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PHARMACOKINETICS, DISPOSITION, AND METABOLISM OF BICIFADINE IN THE MOUSE, RAT, AND MONKEY

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Running Title: *In vivo* Metabolism of Bicifadine

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Abbreviations:

BIcifadine, DOV 220,075 [(±)-1-(4-methylphenyl)-3-azabicyclo[3.1.0]hexane HCl]; DOV 21,947, [(+)-1-(3,4-chlorophenyl)-3-azabicyclo[3.1.0]hexane HCl]; amu, DOV 255,828, 5-(4-methylphenyl)-3-azabicyclo[3.1.0]hexan-2-one; atomic mass unit; BDC, bile duct cannulated; ESP, electrospray; HPLC, high performance

liquid chromatography; IS, internal standard; LC/MS, liquid chromatography/mass spectrometry; LC/MS/MS, liquid chromatography with tandem mass spectrometric detection; LSC, liquid scintillation chromatography; MAO, monoamine oxidase; MRM, multiple reaction monitoring; *m/z*, mass to charge ratio; C_{max}, maximum plasma concentration; T_{max}, time to reach maximum plasma concentration; AUC_{0-t}, area under the plasma concentration versus time curve from time zero to the last measurable concentration; $AUC_{0-\infty}$, area under the plasma concentration versus time curve from time zero to infinity; AUC_{oral}, area under the plasma concentration versus time curve from time zero to infinity after the oral dose; AUC_{IV}, area under the plasma concentration versus time curve from time zero to infinity after the intravenous dose; λ_z, terminal phase rate constant; V_z, terminal phase volume of distribution; CL, clearance; F, absolute bioavailability.

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ABSTRACT

Bicifadine [DOV 220,075; (±)-1-(4-methylphenyl)-3-azabicyclo[3.1.0]hexane HCl)] is a non-narcotic analgesic that is effective in animal models of acute and chronic pain. In this study, the pharmacokinetics, disposition, and metabolism of bicifadine were determined in male and female mice, rats, and cynomolgus monkeys following single oral and i.v. doses. [^{14}C]Bicifadine was well absorbed in all 3 species. The oral bioavailability of bicifadine in mice and rats was 50-63% and 79-85%, respectively, and slightly lower in monkeys (33-42%). Based on the values of the area under the concentration time curves, unchanged bicifadine comprised 7-12% of the plasma radioactivity after the oral dose and 14-26% after the i.v. dose in all 3 species. The major plasma metabolites were the lactam (M12), the lactam acid (M9), and the acid (M3) plus its glucuronide conjugate. At 0.5 h after the oral dose to rats, 63-64% of the radioactivity in the rat brain was bicifadine and the remainder was the lactam. Most of the radioactivity after oral and i.v. dosing to the 3 species was recovered in the urine. The lactam acid was the major urinary metabolite in all species; bicifadine and the lactam were either not detected or were minor components in urine. Fecal radioactivity was due to the acid and lactam acid in the 3 species. Rat bile contained mainly the lactam acid and the acid plus its acyl glucuronide. Plasma protein binding of [^{14}C]bicifadine was moderate in the mouse (80-86%) and higher in the rat and monkey (95-97%). In summary, bicifadine was well absorbed, extensively metabolized, and excreted via the urine and feces as metabolites.

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Bicifadine [(±)-1-(4-methylphenyl)-3-azabicyclo[3.1.0]hexane HCl; DOV 220,075, Figure 1] is an inhibitor of norepinephrine and serotonin uptake that is being developed for the treatment of acute and chronic pain. The compound is a non-narcotic analgesic (Epstein et al., 1982) that is active in preclinical models of neuropathic pain. In animals, bicifadine has been shown to inhibit dopamine uptake, thus making it a functional triple reuptake inhibitor with antinociceptive and anti-allodynic activity in acute, persistent, and chronic pain models (Basile et al., 2007). Clinically, it has been shown to be effective in the treatment of acute dental (Stern et al., 2005) and bunionectomy pain (Riff et al., 2006).

The metabolites of bicifadine formed by mouse, rat, monkey, and human hepatocytes and microsomes were reported previously (Erickson et al., 2007). The two main metabolic pathways in all 4 species were formation of a lactam and the oxidation of the tolylmethyl group. When tested in human *in vitro* systems, MAO-B was the principal enzyme that formed the lactam while CYP2D6 was responsible for the initial hydroxylation of the tolylmethyl group.

The present studies were performed to determine the pharmacokinetics, metabolism, and disposition of bicifadine in the mouse, rat, and monkey, the species used in toxicological and carcinogenicity studies.

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MATERIALS AND METHODS

Chemicals and Reagents. [^{14}C]Bicifadine was synthesized by ViTrax Company (Placentia, CA). The specific activity of the HCl salt was approximately 1.0 mCi/mmol and its radiochemical purity was >99%. Carbo-Sorb, Perma Fluor, Ultima-Flo M scintillation fluids, and [^{14}C]caffeine (51.2 mCi/mmol) were purchased from PerkinElmer Life and Analytical Sciences, Inc. (Waltham, MA). [^{14}C]Warfarin (56.0 mCi/mmol) was purchased from Amersham Biosciences (Piscataway, NJ). Pooled, heparinized plasma from male CD-1 mice, Sprague Dawley rats, cynomolgus monkeys, and humans was purchased from BIochemed Pharmacologicals, Inc. (Winchester, VA). Other reagents were of analytical grade and were purchased commercial sources.

Animal Studies. All experiments were approved by the Covance Institutional Animal Care and Use Committee. CD-1 mice (20-50 g) and Sprague Dawley rats (250-300 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN); BDC Sprague Dawley rats (310-359 g) were purchased from Hilltop Lab Animals, Inc (Scottdale, PA). Rodents were acclimatized for at least 4 days prior to dosing. Cynomolgus monkeys (2.0-3.2 kg) were from the Covance stock colony. The vehicles were deionized water for the oral dose and sterile saline for injection for the i.v. dose. Animals were fasted overnight before dosing and food was returned 4 h later; water was provided *ad libitum*.

Dosing and Sample Collection. Mice (9/sex/time point) and rats (3/sex/time point) were administered [^{14}C]bicifadine as a single oral (20 mg/kg) or i.v. (5

mg/kg) dose and blood samples were collected under isoflurane anesthesia by cardiac puncture using tripotassium EDTA as the anticoagulant. Blood from 9 mice per time point was pooled to form 3 samples, with equal volumes from the 3 mice in each pool, in order to have sufficient sample for analyses. Brains were collected from rats that were euthanized on the first day after dosing and were rinsed with cold saline, blotted dry, weighed, and placed on dry ice before being stored at -80ºC. On the day before dosing, BDC rats were lightly anesthetized and the bile and duodenal cannulas were externalized. A solution of 4 mM sodium taurocholate in isotonic saline was infused into the duodenal cannula at a rate of 0.9 ml/h; the infusion continued until the time of sacrifice. Samples were collected for 72 h after dosing. Monkeys (3/sex) were dosed by i.v. bolus at 4 mg/kg via a saphenous vein and blood samples were collected from a femoral vein. Six weeks later, the monkeys were dosed orally by gavage at 8 mg/kg and blood samples were collected. Urine and feces were collected for 168 h from mice (3/cage), rats, and monkeys that were housed in metabolism cages; cages were rinsed at least every 24 h and wiped at the end of the collection period.

Radioactivity Analysis. Blood and plasma samples (0.1 ml), urine samples (0.2 g), and cage rinses (0.5 g) were added to Ultima Gold XR scintillation cocktail. Feces were homogenized in water and samples (0.2 g) were combusted using a Packard Model 307 Sample Oxidizer (PerkinElmer Life and Analytical Sciences). Cage wipes were extracted in water using gentle shaking and samples (0.5 g) were added to the scintillation fluid. The pooled mouse carcasses (3/sample) were cut into pieces and digested using 1 N NaOH and

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samples (0.5 g) were weighed and decolorized using 30% H_2O_2. Ultima Gold XR scintillation cocktail was added and the samples were counted after setting overnight at room temperature. Each rat brain was homogenized using a probe-type homogenizer at a 1:1 ratio (w:w) with a solution of 36 μM tranylcypromine, an MAO inhibitor, before samples were combusted. All samplers were analyzed by LSC using Packard Model 2900TR liquid scintillation counters.

Bioanalytical Analysis for Bicifadine and M12. Plasma samples were analyzed for bicifadine and the lactam metabolite, M12 (DOV 255,828), using a validated LC/MS/MS method at WIL Research Laboratories (Ashland, OH). Samples (0.2 ml) were transferred to glass tubes containing 50 μl of 0.5 M KOH and the IS (DOV 21,947) in 25 μl methanol:water (v:v, 1:1). Diethyl ether (8 ml) was added to each tube, the tubes were shaken for 15 min, and centrifuged. The organic layer was evaporated to dryness and 1 ml of diethyl ether was added. The tubes were mixed by vortexing and the samples were dried under a stream of nitrogen. The samples were reconstituted in 200 μl of methanol and transferred to autosampler vials. The extracts were injected onto a Hypersil C18-BDS column (50 x 4.6 mm, 3 μm particle size) with a C18 guard column (Agilent Technology, Santa Clara, CA). The mobile phase was 30% acetonitrile/35% methanol/0.5% formic acid/34.5% 5 mM ammonium acetate (v/v/v/v) with a flow rate of 0.4 ml/min. The mass spectrometry was done using a Micromass Quattro Micro™ tandem mass spectrometer equipped with an APCI+ interface (Waters Corporation, Milford, MA). The cone voltage was 25-35 V, depending on the analyte. The source and desolvation temperatures were 130°C and 400°C,

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respectively; the cone and desolvation gas flows were approximately 100 l and 700 l N_2/h, respectively. The collision gas was argon and the collision energy was 17-22 eV. Quantification was done by monitoring the mass transitions of *m/z* 174→133, *m/z* 188→105, and *m/z* 228→159 for bicifadine, M12, and the IS, respectively. The peak area ratios of bicifadine/IS, M12/IS, and the theoretical concentrations of the calibration standards were fit to a quadratic function with $1/x^2$ weighting, excluding the origin. The assay range for bicifadine in mouse, rat, and monkey plasma was 4.13 to 1652 ng/ml and 5 to 2000 ng/ml for M12.

Metabolite Profiling and Identification. Pooled plasma samples collected 1 and 4 h postdose were extracted twice with 3 volumes of acetonitrile and centrifuged. The extracts were combined, evaporated to dryness under a stream of nitrogen, and resuspended in the reconstitution fluid (water:acetonitrile, 1:1). Urine samples from the 0-24 h mouse and rat and 0-48 h monkey collections were pooled such that the pools constituted a fixed percent of the volume from each sample (10% mouse and rat; 5% monkey). The urine was centrifuged in a filtration device (Millipore 0.5 ml Ultrafree-MC 0.45 μm; Billerica, MA) and used for metabolite profiling without further processing. Ten percent of each fecal sample homogenate was pooled to form a single pool for each group (0-48 h for mice and rats; 0-72 h for monkeys). Samples were extracted twice with approximately 3 volumes of acetonitrile and centrifuged. The supernatants were combined, evaporated to dryness under a stream of nitrogen, and resuspended. Ten percent of each rat bile sample from the 0-24 h collection period was pooled, according to sex, clarified using a centrifugal filtration device, and profiled without

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any further purification. Brain homogenates were extracted with 2 volumes of acetonitrile. The extracts were combined, dried under a gentle stream of nitrogen, and resuspended. Aliquots of all samples were taken for HPLC or LC/MS analysis. Corrections were made for extraction and reconstitution recoveries to calculate metabolite concentrations in plasma, feces, and brain.

LC/MS was performed using a Shimadzu SCL-10A VP LC system controller, LC-10AD VP pumps, and SIL-10AD VP injector (Shimadzu Corp., Columbia, MD). The HPLC column was a Zorbax SB-phenyl column (250 mm x 4.6 mm, 5 μm; Agilent Corp., Santa Clara, CA) with a Phenomenex propylphenyl guard column (4 mm x 3.0 mm; Phenomenex Inc., Torrance, CA). The ultraviolet detector was a Shimadzu model SPD-10A set at 254 nm and the radiochemical detector was a Packard Radiomatic series 500 with Flo-One version 3.65 software (Packard Instrument Co., Meriden, CT). The mass spectrometer was a Micromass Quattro II with an ESP Z-spray source and MassLynx version 3.4 software (Waters Corporation). The mobile phase solvents used in the gradient elution were 0.10% formic acid in reverse osmosis water and acetonitrile; the flow rate was 1.0 ml/min. Ultima-Flow M LSC cocktail was used as the scintillant (2.1 ml/min). After passing through the column, 30% of the flow was diverted to the mass spectrometer and 70% to the radiometric detector. The mass spectrometer was used in the electrospray positive mode with cone voltage of 10 to 28 volts. The mass range was 100 to 660 amu with a scan time of 0 to 55 min. The source block temperature was 130ºC and the desolvation temperature was 325ºC. The ESI nebulizer and bath gas was N_2 (15 l/h and 425 l/h, respectively).

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For the product ion analyses, the cone voltage and collision energy were variable and argon was used as the collision gas. Multiple reaction monitoring used the same instrumentation and conditions as the LC/MS analyses with a dwell time of 0.2 sec and argon as the collision gas. The contribution of the [^{14}C]tracer to the mass of the metabolites was low enough (ca. 1.6%) so that it did not contribute to the *m/z* ratios as determined by mass spectrometry.

Pharmacokinetic Analysis. Calculations were performed on mean plasma concentrations of bicifadine and M12 in the mouse and rat and on data from individual monkeys. Noncompartmental analysis was done using WinNonlin, version 4.1 (Pharsight Corporation, Mountain View, CA). C_{max} and T_{max} were the observed values. The terminal phase t½ was calculated as ln (2)/λ_z. AUC_{0-t} was calculated using the linear trapezoidal method; $AUC_{0-\infty}$ was calculated as $AUC_{0-t} + C_t/\lambda_z$, where C_t is the last measurable concentration. Systemic clearance (CL) of bicifadine was calculated as the bolus i.v. dose divided by $AUC_{0-\infty}$ and the volume of distribution of the elimination phase (V_z) was calculated as CL/λ_z. Absolute bioavailability (F) of bicifadine was calculated as $AUC_{oral} \times Dose_{IV}/(AUC_{IV} \times Dose_{oral})$.

Protein Binding. The extent of protein binding of bicifadine was determined using an ultrafiltration method. Radiolabeled bicifadine and the control compounds, [^{14}C]caffeine and [^{14}C]warfarin, were dissolved in ethanol and added to plasma; the volume of ethanol was <1% of the final incubation volume. After incubation for 15 min at 37ºC, duplicate aliquots were taken and radioactivity was determined by LSC. Plasma was added to a Multiscreen Ultracel®-PPB unit (0.5

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ml, 10,000 DA cutoff) ultrafiltration device (Millipore Corp.) and centrifuged at 200*g* at 37ºC until ≤20% of the volume was collected in the receiving cup. Aliquots of the ultrafiltrate were counted by LSC. The percent of free drug was calculated as the concentration of radioactivity in the ultrafiltrate divided by the concentration of radioactivity in the unfiltered plasma. There was no measurable nonspecific binding of ^{14}C-bicifadine to the ultrafiltration devices. The control compounds [^{14}C]caffeine (6000 ng/ml) and [^{14}C]warfarin (4000 ng/ml) were 8.9 ± 4.5% and 96.5 ± 0.1% plasma-protein bound, respectively, in human plasma under these conditions.

RESULTS

Pharmacokinetic Parameters T_{max} of plasma radioactivity and unchanged bicifadine occurred at 30 min and 1 h after the oral dose of [^{14}C]bicifadine to mice and rats, respectively (Tables 1 and 2; Figure 2), and slightly later in monkeys (Table 3). Both C_{max} and $AUC_{0-\infty}$ for radioactivity and bicifadine were approximately 2-4 times higher in rats compared to mice. The higher plasma concentrations of the parent drug were due to a smaller V_z and CL in the rat compared to the mouse. The absolute bioavailability of bicifadine was 50-63% in the mouse and approximately 79-85% in the rat. In the monkey, C_{max} values of plasma radioactivity and bicifadine were 6200 ng equiv/g and 1300 ng/ml, respectively, after an oral dose of 8 mg/kg. The compound's CL and F in the monkey were the lowest of the 3 species. Bicifadine and its metabolites were not concentrated in red blood cells since the concentration of radioactivity in whole

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blood was ≤ 85% of that in plasma in all 3 species for up to 4 h postdose (data not shown).

Based on values of AUC_{0-t}, unchanged bicifadine accounted for only 7-12% of the total plasma drug equivalents in mice, rats, and monkeys after the oral dose. The percentage increased slightly to 14-26% after the i.v. dose. Most of the plasma radioactivity was associated with metabolites. One of the metabolites, the lactam M12 (DOV 255,828), was assayed by LC/MS/MS. In both mice and monkeys, this metabolite accounted for 3-7% of plasma drug equivalents after the i.v. and oral doses. The contribution in the rat was 40-54% after the oral dose and 16-29% after the i.v. dose. The other metabolites were profiled and identified by LC/MS (below).

Protein Binding. Approximately 14-16% of [^{14}C]bicifadine was unbound in mouse plasma at concentrations of 100 to 1000 ng/ml, increasing to 20% when the concentration was increased to 10,000 ng/ml (Table 4). In both the rat and monkey, only 4-5% was unbound over the concentration range of 100 to 10,000 ng/ml. In comparison, 2.2% to 2.6% of [^{14}C]bicifadine was unbound in human plasma over the concentration range of 100 to 3000 ng/ml.

Excretion of Radioactivity. By 168 h postdose, 63-72% of the oral dose of radioactivity to mice was in urine while 13-24% was in the feces (Table 5). The cage washes and wipes contained another 9%. At the end of the collection period, 0.3% or less of the dose remained in the mouse carcass. Total recovery was 96% and 93% in male and female mice, respectively. In rats, 57-61% was recovered in the urine while another 34-36% was in the feces.

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When [^{14}C]bicifadine was administered orally to the BDC rats, 31% of the radioactivity was in bile and only 3% was recovered in the feces. The percentage in urine was approximately the same as in the intact rats. The results indicate that essentially all of the orally administered [^{14}C]bicifadine was absorbed and fecal radioactivity in intact rats was due to biliary excretion. Only 0.4-0.5% of the radioactivity remained in the carcass at 72 h postdose; total recovery was ≥99%. In monkeys, 57% of the orally-administered radioactivity was in the urine of the males and 33% was in the urine of the females. Only 7% was in the feces of either sex. A larger percentage of the radioactivity was recovered in the cage washes and wipes, which could be due to either urinary or fecal excretion. Total recovery in the monkeys was approximately 91%. In all 3 species, most of the radioactivity was excreted by 48 h postdose and the pattern of excretion after the i.v. dose was similar to the oral dose (data not shown).

Metabolite Profiling. Representative metabolite profiles in the plasma and urine of mice, rats, and monkeys are illustrated in Figures 3 and 4, respectively. The proposed metabolic pathway of bicifadine in mouse, rat, and monkey is presented in Figure 5. The plasma concentrations of the metabolites are presented in Table 6. The percentage of the dose in the urine and feces associated with the metabolites were calculated similarly, but using the percent of the radioactivity excreted by 24, 48, or 72 h, depending on the species and matrix (Table 7).

Mouse Plasma. The major metabolites in male and female mice 1 h after the oral dose of [^{14}C]bicifadine were M9, the lactam acid, and M12, the lactam. The

latter metabolite was slightly higher in concentration in female compared to male mice. Unchanged bicifadine had the next highest concentration. The plasma also contained M3, in which the methyl group had been oxidized to the carboxylic acid. M3 was conjugated to form either a taurine conjugate (M27) or an acyl glucuronide (M14), which was detected as a series of peaks. Acyl glucuronides are known to isomerize under neutral to slightly alkaline pH with the aglycone portion migrating to the 2-, 3- or 4-O positions of the sugar (Hasegawa et al., 1982; Janssen et al., 1982). M19 eluted approximately 1 min before M9 and had a protonated molecular ion and product mass spectrum that were similar to M9. The position of oxidation, though, was at C4 of the pyrrolidine ring rather than at C2. Mouse plasma also contained several minor metabolites that eluted at approximately 44 min and 51 min; their structures were not identified. At 4 h postdose, M9 and M12 were the two predominant metabolites in mouse plasma. The metabolite profiles in mice 1 h after the i.v. dose were similar to the oral profiles; the plasma radioactivity, though, was predominantly associated only with M12 at 4 h (data not shown).

Rat Plasma. At 1 h after the oral dose, the two most prominent peaks in plasma were M12 and bicifadine in both male and female rats. The concentrations of M3 and M9 were similar to those in mice but represented a smaller percentage of the radiochemical profile due to the higher concentration of total drug equivalents. Plasma from female rats also contained an N-sulfate conjugate of bicifadine (M23). There were minor amounts of two unidentified metabolites (M20 and M21). By 4 h postdose, the plasma radioactivity was associated mainly with M12

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and M9 with minor amounts of either M3 or M23. At 0.5 h after the i.v. dose of [^{14}C]bicifadine, the metabolite profile in plasma was composed of M12, bicifadine, M3, and M9 in rats (data not shown).

Rat Brain. Brains from orally and i.v. dosed rats were collected from 0.5 h to 8 h postdose and homogenized in a buffer containing an MAO inhibitor to prevent the postmortem formation of M12 (Erickson et al., 2007). The brain-to-plasma ratio of total drug equivalents was approximately 2 at 0.5 h and decreased to 0.3-0.4 at 8 h for both dose routes of administration (Table 8). Bicifadine accounted for 63-64% of the profiled radioactivity at 0.5 h after the oral dose (Figure 6); the rest was composed of M12 with small but non-quantifiable concentrations of M24 in several samples (Figure 7). Several pooled samples had a small, unidentified peak at approximately 26 min. By 4 h, greater than 90% of the profiled brain radioactivity was M12. Similar profiles were detected after the i.v. dose at 0.5 and 1 h; by 2 h, only M12 was present. The concentration of bicifadine in the brain was approximately 2 and 2-3 times higher than in the plasma following the oral and i.v. doses, respectively (Figure 6). By comparison, M12 concentrations were similar in the plasma and brain after the oral dose but slightly higher in the brain after the i.v. dose. Results for oral and i.v. dosed female rats were similar to the male.

Monkey Plasma. The radioactive peaks in monkey plasma 1 h after the oral dose were identified as M9, M3, bicifadine, M12, and an unknown metabolite that eluted at 4.0 min. By 4 h postdose, the plasma contained only M3 (male and female) and M9 (female only). The metabolite profile after the i.v. dose was

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similar to the oral profile except that the unknown metabolite at 4 min was not detected while the acyl glucuronide conjugate of M3 (M14) was observed as a series of peaks with similar mass spectra (Figure 3). The conjugates of M14 comprised approximately 20-23% of the metabolite profile at 1 h in both male and female monkeys after the i.v. dose.

Urine. The main urinary metabolite in all 3 species was the lactam acid (M9). In mice and rats, it constituted 36-39% of the dose and was also the major urinary metabolite in monkeys (Table 7). Mouse urine contained lesser amounts M27, M3 and its acyl glucuronide (M14A and M14C), M19, M28B, M26, and M12, while bicifadine constituted less than 1% of the dose. Urine from female mice contained a small amount of M17, in which the pyrrolidine ring had been opened. In addition to M9, rat urine contained minor amounts of M3 and its acyl glucuronide (M14A-C), M17, bicifadine (2-5% of the dose), M19, and M12. M2 and M9, each constituting ≤1% of the dose, were detected only in rat urine. Rat urine contained a number of minor unidentified metabolites that each represented ≤1.1% of the dose. The metabolite profile in monkey urine was composed of M9 and its acyl glucuronide (M31A-F), plus M3 and its acyl glucuronide (M14A-F). Minor amounts of the C4 lactam (M19) and M26 were also in male monkey urine.

Bile. The carboxylic acid metabolite M3 in rat bile accounted for 10.7% of the orally administered dose in male rats and 5.6% in female rats; its acyl glucuronide conjugate represented another 12.5-12.6% of the dose (Table 7). The other acid-containing metabolite, M9, was also present in the bile sample

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(Figure 4). M25, the taurine conjugate of M9, was detected only in the bile from male rats. The unidentified metabolite M26 was also in bile. The metabolite profile in urine of the BDC rats was the same as that of intact rats (data not shown).

Feces. Following either oral or i.v. dosing of [^{14}C]bicifadine, mice, rats (intact and BDC), and monkey feces contained predominantly the 2 acid metabolites (M3 and M9). Feces from orally dosed monkeys contained minor amounts of M26. The ratio of M3 to M9 was similar in all 3 species when [^{14}C]bicifadine was administered by either dose route.

Metabolite Identification. *Bicifadine, M9, and M12.* The full scan mass spectra of bicifadine, the lactam M12, and lactam acid M9 were essentially identical to standards of bicifadine, M12, and M9, respectively (Table 9). In addition, they had the same HPLC retention times as the authentic standards.

M2 (hydroxymethyl bicifadine). M2 had a $[M + H]^+$ at *m/z* 190, 16 amu (oxygen) higher than that of bicifadine. The presence of ions at *m/z* 149 and 121 indicated that hydroxylation had occurred on the methyl group.

M3 (bicifadine carboxylic acid). The full scan LC/MS mass spectrum of M3 had a $[M + H]^+$ at *m/z* 204, 30 amu (+2 oxygen - 2 hydrogen atoms) higher than bicifadine. The presence of ions at *m/z* 163 and 135 in the ions product mass spectrum indicated that the methyl group had been oxidized to the carboxylic acid.

M5 (N-acetyl M9). M5 had a $[M + H]^+$ at *m/z* 260, 42 amu (acetyl) higher than that of M9. The ions at *m/z* 163 and 135 indicated that the methyl group was oxidized

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to the carboxylic acid while the ion at m/z 218 showed that an acetyl group was present. It could not be determined in these studies whether the acetyl group was added before or after lactam formation.

M10 (hydroxy bicifadine). M10 had a $[M + H]^+$ at *m/z* 190, 16 amu (oxygen) higher than bicifadine. The presence of ions at *m/z* 105 and 91 indicated that hydroxylation had not occurred on the methyl group, while ions at *m/z* 57 and 134 indicated that oxidation had occurred at the C2 position of the pyrrolidine ring. Hydroxylation at C2, rather than another carbon, was supported by comparison of the product mass spectrum of M10 with the same metabolite formed in hepatocyte incubations using bicifidine labeled at the C2 position almost exclusively with ^{14}C, rather than ^{12}C (Erickson et al., 2007). Only the unchanged parent drug and proposed fragments that contained the highly enriched $^{14}C2$ atom in the *in vitro*-generated metabolite had *m/z* ratios that were increased by 2 amu when compared to M10 formed *in vivo* using bicifadine labeled at a much lower specific activity. The rest of the proposed fragments, which did not contain the C2 atom, had the same *m/z* ratios between the two spectra.

M14A-F (M3 acyl glucuronides). A series of peaks eluted between 6.8 and 9 min, all of which had a full scan $[M + H]^+$ at *m/z* 389, 176 amu (glucuronic acid) higher than M3. The ion at *m/z* 204 was due to the loss of the glucuronic acid moiety, which was the base peak in the product mass spectrum for some of the glucuronides.

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M17 (pyrrolidine ring-opened bicifadine). M17 had a full spectrum $[M + H]^+$ at *m/z* 206, 32 amu (2 oxygen atoms) higher than bicifadine. The ions at *m/z* 105 and 91 indicated that hydroxylation had not occurred on the methyl group. The loss of water, CO, and formic acid from the *m/z* 189 fragment ion indicated that a terminal carboxylic acid was present. The addition of water across the C1 and C2 position of the pyrridoline ring of bicifadine could result in a ring-opened metabolite containing both a terminal amine and carboxylic groups.

M19 (C4 lactam acid). The full spectrum $[M + H]^+$ of M19 was at *m/z* 218, the same molecular weight as M9. The ion at *m/z* 135 indicated that the methyl group was oxidized to the carboxylic acid. The differences in the way that M19 fragmented with respect to M9, though, demonstrated that oxidation had occurred at the C4 position on the pyrrolidine ring.

M23 (bicifadine N-sulfate). The full spectrum $[M + H]^+$ of M23 was 80 amu (sulfate) higher than bicifadine. The product ion mass spectrum of the acetonitrile adduct of M23 (*m/z* 295) gave ions that included *m/z* 174 (-sulfate) and three ions that were similar to those in bicifadine (*m/z* 157, 142, and 105).

M24 (bicifadine C4 lactam). The full spectrum $[M + H]^+$ of this metabolite was at *m/z* 188, 14 amu (oxygen - 2 hydrogen) higher than that of bicifadine. The ion at *m/z* 105 indicated that the methyl group was not modified. The differences in the fragmentation of M24 with respect to M12 indicated that the oxidation had occurred on the C4 position of the pyrrolidine ring.

M25 (M9 taurine conjugate). M25 was detected only in urine and bile from male rats. Its $[M + H]^+$ was at *m/z* 325, 107 amu (taurine) higher than that of M9 and

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its product ion mass spectrum gave an ion at *m/z* 200, indicating the loss of the taurine moiety.

M27 (M3 taurine conjugate). The $[M + H]^+$ of M27 was 107 amu higher than that of M3 and its base peak (*m/z* 186) corresponded to the loss of the taurine moiety.

M29A-B. The $[M + H]^+$ of both M29A and M29B was at *m/z* 436, 262 amu higher than bicifadine. Their product ion mass spectra were similar with minor differences. They were detected only in mouse plasma; a structure has not been proposed for either metabolite.

M31A-F (M9 acyl glucuronides). The metabolites M31A-F all had $[M + H]^+$ at *m/z* 394, 178 amu (glucuronic acid) higher than that of M9. They all had ions at *m/z* 218 (-glucuronic acid), 200 (*m/z* 218 - water), and 171.

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DISCUSSION

The oral bioavailability of bicifadine was high in mice (50-63%) and rats (79-85%), indicating that first pass metabolism of the compound was low. In monkeys, bioavailability was slightly lower (33-42%). Plasma clearance after the IV dose, though, was either similar or higher than can be accounted for only by the liver. Based on the blood flows and hematocrit in the 3 species (Davies and Morris, 1993), liver plasma flow in the mouse, rat, and monkey is 50, 30, and 26 ml/min/kg, respectively. In the 2 rodent species, bicifadine clearance was 25% greater than these values. Oxidation to the lactam, M12, by MAO-B was the major primary metabolite in human hepatocytes (Erickson et al., 2007); this pathway also appears to be important in animals. Since MAO-B is widely distributed in the body (Cesura and Pletscher, 1992), extrahepatic clearance of bicifadine is likely.

Bicifadine readily penetrated into the rat brain. The concentration of drug-derived radioactivity was greater in brain compared to plasma up to 2 h following the oral dose with essentially all of the radioactivity either unchanged bicifadine or M12 in both males and females. At 0.5 h following the oral and IV doses, the brain-to-plasma ratio of bicifadine was approximately 3. The concentrations of lactam in the brain and plasma after the oral dose were similar and slightly higher in the brain after the i.v. dose. The lactam in the brain could have come either from plasma or by *in situ* oxidation of bicifadine by MAO.

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Elimination of drug-related radioactivity was mainly via the urine. Radioactivity in the feces of intact rats was due predominantly to biliary excretion. Only 3% of the orally-administered radioactivity was recovered in the feces from BDC rats, showing that essentially all of the orally administered [^{14}C]bicifadine was absorbed. The carcasses of mice and BDC rats contained 0.5% or less of the dose at 7 and 3 days, respectively.

Metabolic clearance of bicifadine in the mouse, rat, and monkey is due mainly to two pathways. One is the formation of the lactam, M12 (Erickson et al., 2007). Initial oxidation by MAO leads to the formation of an imine (Belleau and Moran, 1963); the enzyme responsible for further oxidation to the lactam has not been delineated. The other main pathway is oxidation of the methyl group, via M2, to the carboxylic acid M3. This metabolite undergoes conjugation to the acyl glucuronide in all 3 species and to the taurine conjugate in mice.

Approximately half of the radioactivity was excreted in the urine and feces of all 3 species as the lactam acid, M9, and its acyl glucuronide conjugate(s), M31A-F. Another 17 to 25% was excreted as the other acid metabolite, M3, and its acyl glucuronide(s). In monkeys, the only other excreted metabolites were the alternative lactam acid, M19, and a minor unidentified metabolite. Unchanged bicifadine was either a minor component or absent in urine. While the lactam, M12, is a major metabolite in plasma, it constituted less than 1% of the dose found in the excreta. The data suggest that oxidation of the lactam's methyl group to the carboxylic acid is required before it can be excreted. Alternatively, bicifadine is oxidized to the zwitterionic metabolite M3 before it is excreted. Like

the urine, the metabolites in rat bile were either carboxylic acids or their conjugates with no detectable concentrations of the unchanged parent or M9.

M9 is formed by oxidation of both the ring C2 carbon and the methyl group. Experiments using mouse and monkey hepatocytes suggested that the lactam is formed first, followed by oxidation of the methyl group (Erickson et al., 2007). The concentration of M12 was higher at 1 h of incubation and then declined at 4 h, indicating a precursor-product relationship between M12 and M9. In contrast, there was a time-dependent increase in the concentration of M3 from 1 h to 4 h of incubation.

Multiple peaks with the appropriate molecular weight and fragmentation for acyl glucuronides of M3 were observed in the plasma of mice after both i.v. and oral dosing and in plasma from i.v.-dosed, but not orally-dosed, monkeys (Figure 3). They were also detected in urine from all 3 species and in rat bile. Acyl-substituted glucuronides of M9 were in urine from monkeys but not from mice and rats. Rearrangement of the initial β-O-acyl glucuronides to other isomers is a well-established phenomenon, such as glucuronide conjugates of bilirubin-IX alpha (Compernolle et al, 1978), zomepirac (Hasegawa et al., 1982), and 3-(*p*-chlorophenyl)thiazolo[3,2-*a*]benzimidazole-2-acetic acid (Janssen et al, 1982).

In conclusion, bicifadine was readily absorbed by the mouse, rat, and monkey. Most of the drug-derived plasma radioactivity was associated with metabolites in the 3 species. Bicifadine readily penetrated into to the rat brain. Clearance was due to metabolism with the formation of the lactam and oxidation

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of the methyl group as the two main metabolic routes. The lactam acid and its acyl glucuronides accounted for approximately half of the excreted dose.

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Footnote

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Figure Legends

Fig. 1. Structure of Bicifadine.

The asterisk indicates the location of the [^{14}C]radiolabel.

Fig. 2. Plasma concentration of radioactivity, bicifadine, and the lactam metabolite M12 following a single oral dose of [^{14}C]bicifadine to male mice (20 mg/kg), rats (20 mg/kg), and monkeys (8 mg/kg).

Data are mean ± SD; N = 3 groups of pooled mice (N = 3/group), N = 3/time point for rats, and N = 3 for monkeys.

Fig. 3. Profile of plasma radioactivity from female mice (20 mg/kg PO), male rats (20 m/kg PO) and female monkeys (4 mg/kg IV).

All profiles are from pooled plasma collected 1 h after dosing.

Fig. 4. HPLC chromatogram of radioactivity in urine (0-24 h) (top panel) and bile (0-24 h) (bottom panel) of male rats dosed orally with 20 mg [^{14}C]bicifadine/kg.

Fig. 5. Proposed Metabolic Scheme of Bicifadine in Mice, Rats, and Monkeys.

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Fig. 6. Concentrations of bicifadine and the lactam metabolite M12 in the brain and plasma of male rats dosed orally (20 mg/kg; top) and i.v. (5 mg/kg; bottom) with [^{14}C]bicifadine.

Brain concentrations of bicifadine and M12 were derived from the HPLC radiochromatograms while plasma concentrations were from the LC/MS/MS assay.

Fig. 7. HPLC chromatogram of radioactivity in the brain of a male rat at 0.5 h following an oral dose of [^{14}C]bicifadine.

Table 1

Pharmacokinetics of Radioactivity, Bicifadine, and M12 in Mice Following Oral (20 mg/kg) and Intravenous (5 mg/kg) Doses of [^{14}C]Bicifadine

	Male Mice			Female Mice		
Parameter (units)	Radioactivity[a]	Bicifadine	M12	Radioactivity	Bicifadine	M12
C_{max} (ng/ml)	4890	1090	757	3990	602	581
T_{max} (h)	0.5	0.5	0.5	0.5	0.5	0.5
$AUC_{0-\infty}$ Oral (ng·h/ml)	20,800	2210	1290	27,700	2490	1650
$AUC_{0-\infty}$ IV (ng·h/ml)	3900	1030	249	6940	1030	373
t½ Oral (h)	-	2.1	1.83	-	4.6	3.9
t½ IV (h)	-	0.40	0.35	-	0.58	0.35
F(%)	-	50.1	-	-	62.5	-
CL IV (ml/min/kg)	-	62.5	-	-	63.2	-
V_z IV (l/kg)	-	2.15	-	-	3.19	-

a Units for radioactivity are expressed as ng equiv
N = 3 pooled groups/time point, each composed of 3 mice

Table 2

Pharmacokinetics of Radioactivity, Bicifadine, and M12 in Rats Following Oral (20 mg/kg) and Intravenous (5 mg/kg) Doses of [^{14}C]Bicifadine

	Male Rats			Female Rats		
Parameter (units)	Radioactivity[a]	Bicifadine	M12	Radioactivity	Bicifadine	M12
C_{max} (ng/ml)	16,500	2620	9390	15,300	3530	10,000
T_{max} (h)	1.0	0.5	1.0	1.0	1.0	2.0
$AUC_{0-\infty}$ Oral (ng·h/ml)	76,400	5470	30,200	80,400	7440	44,000
$AUC_{0-\infty}$ IV (ng·h/ml)	12,900	1770	2030	13,900	2240	4020
t½ Oral (h)	-	0.74	0.60	-	0.78	0.73
t½ IV (h)	-	0.32	0.54	-	0.52	0.51
F(%)	-	79.3	-	-	84.9	-
CL IV (ml/min/kg)	-	39.2	-	-	30.8	-
V_z IV (l/kg)	-	1.08	-	-	1.40	-

a Units for radioactivity are expressed as ng equiv
N = 3/timepoint

Table 3

Pharmacokinetics (Mean ± SD) of Radioactivity, Bicifadine, and M12 in Monkeys Following Oral (8 mg/kg) and Intravenous (4 mg/kg) Doses of [^{14}C]Bicifadine

	Male Monkeys (N = 3)			Female Monkeys (N = 3)		
Parameter (units)	Radioactivity[a]	Bicifadine	M12	Radioactivity	Bicifadine	M12
C_{max} Oral (ng/ml)	6200 ± 2470	1300 ± 930	369 ± 158	6970 ± 1790	1530 ± 700	374 ± 1 23
T_{max} Oral (h)	1.67 ± 0.58	1.33 ± 0.58	1.67 ± 0.58	1.33 ± 0.58	0.83 ± 0.29	1.33 ± 0.58
$AUC_{0-\infty}$ Oral (ng·h/ml)	20,900 ± 3200	1830 ± 710	637 ± 85	17,300 ± 600	2140 ± 450	630 ± 20
$AUC_{0-\infty}$ IV (ng·h/ml)	14,200 ± 600	2810 ± 890	997 ± 14	11,500 ± 300	2600 ± 120	7474 ± 104
t½ Oral (h)	-	0.65 ± 0.26	ND	-	0.49 ± 0.08	0.50 ± 0.06
t½ IV (h)	-	0.72 ± 0.22	0.47 ± 0.12	-	0.84 ± 0.29	0.40 ± 0.09
F Oral (%)	-	32.8 ± 9.1	-	-	42.2 ± 11.4	-
CL IV (ml/min/kg)	-	19.8 ± 5.8	-	-	20.3 ± 1.0	-
V_z IV (l/kg)	-	1.24 ± 0.50	-	-	1.48 ± 0.54	-

a Units for radioactivity are expressed as ng equiv
ND Not determined due to variability

Table 4

Plasma Protein Binding of [^{14}C]Bicifadine in the Mouse, Rat, and Monkey

Concentration (ng/mL)	Percent Bound ± SD[a]		
	Mouse	Rat	Monkey
100	85.7 ± 0.3	95.5 ± 0.2	95.6 ± 0.2
300	84.8 ± 0.1	96.0 ± 0.2	96.3 ± 0.0
1000	84.4 ± 0.1	96.3 ± 0.2	96.5 ± 0.1
10,000	79.6 ± 0.1	94.8 ± 0.1	94.8 ± 0.3

a Mean and standard deviation of 3 determinations

Table 5

Recovery of Radioactivity (Mean Percent ± SD) in Mice, Rats, and Monkeys Following an Oral Dose of [^{14}C]Bicifadine

Matrix	Mice (20 mg/kg)		Rats (20 mg/kg)				Monkeys (8 mg/kg)	
	Male	Female	Male		Female		Male	Female
			Intact	BDC	Intact	BDC		
Urine	62.9 ± 7.8	71.5 ± 5.0	60.9 ± 3.6	62.4 ± 1.0	57.2 ± 3.6	65.2 ± 3.3	56.9 ± 5.8	33.0 ± 37.0
Feces	24.0 ± 5.9	12.8 ± 0.5	34.3 ± 2.3	3.4 ± 0.3	36.1 ± 3.0	2.5 ± 0.3	6.7 ± 1.2	7.3 ± 0.7
Bile	-	-	-	31.0 ± 1.2	-	30.6 ± 2.9	-	-
Cage Residue	9.0 ± 0.2	8.6 ± 1.8	2.4 ± 0.4	1.9 ± 0.3	5.1 ± 2.6	1.0 ± 0.3	27.0 ± 3.7	50.9 ± 34.7
Carcass	0.3 ± 0.1	0.2 ± 0.1	ND	0.4 ± 0.0	ND	0.5 ± 0.2	-	-
Total	96.2 ± 2.7	93.1 ± 3.0	97.7 ± 2.0	99.0 ± 0.6	98.4 ± 1.7	99.7 ± 0.6	90.5 ± 1.1	91.2 ± 3.1

Radioactivity in rat carcasses not determined (ND) since >95% of administered radioactivity was recovered

Table 6

Plasma Concentrations (ng equiv/g) of Metabolites in Mice, Rats, and Monkeys Following a Single Oral Dose of [^{14}C]Bicifadine

Metabolite	Mice (20 mg/kg)				Rat (20 mg/kg)				Monkey (8 mg/kg)			
	Male		Female		Male		Female		Male		Female	
	1 hr	4 hr	1 hr	4 hr	1 hr	4 hr	1 hr	4 hr	1 hr	4 hr	1 hr	4 hr
Unknown	-	-	-	-	-	-	-	-	214	-	444	-
M14D	79.8	-	153	-	-	-	-	-	-	-	-	-
M14A	-	10.8	70.6	-	-	-	-	-	-	-	-	-
M14B, M27	205	51.3	266	52.1	-	-	-	-	-	-	-	-
M3	113	21.6	174	41.1	94.7	80.1	225	-	459	353	1083	266
M10	-	-	-	-	1046	-	130	-	-	-	-	-
Bicifadine	402	64.8	422	60.3	2487	-	3112	-	433	-	513	-
M19	120	105	16.6	13.7	-	-		-	-	-	-	-
M9	1042	281	1178	240	452	2000	746	1169	1268	-	1862	69.9
M20	-	-	-	-	96.1	-	-	-	-	-	-	-
M21	-	-	-	-	174	-	-	-	-	-	-	-
M23	-	-	-	-	-	-	458	148	-	-	-	-
M29A, M29B	28.6	56.7	179	233	-	-	-	-	-	-	-	-
M12	715	385	1480	785	9352	2960	8238	5181	349	-	662	-
Unknown	38.5	-	36.5	-	-	-	-	-	-	-	-	-

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Table 7

Percent of Administered Dose as Metabolites in Excreta Following a Single Oral Dose of [^{14}C]Bicifadine to Mice, Rats, and Monkeys

	Mice (20 mg/kg)		Rats (20 mg/kg)		Monkeys (8 mg/kg)	
Metabolite	Male	Female	Male	Female	Male	Female
			Urine			
Bicifadine	0.8	-	2.1	5.2	-	-
M2	-	-	1.0	-	-	-
M3	7.5	6.2	6.5	4.4	4.3	3.5
M14A-F	-	9.7	2.3	1.2	11.9	6.3
M27	3.6	3.7	-	-	-	-
M12	0.4	0.5	0.5	0.2	-	-
M9	38.2	39.1	36.4	38.2	31.2	18.3
M31A-F	-	-	-	-	5.8	2.3
M19	4.5	3.6	4.4	1.5	0.2	
M5	-	-	0.8	0.3	-	-
M17	-	0.5	0.5	0.5	-	-
M15	-	-	1.1	0.5	-	-
M16	-	-	0.5	0.4	-	-
M18	-	-	0.4	0.2	-	-
M22	-	-	0.5	0.3	-	-
M28B	0.4	-	-	-	-	-
M26	0.4	1.0	-	-	0.6	-
			Feces			
M3	6.3	3.7	16.2	13.9	3.3	2.5
M9	9.2	4.3	9.7	13.0	2.5	1.7
M26	-	-	-	-	0.3	0.2
			Bile (BDC Rats Only)			
M3			10.7	5.6		
M14A-F			12.6	12.5		
M9			4.1	9.8		
M25			0.9	-		
M26			1.3	1.5		
			Cage Rinse			
M3					3.7	7.8
M14A,D,E,F					-	5.0
M9					15.6	29.1

- Not detected

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Table 8

Concentrations (Mean ± SD) of Radioactivity in the Plasma and Brain of Male Rats Following a Single Oral and IV Administration of [^{14}C]Bicifadine

Time point	Oral (20 mg/kg)		IV (5 mg/kg)	
(h)	Plasma	Brain	Plasma	Brain
0.5	12,400 ± 1900	22,800 ± 2400	3910 ± 250	8510 ± 630
1	16,500 ± 2600	23,400 ± 3500	3770 ± 800	5340 ± 470
2	14,000 ± 2400	20,100 ± 3800	867 ± 58	994 ± 538
4	5950 ± 3950	4620 ± 3350	586 ± 118	323 ± 175
8	661 ± 389	255 ± 225	145 ± 58	44 ± 6

Note Concentrations are ng equiv/g plasma and brain.

TABLE 9

Major Product Ions of Metabolites of [^{14}C]Bicifadine

Metabolite	$[M+H]^+$	Major Fragment Ions (*m/z*)
Bicifadine Standard	174	157 (-NH_3), 142, 133 (base peak, -C_2H_3N), 129, 118, 105 (*m/z* 133-C_2H_4), 91 (tropylium ion), 82
M9 Standard	218	201 (-NH_3), 200 (-H_2O), 182, 171, 164, 146, 135 (base peak), 129, 118, 107, 96, 92, 83, 55
M12 Standard	188	171 (-NH_3), 170 (-H_2O), 143, 134, 105 (base peak), 96, 55
Bicifadine	176	157 (-NH_3), 142, 133 (base peak, -C_2H_3N), 129, 118, 105 (*m/z* 133-C_2H_4), 91 (tropylium ion), 82
M2	190	173 (-NH_3), 160, 155, 149 (base peak, -C_3H_3N), 143 (*m/z* 175-C_2H_6), 128, 121 (*m/z* 149-C_2H_4), 93
M3	204	187 (-NH_3), 169, 163 (base peak, -C_2H_3N), 143 (*m/z* 189-CO_2), 135 (*m/z* 163-C_2H_4), 128, 117
M5	260	242 (-H_2O), 218 (-acetyl), 202 (base peak), 200, 163, 146, 135, 97
M9	218	201 (-NH_3), 200 (-H_2O), 182, 171, 164, 146, 135 (base peak), 129, 118, 107, 96, 92, 83, 55
M10	190	172 (-H_2O), 146, 134 (base peak, -C_3H_4O), 131, 117, 105, 91 (tropylium), 72, 57
M12	190	171 (-NH_3), 170 (-H_2O), 143, 134, 105 (base peak), 96, 55
M14A,B	380	363 (-NH_3), 339 (-C_2H_3N), 204 (-glucuronic acid), 186 (base peak-H_2O), 169, 157, 143
M14C	380	204 (-glucuronic acid), 187 (base peak-NH_3), 186 (*m/z* 204-H_2O), 175, 163, 143
M14D	380	363 (-NH_3), 362 (-H_2O), 339 (-C_2H_3N), 204 (-glucuronic acid), 186 (base peak-H_2O), 169, 157, 146, 143
M14E	380	363 (-NH_3), 362 (-H_2O), 204 (base peak, -glucuronic acid), 186 (*m/z* 204-H_2O), 175, 157, 143, 113, 85
M14F	380	363 (-NH_3), 362 (-H_2O), 339 (-C_2H_3N), 204 (-glucuronic acid), 186 (base peak, *m/z*-H_2O), 175, 169, 141, 113, 85
M15	208	191 (-NH3), 173 (*m/z* 191-H_2O), 145, 131 (base peak), 105
M17	206	189 (-NH3), 171 (*m/z* 189-H_2O), 161 (*m/z* 189-CO), 143 (base peak, *m/z* 189-formic acid), 128, 119, 117, 105, 91, 73, 55
M19	218	201 (-NH_3), 200 (-H_2O), 156, 135 (base peak), 129, 107, 96, 80
M22	220	202 (-H_2O), 161, 157, 135, 131, 119 (base peak), 117, 91, 84, 55
M23	254	295 (ACN adduct), 174 (-sulfate), 157 (base peak, *m/z* 174-NH_3), 145, 142, 131, 110, 105

M24	188	717 (-NH_3), 170 (-H_2O), 156, 105 (base peak), 93, 80
M25	325	200 (base peak, -$NH(CH_2)_2SO_3H$), 171 (*m/z* 200-NH_3), 157 (*m/z* 200-NHCO)
M27	311	294 (-NH_3), 270, 186 (base peak, -$NH(CH_2)_2SO_3H$), 169 (*m/z* 186-NH_3), 157 (*m/z* 186-$NHCH_2$), 143, 119
M28A,B	380	204 (base peak, -glucuronic acid), 186 (*m/z* 204-H_2O), 159, 143, 141, 113, 105, 85
M29A	436	419 (-NH_3), 383, 365, 308, 243 (base peak), 219, 177, 159, 131, 123,107
M29B	436	383, 365, 308, 267, 243 (base peak), 219, 177, 159, 131, 123,107
M30	205	187 (-H_2O), 164, 159, 144, 141, 131 (base peak), 105, 91, 85, 73
M31C	394	218 (-glucuronic acid), 200 (base peak-H_2O), 171, 113, 85
M31D,E,F	394	218 (-glucuronic acid), 200 (base peaK-H_2O), 171

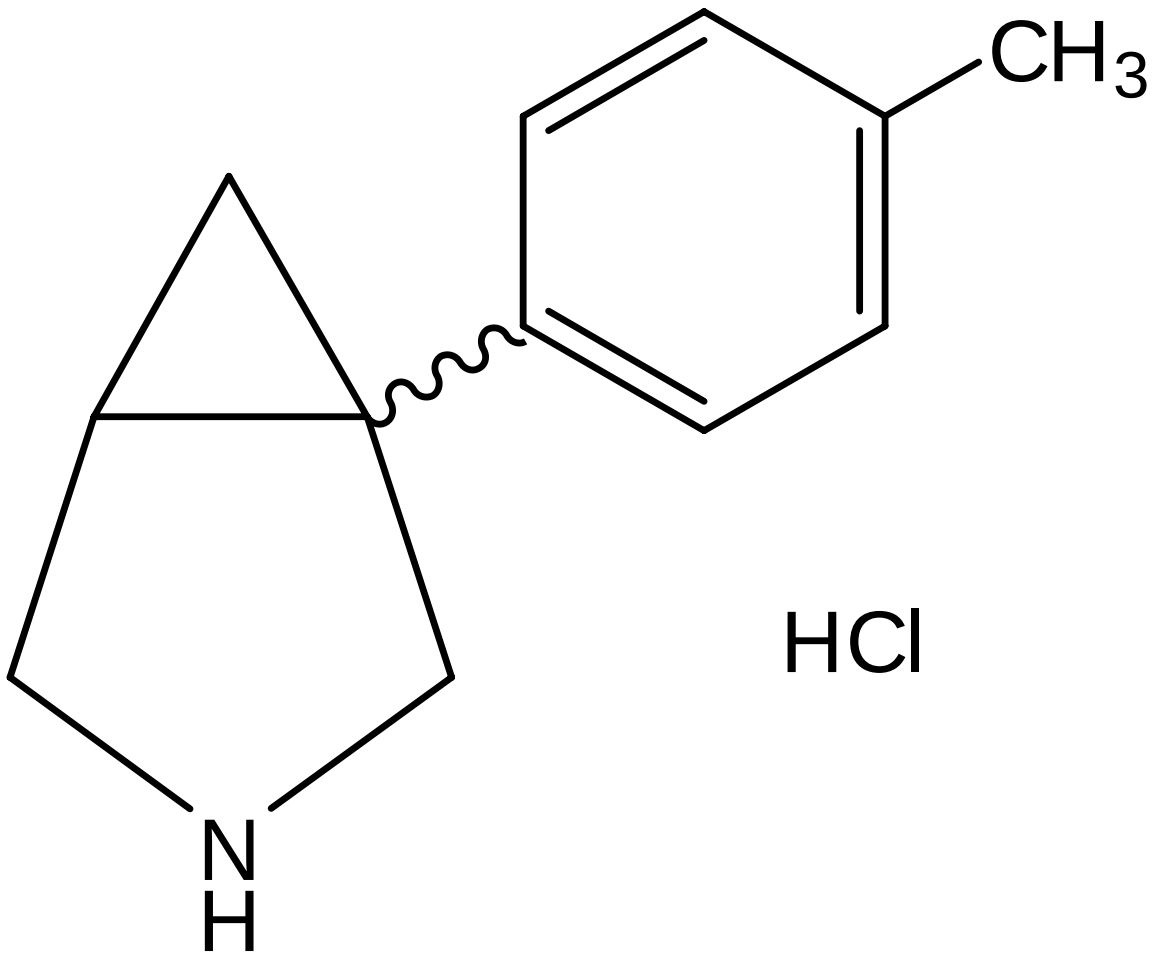
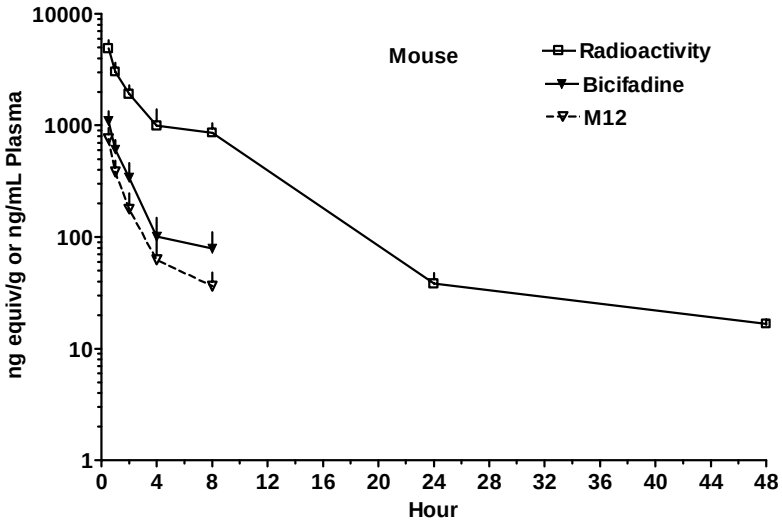
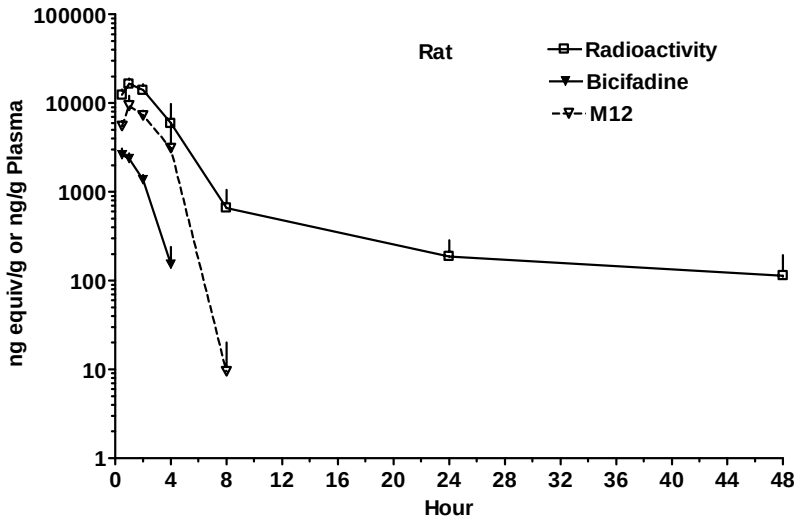


Figure 1





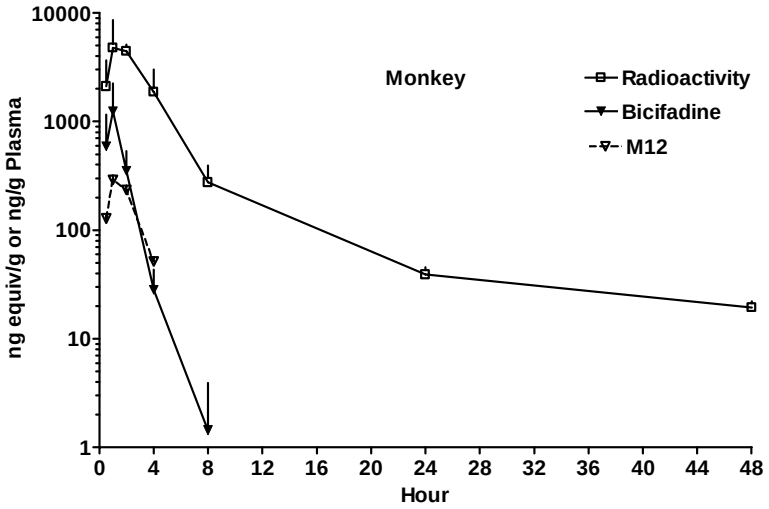
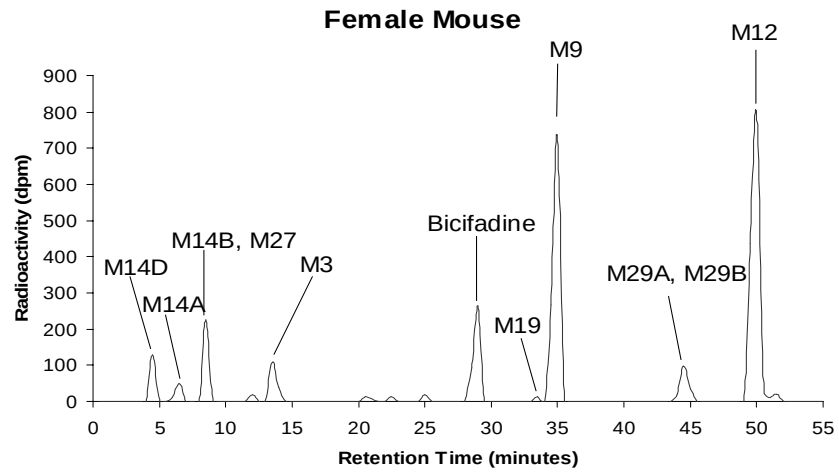
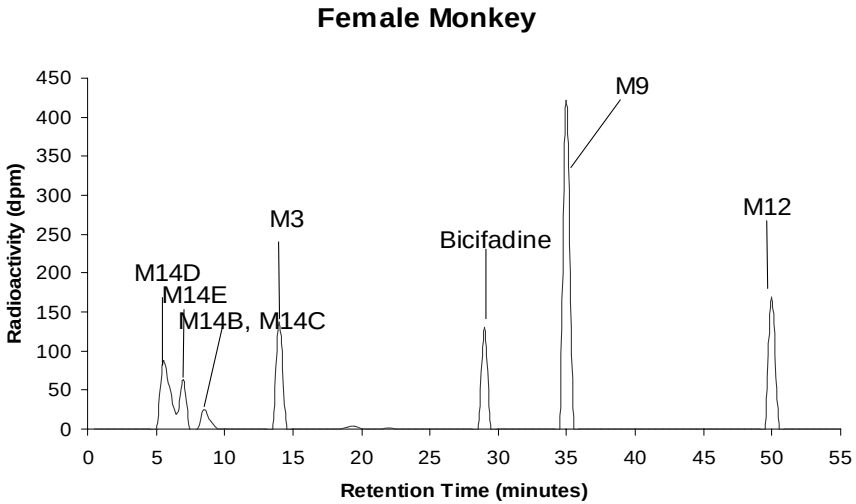


Figure 2





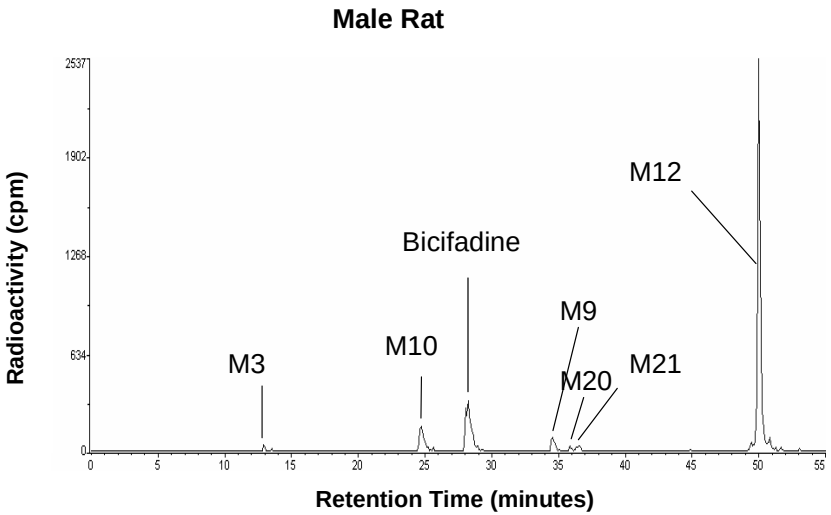


Figure 3

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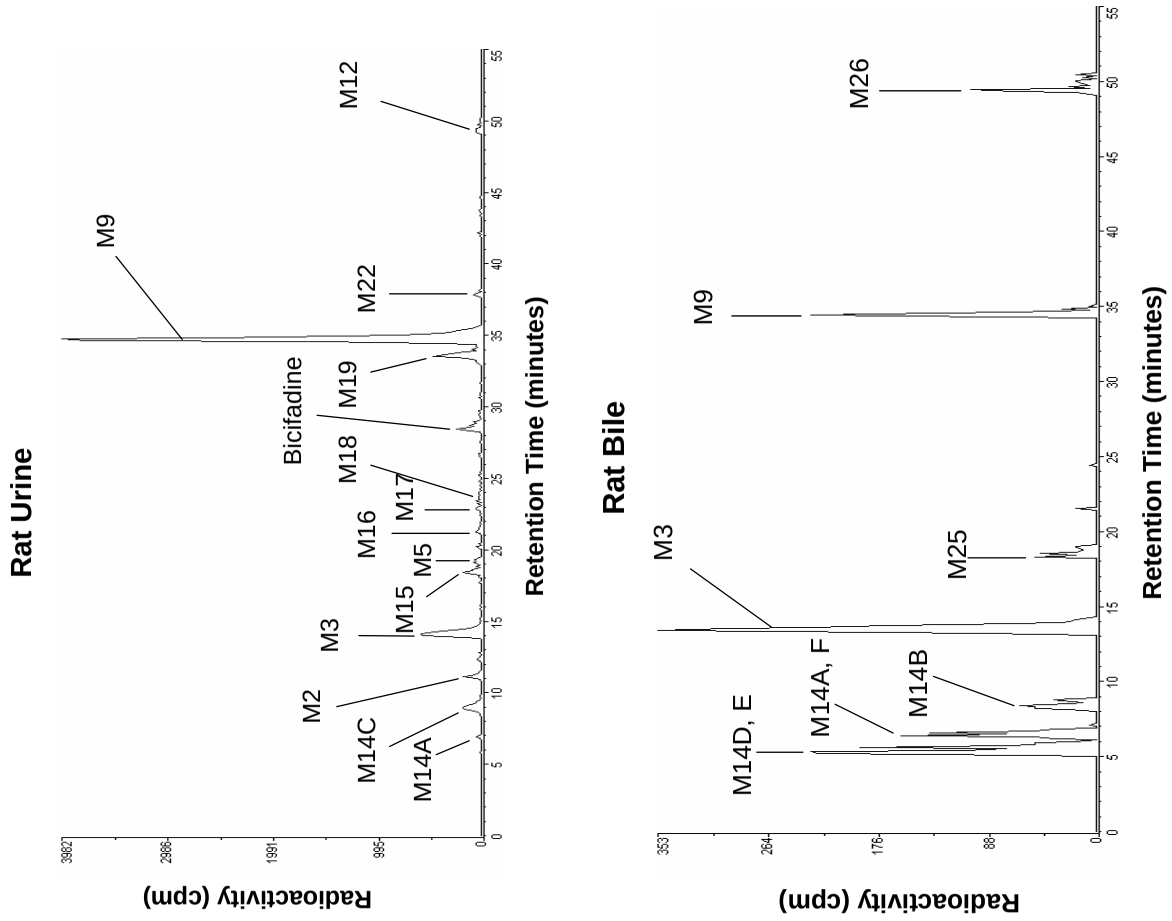
Figure 4



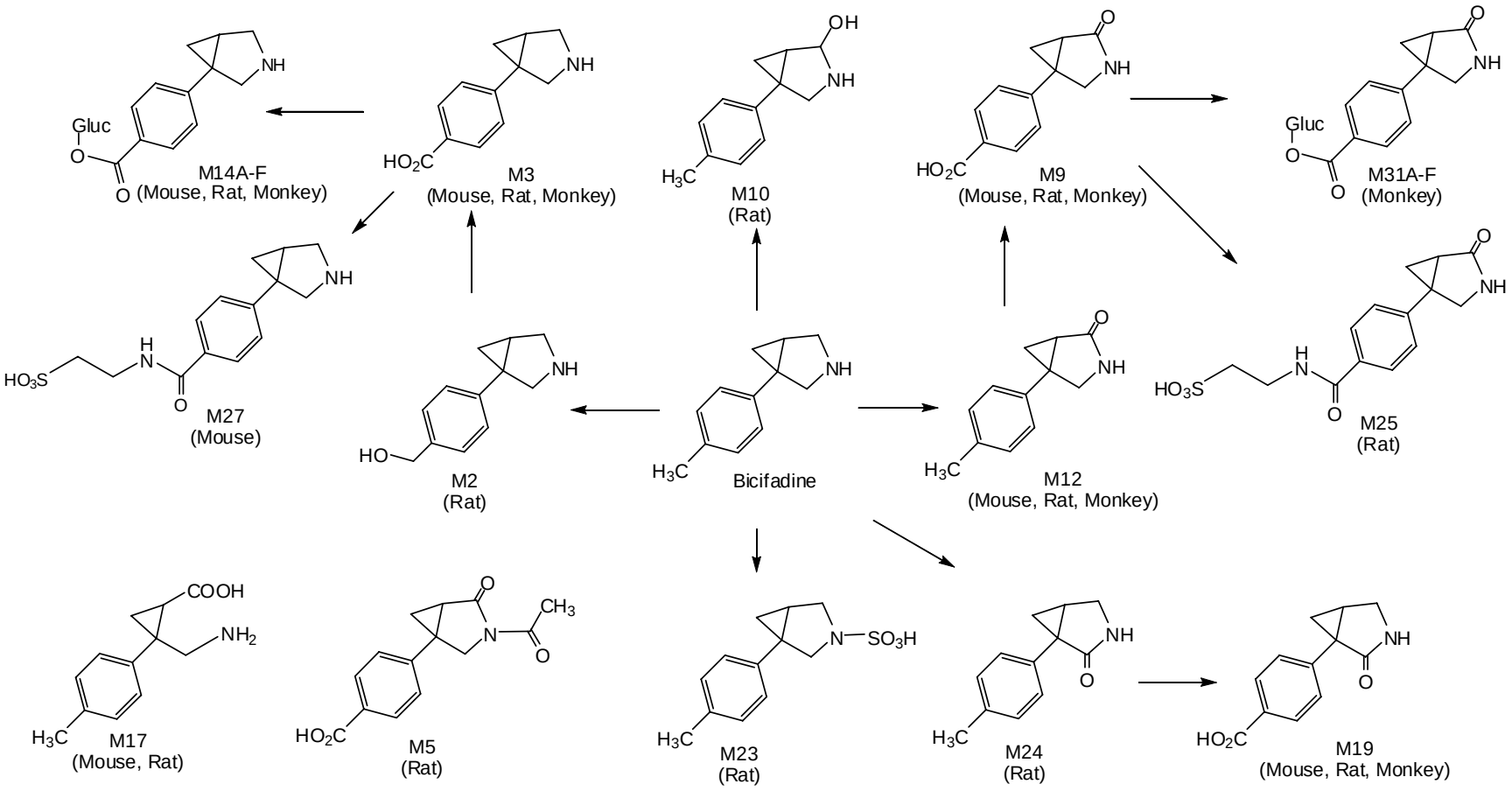
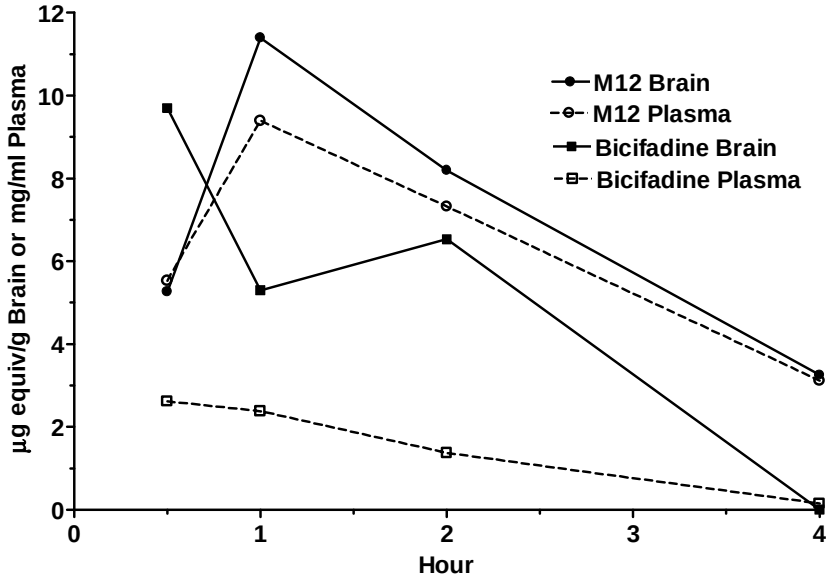


Figure 5



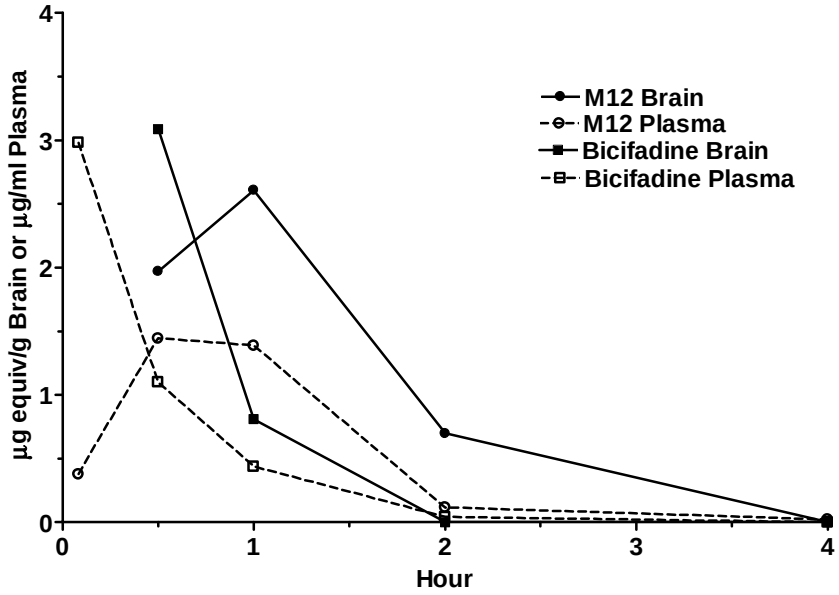


Figure 6

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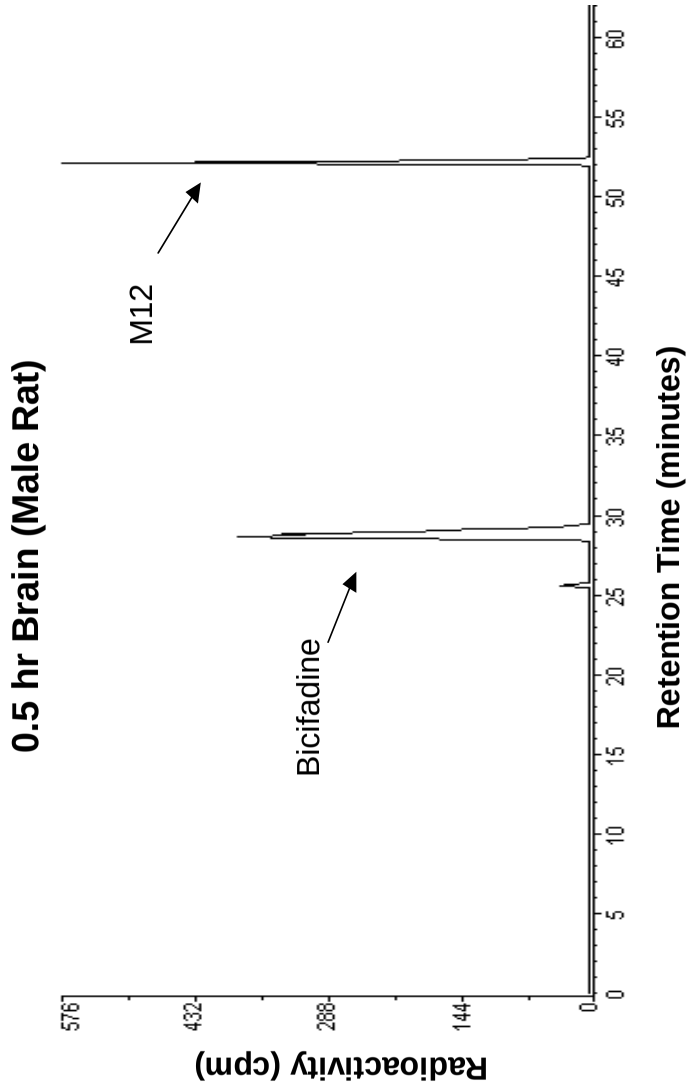

Figure 7