which were observed in the CID spectrum of 1, suggested that $M_7$ was structurally very similar to 1. Fragment ion m/z 239, which is postulated to be an isocyanate, is unique to $M_7$ compared with 1, and it is believed to form by sequential dehydration and anilide cleavage of [$M_7 + \text{H}^+$]. Thermal degradation of carbamic acids and carbamates to isocyanates is well known (Fishbein and Zielinski, 1969; Honing et al., 1994; Liska and Slobodnik, 1996; Sanda et al., 1997).

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TABLE 4  
Proton NMR data at 298 K for 1

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* The single proton at 7.50 ppm integrated to 0.3H due to slow relaxation and short relaxation delay.
The presence of the m/z 239 fragment and the significantly diminished amount of the m/z 86 fragment, which would arise from m/z 374 upon loss of CO₂ from [M₇ + H]⁺, relative to that observed in the CID spectrum of 1, portends the addition of CO₂ to the nitrogen of the 2-aminoproplethoxy side chain, which is the most basic nitrogen (pKₐ 8.2) within 1. Accordingly, M₇ was identified as a putative carbamic acid metabolite of 1.

M₈. M₈ had a protonated molecular ion of m/z 594, 220 amu greater than 1, and an LC tᵣ of ca. 21.5 min. The CID product ion spectrum of M₈, whose molecular ion corresponded to 1 + CO₂ + glucuronic acid, included fragment ions with m/z 418, 400, 374, 239, 213, 180, and 162 (Fig. 8), all of which were the fragment ions observed in the CID spectrum of M₇ (Fig. 7). Fragment ion m/z 418 (M₈ + H – 176) indicated loss of a glucuronide functionality to afford the aforementioned carbamic acid ion; m/z 594 was also observed as a single peak in a neutral loss of 176 scan. Fragment ion m/z 374, which corresponded to 1, was formed upon loss of both the glucuronide and CO₂ from the protonated parent ion, a diagnostic fragmentation cascade for N-carbamoyl glucuronides (Straub et al., 1988; Tremaine et al., 1989; Schaefer et al., 1992; Liu et al., 2001; Beconi et al., 2003; Edlund and Baranczewski, 2004). The presence of fragment ion m/z 239 (i.e., M₈ + H – 176 – 18), the isocyanate fragment unique to the CID spectrum of M₇ and an indicative (yet very minor) fragment also observed but not specifically cited in previously reported CIDs of N-carbamoyl glucuronides (Straub et al., 1988; Tremaine et al., 1989; Schaefer et al., 1992; Liu et al., 2001; Edlund and Baranczewski, 2004), suggested that carbamoyl glucurononidation of 1 occurred at its 2-aminoproplethoxy nitrogen rather than at its 4-oxotetrahydroindole nitrogen. To confirm the proposed structure for M₈, an N-carbamoyl glucuronide of 1 was generated in RLMs (Schaefer, 1992) for both NMR analysis and as a biosynthetic reference standard. Although M₈ was observed in both rat and monkey bile, only RLMs were used as a model system to generate an adequate amount of M₈ for further characterization. Both the biosynthesized N-carbamoyl glucuronide and M₈ had indistinguishable LC tᵣ values and CID spectra. Treatment of [¹⁴C]M₈ with β-glucuronidase resulted in its rapid, quantitative conversion to 1, consistent with past reports (Elvin et al., 1980; Tremaine et al., 1989; Schaefer et al., 1992). Based on these data, M₈ was identified as an N-carbamoyl glucuronidone of 1.

The NMR data for M₈ support its proposed structure (Table 5); two-dimensional NMR data (not shown) were consistent with one-dimensional NMR data and aided in confirming atom connectivity. Comparison of the spectral data for 1 and M₈ indicated that all protons within 1 are also present in M₈, which excludes the possibility of glucuronidation at any carbon atom. Unique to the spectrum of M₈, five resonances consistent with a glucuronide are present: δ 5.3 ppm (d, J = 8 Hz, 1H at 338 K) denotes the anomeric proton, and four signals with δ 3.3 to 3.9 ppm correspond to the other protons of the glycone backbone. Furthermore, the [M + D]⁺ detected by MS for M₈ deuterated solvents was 7 amu greater than its [M + H]⁺ in nondeuterated solvents, consistent with six exchangeable protons within M₈. Similarly, three exchangeable protons were observed for 1.

Several resonances in the ¹H NMR spectrum of M₈ at 298 K have unexpected splitting patterns. The anomeric ¹H signal (5.34 ppm) appears as a triplet or doublet of doublets at 298 K, but collapses into a doublet upon heating to 338 K (Fig. 9). Several other signals (3.92 ppm, 4.17 ppm, and 7.06 ppm) behave similarly. This reversible change in the splitting patterns upon heating and cooling indicates an equilibrium between two forms of M₈ in solution, which may be the two rotomers of M₈ arising from slow rotation about the C–N bond of the carbamate at lower temperatures (Hilal et al., 1986; Wildenberg et al., 1990; Schaefer et al., 1992).

Discussion

An overview of the metabolism of 1 in SD rats and cynomolgus monkeys is presented in Fig. 10. On average, in rats, 94.1% of administered radioactivity was recovered (10.3% in urine, 81.8% in feces). On average, in monkeys, 91.9% of the dose was recovered (21.9% in urine, 64.5% in feces). Within both species, 1 underwent three main biotransformation routes: aliphatic hydroxylation, N-dealkylation (followed by oxidation or reduction), and direct conjugation.

Aliphatic hydroxylation of 1 on its 4-oxotetrahydroindole moiety led to three regioisomers in rats (M₁, M₂, and M₃), but only two in monkeys (M₁ and M₃). Although the precise site of monohydroxy-
dehydration of fragment ion of the 4-oxotetrahydroindole core, which would allow for facile are believed to be hydroxylated at the same carbon, either C-6 or C-7 unique to monkeys, also contain delay.

gests that 4-oxotetrahydroindole hydroxylation to afford occur at C-5, C-6, or C-7 of the 4-oxotetrahydroindole moiety. The lation within these metabolites was not determined, it is believed to occur at C-5, C-6, or C-7 of the 4-oxotetrahydroindole moiety. The CID fragmentation patterns of M1 and M3 are identical to each other, yet distinct from that of M2, which contains a unique fragment ion of m/z 160. Interestingly, the CID spectra of M4, M5, and M6, which are unique to monkeys, also contain m/z 160. Thus, M2, M4, M5, and M6 are believed to be hydroxylated at the same carbon, either C-6 or C-7 of the 4-oxotetrahydroindole core, which would allow for facile dehydration of fragment ion m/z 178 to m/z 160 observed in their CID spectra. Since M2 was not observed in monkeys, this strongly suggests that 4-oxotetrahydroindole hydroxylation to afford M4, M5, or M6 occurs after N-dealkylation of 1 (to 2 or 3) rather than before (via M2). This hypothesis seems substantiated by the fact that in both RLMs and MLMs, M4 arises directly from 2, and M5 and M6 from 3. A similar in vitro experiment with M2, to determine whether M4, M5, or M6 arises from it, was never undertaken.

Oxidative deamination at the 2-aminopropylethoxy nitrogen of 1 was found to be a more predominant metabolic clearance pathway in monkeys than in rats. It was not determined whether this is due to the greater role of MAO- versus P450-mediated N-dealkylation in monkeys than in rats. Oxidative deamination of 1 resulted in a putative aldehyde intermediate, which was never detected, that underwent one of two fates: oxidation to carboxylic acid 2 or reduction to alcohol 3. It is presumed that the enzymes responsible for the conversion of the putative aldehyde intermediate to either 2 or 3 are aldehyde dehydrogenase and alcohol dehydrogenase, respectively. It is well known that carboxylic acid formation via aldehyde dehydrogenase is irreversible, whereas alcohol formation via alcohol dehydrogenase is a reversible pathway, which can ultimately lead to formation of the carboxylic acid from the alcohol metabolite. Interestingly, the much greater proportion of 2 (and its hydroxylated metabolite M4) to 3 (and its hydroxylated metabolites M5 and M6) observed in both species may be due to this reversibility in vivo.

The third, and most interesting, route of 1 biotransformation in rats and monkeys is direct conjugation to one of two N-carbamoyl metabolites. Ironically, the more stable N-carbamoyl metabolite M8 can only be formed following formation of its less stable prerequisite M7. This suggests that carbamic acid M7, which would intuitively be predicted to be quite unstable at varying pH due to its seemingly favorable degradation to CO₂ and 1, exists long enough under physiological conditions to be conjugated by a UGT. Such a view has been echoed in a recent report investigating the UGT responsible for the N-carbamoyl glucuronidation of the aliphatic secondary amine sertraline (Obach et al., 2005). This implied stability of M7, which may be thermodynamically favorable under certain conditions (Aresta et al., 2000), is legitimated by its distinct radiochromatographic peak (well resolved from M8) that coelutes with m/z 418, a drug-dependent ion not observed in predose samples, in acidic (pH 3.5) aqueous mobile phase within concentrated rat plasma extracts and monkey bile. The detection of a unique radiochromatographic peak suggests strongly that M7 is not a solvent- or ion source-generated artifact as reported

![Proton NMR data at 298 K for isolated metabolite M8](image)

**TABLE 5**

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<th>Chemical Shift (ppm)</th>
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- Multiplicity simplifies upon heating.
- Exact value was not discernible due to signal overlap and/or complexity.
- Partially obscured due to acetonitrile.
- Seven protons were integrated between 3.20 and 3.75 ppm.
- The single proton at 7.49 ppm integrated to 0.3H due to slow relaxation and short relaxation delay.

![Collision-induced dissociation spectrum of protonated M8 (m/z 594) and its proposed fragmentation pathways](image)
(Edlund and Baranczewski, 2004) for the carbamic acid precursor of an N-carbamoyl glucuronide metabolite which was only minimally observed upon direct MS infusion of the parent amine in 20 mM bicarbonate buffer. In these in vitro studies, the carbamic acid was never detected, although the N-carbamoyl glucuronide was generated readily, consistent with our experience with 1. Although the proposed structure of M7 may be considered instinctively speculative due to the anticipated instability of such a molecule during sample work-up, in acidic mobile phase and under high-temperature MS conditions, similar carbamic acid metabolites have been observed under analogous conditions by LC-MS (Beloborodov et al., 1989; Wang et al., 1991), and some were stable enough to be isolated for 1H NMR analysis (Delbressine et al., 1990; Richards et al., 1997; Pothuluri et al., 2000).

It is unknown whether M7 is formed enzymatically (e.g., via carbonic anhydrase) or nonenzymatically in vivo. It is currently believed that carbamic acid formation from amine-containing molecules in vivo occurs via a solution equilibrium between the amine and dissolved CO2-equivalents (CO2(d); White et al., 1968; Morrow et al., 1974; Davis et al., 1993); hemoglobin transport of some CO2 occurs by carbamate formation between its free α-amino groups and CO2(d) (Kilmartin and Rossi-Bernardi, 1973). Based on this scenario, M7 could form by direct nucleophilic attack of CO2(d) by 1, resulting in an equilibrium between M7 and CO2(d) and 1. If M7 is formed in close proximity to a UGT, such as in a metabolic enzyme-rich environment like liver tissue, glucuronidation of M7 to stable M8 would cause further formation of M7, resulting in the adjustment of the various equilibriums within the total carbonic acid pool. In tissues such as the liver, where CO2 concentrations are elevated and UGTs are ubiquitous, the 1 and CO2(d)-M7 equilibrium will be driven toward M7, resulting in further generation of M8. Complete biliary clearance of M8 from hepatic tissues may explain why M8 is only detected in the bile of rats and monkeys. This last fact suggests that M8 is formed in both species by hepatobiliary clearance of 1; this is supported by the generation of M8 in hepatic microsomes. It is postulated that once the bile, of which M8 is the major drug-related component in both the rat and monkey, is secreted into the upper gastrointestinal, M8 is degraded within the gut microflora by β-glucuronidase directly to 1, which is either reabsorbed or excreted in the feces as unchanged or metabolized drug. As observed in vitro when M8 is subjected to β-glucuronidase, M7 is not detected in feces, suggesting that M7 is not stable within the small intestine, possibly due to its pH 6.8 environment.

It is hypothesized that M7 was only detected within venal plasma
because this was the only analyzed biological matrix in which 1 could form the equilibrium with CO$_2$(d) necessary for M7 formation (as described above). In humans, total bicarbonate concentrations in plasma tend to be ca. 2-fold greater than that in tissues (Davis et al., 1993), with a fraction (0.5 mM) of plasma CO$_2$ in carbamate bonds with plasma proteins (White et al., 1968). Furthermore, human venous blood carries approximately a 2-fold greater amount of CO$_2$ within carbamino bonds than does arterial blood (White et al., 1968). Hence, the conditions may be most optimal for M7 formation in venal plasma. However, it remains unclear why M7 is present in rat, but not monkey, plasma. This difference may be due to species differences in plasma pH and/or bicarbonate concentrations (Kilmartin and Rossi-Bernardi, 1973), but may be more readily explained by the significantly higher systemic exposures of total drug-related material in rats (AUC$_{0-24}$ of 56,500 ng-Eq·h/ml) versus monkeys (AUC$_{0-24}$ of 6380 ng-Eq·h/ml), which might have afforded greater absolute quantities of M7 in rat plasma that were well above its analytical limit of detection.

M7 may also arise upon loss of the sugar moiety within M8. However, the rapid, quantitative conversion of M8 to 1 directly by β-glucuronidase in vitro without the detection of M7 suggests that this is a highly unlikely scenario. This conclusion is also supported by the in vivo observation in both species that M8 is only detected within the bile, suggesting that M7 is not stable in the upper gastrointestinal once M8 is deconjugated following its biliary excretion. In addition, the absence of M8 in circulation suggests that M7 does not arise from M8 in plasma. The process by which M7 is generated physiologically within circulation and/or tissues remains to be determined. Further studies to better understand the equilibrium between 1 and M7 in biologically relevant solutions of varying bicarbonate concentration and pH are currently underway within our laboratory.

The detection of a stable carbamic acid metabolite of an aliphatic secondary amine in plasma highlights the caveat of the potential simultaneous overestimation of unchanged drug and underestimation of its carbamic acid to the contribution of systemic exposures to total drug-related material. The generally accepted intrinsic instability of carbamic acids suggests that their degradation may be possible upon sample work-up that would inadvertently result in erroneous overestimation of parent pharmacokinetic parameters. However, previous reports of carbamic acids arising from both endogenous and exogenous amine-containing compounds, the commercial availability of certain highly stable carbamic acids (e.g., α-naphthyl-N-methylcarbamic acid), and our ability to detect M7 within acidic aqueous solutions and after

**Fig. 10.** An overview of the cross-species metabolic pathways for 1 in vivo.
high-temperature vaporization should prompt a greater appreciation for such chemical species as metabolites influencing the disposition of xenobiotic amines.

Acknowledgments. We acknowledge Neurogen Corporation for significant contribution to the collaborative development of 1 with Pfizer Inc., and for supplying 1, 2, and 3. We also thank Dr. Klaus Schildknecht of the Radiosynthesis Group at PGRD, Groton, CT for the synthesis and purification of [14C]1.

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