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THE METABOLIC DISPOSITION OF APREPITANT, A SUBSTANCE P RECEPTOR ANTAGONIST, IN RATS AND DOGS

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ABSTRACT:

The absorption, metabolism, and excretion of [^{14}C]aprepitant, a potent and selective human substance P receptor antagonist for the treatment of chemotherapy-induced nausea and vomiting, was evaluated in rats and dogs. Aprepitant was metabolized extensively and no parent drug was detected in the urine of either species. The elimination of drug-related radioactivity, after i.v. or p.o. administration of [^{14}C]aprepitant, was mainly via biliary excretion in rats and by way of both biliary and urinary excretion in dogs. Aprepitant was the major component in the plasma at the early time points (up to 8 h), and plasma metabolite profiles of aprepitant were qualitatively similar in rats and dogs. Several oxidative metabolites of aprepitant, derived from *N*-dealkylation, oxidation, and opening of the morpholine ring, were detected in the plasma. Glucuronidation represented an important pathway in the metabolism and excretion of aprepitant in rats and dogs. An acid-labile glucuronide of [^{14}C]aprepitant accounted for ~18% of the oral dose in rat bile. The instability of this glucuronide, coupled with its presence in bile but absence in feces, suggested the potential for enterohepatic circulation of aprepitant via this conjugate. In dogs, the glucuronide of [^{14}C]aprepitant, together with four glucuronides derived from phase I metabolites, were present as major metabolites in the bile, accounting collectively for ~14% of the radioactive dose over a 4- to 24-h period after i.v. dosing. Two very polar carboxylic acids, namely, 4-fluoro-α-hydroxybenzeneacetic acid and 4-fluoro-α-oxobenzeneacetic acid, were the predominant drug-related entities in rat and dog urine.

Substance P, an endogenous undecapeptide neurotransmitter, binds with high affinity (K_d = 125 pM) to the neurokinin-1 (NK_1)[1] receptor, a member of the tachykinin receptor family of G-protein-coupled receptors (Saria, 1999; Severini et al., 2002). More recently, the activation of NK_1 receptors has been linked to the pathoetiology of emesis (Bountra et al., 1993; Beattie et al., 1995; Hale et al., 1998; Rupniak and Kramer, 1999; Diemunsch and Grelot, 2000; Tattersall et al., 2000), pain (Gonzalez et al., 1998; Urban and Fox, 2000), anxiety (Griebel, 1999; Ballard et al., 2001; Cheeta et al., 2001), and depression (Kramer et al., 1998; Maubach et al., 1999). The discovery of nonpeptide, substance P receptor antagonists has led to the development of therapeutic agents for treatment of chemotherapy-induced emesis. Subsequently, the ability of these antagonists to penetrate the brain has been shown to be essential for their antiemetic efficacy (Watson et al., 1995; Gonsalves et al., 1996; Rudd et al., 1996; Rupniak et al., 1997; Singh et al., 1997; Zaman et al., 2000; Harrison et al., 2001).

Aprepitant (MK-0869; 5-[[(2*R*,3*S*)-2-[(1*R*)-1-[3,5-bis(trifluoromethyl)phenyl]ethoxy]-3-(4-fluorophenyl)-4-morpholinyl]methyl]-1,2-dihydro-3*H*-1,2,4-triazol-3-one; Fig. 1), a potent and selective human NK_1 receptor antagonist with a mean IC_{50} value of 0.12 nM, has been approved recently in the United States for clinical evaluation for the treatment of chemotherapy-induced nausea and vomiting (Navari et al., 1999; Diemunsch and Grelot, 2000; Hale et al., 2000; Campos et al., 2001; Cocquyt et al., 2001). This article describes the absorption, metabolism,

[1] Abbreviations used are: NK_1, neurokinin 1; aprepitant (MK-0869), 5-[[(2*R*,3*S*)-2-[(1*R*)-1-[3,5-bis(trifluoromethyl)phenyl]ethoxy]-3-(4-fluorophenyl)-4-morpholinyl]methyl]-1,2-dihydro-3*H*-1,2,4-triazol-3-one; SD, Sprague-Dawley; M-1, (2*R*,3*S*)-2-[(1*R*)-1-[3,5-bis(trifluoromethyl)phenyl]ethoxy]-3-(4-fluorophenyl)morpholine; M-2, (2*R*)-2-[(1*R*)-1-[3,5-bis(trifluoromethyl)phenyl]ethoxy]-3-(4-fluorophenyl)-5,6-dihydro-2*H*-1,4-oxazine; M-3, (5*S*,6*R*)-6-[(1*R*)-1-[3,5-bis(trifluoromethyl)phenyl]ethoxy]-5-(4-fluorophenyl)-3-morpholinone; M-4, (6*R*)-6-[(1*R*)-1-[3,5-bis(trifluoromethyl)phenyl]ethoxy]-5-(4-fluorophenyl)-5-hydroxy-3-morpholinone; M-5, [(1*R*)-1-[(1*R*)-1-[3,5-bis(trifluoromethyl)phenyl]ethoxy]-2-(4-fluorophenyl)-2-oxoethoxy] acetic acid; M-6, [(1*R*)-1-[(1*R*)-1-[3,5-bis(trifluoromethyl)phenyl]ethoxy]-2-(4-fluorophenyl)-2-hydroxyethoxy]acetic acid; M-8, (2R,3S)-2-[(1*R*)-1-[3,5-bis(trifluoromethyl)phenyl]ethoxy]-3-(4-fluorophenyl)-4-morpholineacetic acid; M-9, α-[(*R*)-[(1*R*)-[3,5-bis(trifluoromethyl) phenyl]ethoxy](2-hydroxyethoxy) methyl]-4-fluorobenzenemethanol; M-10, 4-fluoro-α-hydroxybenzeneacetic acid; M-11, 4-fluoro-α-oxobenzeneacetic acid; M-12, 4-fluorophenylglycine; M-13, (α*S*)-4-fluoro-α-[[(2,5-dihydro-5-oxo-1*H*-1,2,4-triazol-3-yl)methyl] amino]benzeneacetic-carboxy acid; M-14, 5-[[(1*S*)-1-(4-fluorophenyl)-2-hydroxyethyl-2-amino]methyl]-1,2-dihydro-3H-1,2,4-triazol-3-one; HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; TFA, trifluoroacetic acid; MEK, methyl ethyl ketone; NOE, nuclear Overhauser effect.

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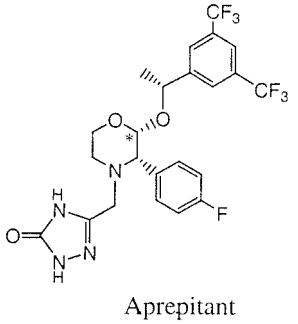

Aprepitant

FIG. 1. *Structure of aprepitant.*

* denotes position of ^{14}C label.

and elimination of [^{14}C]aprepitant in adult male Sprague-Dawley (SD) rats and beagle dogs, after a single i.v. or p.o. dose of [^{14}C]aprepitant.

Previously, several metabolites of aprepitant were identified in primary hepatocyte cultures from rats and humans (S. Huskey, R. I. Sanchez, G. Doss, B. Arison, B. J. Dean, J. Pang, K. Leung, B. Zhu, M. Braun, P. Finke, D. Luffer-Atlas, T. A. Baillie, and S. H. L. Chiu, unpublished data). As shown in Fig. 9, these metabolites included a des-triazolone derivative (M-1), an imine derivative of the morpholine ring (M-2), a lactam derivative (M-3), two epimers of a hydroxylactam derivative (M-4), a morpholine ring-opened keto acid (M-5), and the corresponding hydroxyacid (M-6). In the present investigation, we have extended the in vitro studies to examine the profiles of aprepitant metabolites present in the plasma, urine, bile, and feces of rats and dogs. It was found that, in addition to the above oxidative metabolites, glucuronides derived from aprepitant and its metabolites comprised the major metabolites in bile of rats and dogs. Most notably, two very polar novel metabolites, 4-fluoro-α-hydroxybenzeneacetic acid and 4-fluoro-α-oxobenzeneacetic acid, were isolated and identified from the urine of both rats and dogs.

Materials and Methods

Chemicals. Aprepitant (MK-0869; Fig. 1) was prepared by Merck Process Research (Rahway, NJ.). [Morpholine-2-^{14}C]aprepitant (specific activity 29.83 μCi/mg) was synthesized by the Merck Labeled Compound Synthesis Group (Rahway, NJ). Several metabolites, including M-1, M-3, M-4, M-5, M-6, M-8, M-9, M-11, M-12, M-13, and M-14 (Figs. 1 and 9) were synthesized by Merck Medicinal Chemistry (Rahway, NJ), and metabolite M-2 was synthesized by Merck Medicinal Chemistry (Terlings Park, UK) (PCT Patent publication number: WO 02/34699, published in May, 2002). 4-Fluoro-α-hydroxybenzeneacetic acid (M-10; Fig. 9) was purchased from Lancaster Synthesis Inc. (Windham, NH). β-Glucuronidase from *Escherichia coli* was purchased from Sigma-Aldrich (St. Louis, MO). All chemicals were of the highest analytical purity available.

Synthesis of the Triacetyl Methyl Ester Glucuronide of Aprepitant. To a mixture of aprepitant (200 mg, 0.37 mmol), the thioethyl sugar (205 mg, 0.56 mmol), and type 4A crushed molecular sieves (1 g) in 1,2-dichloroethane (6 ml), at 0°C were added *N*-iodosuccinimide (126 mg, 0.56 mmol) and trifluoromethane sulfonic acid (5.0 μl, 0.056 mmol). The mixture was stirred at room temperature for 18 h and then quenched by the addition of 0.25 M $Na_2S_2O_3$, saturated aqueous $NaHCO_3$, and ethyl acetate (20 ml each, 1:1, by volume). The organic layer was separated, followed by filtration through a 0.2-μm PTFE filter, and the filtrate was concentrated. The resulting residue was purified by column chromatography (40 g of SG 60 silica, 34-mm column diameter, 2.5–5.0% MeOH/CH_2Cl_2), affording a colorless glass. This glass was redissolved in acetonitrile (2 ml), filtered through a 0.2-μm PTFE filter, and purified by reverse phase HPLC (RXC-8 column, 20 × 250 mm, 60% CH_3CN/40% H_2O, 12 ml/min, 500-μl injection). The final product (8.2 mg) was isolated as the protected glucuronide (retention time = 28.6 min) as a colorless glass.

Synthesis of the Glucuronide of Aprepitant (M-7). Triethylamine (50 μl) was added to the triacetyl methyl ester glucuronide of aprepitant (2.0 mg in 450 μl of 50% aqueous MeOH) at room temperature. After stirring for 0.5 h, the reaction mixture was concentrated to yield the free glucuronide as a colorless glass.

Animals. Male SD rats (~230–300 g) were obtained from Charles River Laboratories, Inc. (Wilmington, MA), and male beagle dogs (~10–14 kg) were purchased from Marshall Farms USA Inc. (North Rose, NY). The animals were housed under standard conditions, with a 12-h light/dark cycle, in the animal facilities of Merck Research Laboratories (Rahway, NJ). Animals were allowed access to water ad libitum and to commercial chow or dry dog food. They were fasted overnight before dosing, and for 4 h afterward. Animals were allowed unrestricted access to water during the fasting period. All experiments were carried out under the jurisdiction of the Merck Institutional Animal Care and Use Committee (Rahway, NJ).

Dose Preparation. For the total mass balance studies, the i.v. and p.o. doses of [^{14}C]aprepitant were prepared in ethanol/propylene glycol/water (13:58:29, by volume). For the biliary excretion and metabolism studies, i.v. dose was prepared in a solution of ethanol/propylene glycol/water (13:58:29, by volume), and the oral dose was in a suspension of 0.5% aqueous methylcellulose containing 0.02% sodium laurylsulfate. The i.v. dosing solutions for dogs were passed through sterile 0.45-μm filters (Waters, Milford, MA) before dosing.

Total Mass Balance Studies of [^{14}C]Aprepitant in Rats. Six adult male SD rats were housed separately in Nalgene metabolism cages. Rats (n = 3 per dosing route) received either a single i.v. dose of [^{14}C]aprepitant via the lateral tail vein or a single p.o. dose of [^{14}C]aprepitant by gavage at 2 mg/kg (specific activity 10 μCi/mg; 1.2 ml/kg). Urine and feces samples were collected into tared, separate plastic containers surrounded by dry ice, daily for 5 days. Samples were stored at −70°C until analyzed.

Biliary Excretion of [^{14}C]Aprepitant in Rats. Six adult male SD rats had cannulas implanted into their bile ducts and duodena 1 day before dosing with [^{14}C]aprepitant. The cannulas in each animal were connected to allow recirculation of bile overnight, before dosing. Three of the animals had cannulas implanted into the femoral vein for i.v. dosing. After recovery from surgery, all six rats were placed into separate metabolism cages and allowed unlimited access to an aqueous solution containing 5% dextrose, 0.9% NaCl, and 0.05% KCl.

The bile duct-cannulated rats (n = 3 for each dosing route) received either an i.v. dose of [^{14}C]aprepitant via the femoral vein at 2 mg/kg (specific activity 29.83 μCi/mg; 1.2 ml/kg) or an oral dose of [^{14}C]aprepitant by gavage at 5 mg/kg (specific activity 14.87 μCi/mg; 2 ml/kg). After dosing, the rats were infused with a solution of 10 mM sodium taurocholate, 0.9% NaCl, and 0.05% KCl (2 ml/h), via the duodenal catheter, for 48 h. Bile samples were collected into tared plastic containers surrounded by ice, predose and at 0- to 2-, 2- to 4-, 4- to 8-, 8- to 24-, and 24- to 48-h intervals postdose. Urine and feces samples were collected at room temperature, daily, for 2 days. Samples were stored at −70°C until analyzed.

Metabolism of [^{14}C]Aprepitant in Rats. Each of 18 adult male SD rats received an oral dose of [^{14}C]aprepitant, by gavage, at 100 mg/kg (specific activity 1 μCi/mg; 4 ml/kg). Three rats per time point were euthanized (CO_2) at 2, 4, 6, 8, 18, and 24 h postdose, and blood (~10 ml) from each animal was collected into heparinized tubes. Plasma was prepared by centrifugation of the blood at 3000*g* for 10 min at 4°C.

Total Mass Balance Studies of [^{14}C]Aprepitant in Dogs. Each of four adult male beagle dogs received either an i.v. dose of [^{14}C]aprepitant via a cephalic vein at 1 mg/kg (specific activity 5 μCi/mg; 1 ml/kg) or a p.o. dose of [^{14}C]aprepitant by gavage at 2 mg/kg (specific activity 5 μCi/mg; 2 ml/kg). Blood samples (10–15 ml) were collected into heparinized tubes from all animals via the jugular vein at 2, 4, 6, 8, 24, 30, 48, 54, and 72 h postdose. Plasma was obtained by centrifugation of blood samples. Urine samples were collected in plastic containers surrounded by dry ice at 0- to 8-, 8- to 24-, and 24-h intervals up to 7 days postdose. Feces were collected at room temperature daily for 7 days. Samples were stored at −70°C until analyzed.

Biliary Excretion of [^{14}C]Aprepitant in Dogs. Four male beagle dogs underwent bile duct cannulation surgery 2 weeks before the study. Blood samples were collected presurgery and at 9 days postsurgery for assessment of clinical chemistry and hematology parameters. Each dog received either an i.v. dose of [^{14}C]aprepitant via the cephalic vein at 1 mg/kg (specific activity 10 μCi/mg; 1 ml/kg) or a p.o. dose of [^{14}C]aprepitant by gavage at 2 mg/kg

(specific activity 10 μCi/mg; 1 ml/kg). Bile samples were collected in plastic containers surrounded by dry ice, predose and at 0- to 4-, 4- to 8-, 8- to 12-, 12- to 24-, 24- to 48-, 48- to 72-, 72- to 96-, and 96- to 120-h intervals postdose. The bile samples were stored at −70°C until analyzed.

Preparation of Plasma Samples for LC-MS/MS Analysis. Equal volumes of plasma from rats or dogs (1.1–3.2 ml) were combined for each selected time point and proteins were precipitated with 4 volumes of isopropanol. After centrifugation at 3000*g* for 10 min, the organic layers were transferred to clean tubes and evaporated to dryness under N_2. The residues were re-precipitated twice with 5 ml of isopropanol and resuspended in 200 or 400 μl of water/methanol (1:1, v/v). The suspensions were centrifuged at 3000*g* for 10 min (Eppendorf, model 5417C; Fisher Scientific Co., Pittsburgh, PA), and aliquots (20 μl) of the supernatants were analyzed by LC-MS/MS.

To monitor for the very polar metabolites, equal volumes of plasma (0.55–1 ml) were combined, extracted as described above, and subjected to HPLC analysis (see below, method A). The HPLC effluent fractions collected between 3 and 5 min were combined, evaporated to dryness under a stream of N_2, solubilized with 400 μl of 1% aqueous trifluoroacetic acid (TFA), and analyzed by LC-MS/MS.

Preparation of Fecal Samples for LC-MS/MS Analysis. Weighed fecal samples from rats or dogs were homogenized with ~3 volumes of deionized water. Each homogenate (~4 g) was extracted with 20 ml of methyl ethyl ketone (MEK) followed by a second extraction with 10 ml of MEK. The two organic extracts were combined and evaporated to dryness under N_2. The resulting residue was resuspended in 5 ml of isopropanol, placed in a freezer at −20°C for 2 h, and centrifuged at 3000*g* for 10 min, and the organic layer was transferred to a clean glass tube and evaporated to dryness under N_2. Each sample was resuspended in 120 μl of deionized water and 240 μl of acetonitrile, sonicated, vortex-mixed, and centrifuged at 3000*g* for 10 min. An aliquot (200 μl) of the resulting supernatant was analyzed by LC-MS/MS. The radioactive content of a duplicate aliquot (10 μl) was estimated by liquid scintillation counting to determine the efficiency of extraction.

Metabolite Isolation from Dog Feces. For the isolation of metabolites in dog feces, the aqueous fecal homogenate (~40 g from the 0- to 24-h sample) from dogs was extracted with MEK and isopropanol (see above). The dried extract was resuspended in 40 ml of water and 80 ml of acetonitrile, and extracted with 60 ml of *n*-hexane. After centrifugation at 3000*g* for 10 min, the aqueous acetonitrile layer was evaporated to dryness under N_2 and resuspended in 300 μl of water and 600 μl of acetonitrile before HPLC analysis. The isolation and purification procedures were performed sequentially on three different columns (see below, HPLC analysis), and the resulting column fractions were subjected to LC-MS/MS and ^{1}H NMR analyses.

Preparation of Urine Samples for LC-MS/MS Analysis. Combined urine samples from rats or dogs (30 ml) were concentrated under N_2 to 2.3 ml, acidified by the addition of 2.3 ml of 30% TFA, and centrifuged at 3000*g* for 10 min, and aliquots (50 μl) of the resulting supernatants were analyzed by LC-MS/MS.

Preparation of Bile Samples for LC-MS/MS Analysis. For the analysis of biliary metabolites from rats or dogs, samples (30–60 ml) were applied to a Varian Mega Bond Elut C18 cartridge (10 g) (Varian, Inc., Palo Alto, CA). The cartridges were washed with 30 ml of deionized water, and elution of the analytes of interest was accomplished using 10 ml of 90% aqueous methanol followed by 10 to 20 ml of methanol, and the methanol elution process was repeated four times. The combined methanol eluates were evaporated to dryness under N_2, and the residues were redissolved in deionized water for subsequent LC-MS/MS analyses.

Chemical Hydrolysis of Bile Samples. Bile samples from rats were subjected to chemical hydrolysis to evaluate the stability of biliary metabolites. Three aliquots (125 μl) of bile were incubated at 37°C for 16 h, one with 0.1 N HCl (200 μl) and two with 0.1 N NaOH (200 μl). One of the latter incubations was subjected to HPLC analysis directly, and the other was treated further with 0.1 N HCl (400 μl) at 37°C for an additional 16 h and then analyzed by HPLC (see below, method A).

Enzymatic Hydrolysis of Bile Samples. HPLC separation of biliary metabolites from rats or dogs was performed using a Zorbax RX-C8 column (see below, HPLC analysis method A). The column effluent fractions collected from 9 to 14 min were combined, evaporated to dryness under N_2, and reconstituted with 4 ml of 0.1 M phosphate buffer (pH 7.4). An aliquot (3 ml) was incubated in the presence of 0.5 ml of β-glucuronidase (100 units) at 37°C for 16 to 24 h. The reaction was quenched with an equal volume of cold acetonitrile, and the suspension was centrifuged at 3000*g* for 10 min. The supernatant was transferred to a clean tube, concentrated to approximately 3 ml under N_2, and an aliquot (200 μl) was analyzed by HPLC (see below, method A).

Radioactivity Measurements. For an estimate of total radioactivity, duplicate aliquots of urine (100–200 μl), bile (100–500 μl), and column effluent fractions (10–20 μl) were analyzed in a liquid scintillation counter (Beckman Coulter, Fullerton, CA) following the addition of scintillation cocktail (Ultima-FLO M; PerkinElmer Life Sciences, Boston, MA). Duplicate or triplicate aliquots of plasma (0.2 ml) or fecal homogenate (~0.2 g) were pipetted into paper combustion cones and air-dried overnight before combustion. Sample combustions were performed in an oxidizer (model 307; PerkinElmer Life Sciences), followed by liquid scintillation counting. Quench corrections were made by the external standard method.

HPLC Analysis. A Shimadzu HPLC system (Shimadzu Scientific Instruments Inc., Columbia, MD) consisting of two pumps (LC-10AD), a controller (SCL-10A), an autosampler (SIL-10A), a radiomonitor (IN/US Systems Inc., Tampa, FL), and a fraction collector (model FC 204; Gilson, Inc., Middleton, WI) was used for all analyses.

Metabolite Isolation from Dog Feces. The isolation and purification of an unknown fecal metabolite was carried out using three consecutive chromatographic analyses. Three different columns: 1) Zorbax RX-C8 (5 μm; 4.6 × 250 mm; MacMod Analytical, Chadds Ford, PA), 2) Hypersil BDS-C18 (5 μm; 4.6 × 250 mm; MacMod Analytical), and 3) Zorbax SB-phenyl (5 μm; 4.6 × 250 mm; MacMod Analytical) were used for separation. The same mobile phase, consisting of solvent A (10 mM ammonium acetate in water) and solvent B (7.2 mM ammonium acetate in 7.2% methanol/92.8% acetonitrile), was used in all three separations. The flow rate was 1 ml/min. The linear gradients were from 35 to 80% B in 40 min for the Zorbax RX-C8 column (method A), 50 to 60% B in 30 min for the Hypersil BDS-C18 column, and 40 to 60% B in 30 min for the Zorbax SB-phenyl column. The retention times for the fractions containing the radioactive component of interest using HPLC conditions 1, 2, and 3 were ~17, ~7.9, and ~13.1 min, respectively. The fractions containing the radioactive component were evaporated to dryness under N_2 and subjected to LC-MS/MS and ^{1}H NMR analyses.

HPLC Analysis of Urine Samples. The HPLC separation of urinary extracts from rats or dogs was performed using a Zorbax SB-Phenyl column (5 μm; 4.6 × 250 mm). The mobile phase consisted of solvent C (0.1% aqueous TFA) and solvent D (acetonitrile containing 0.1% TFA). Samples were injected onto the column via an autosampler. The flow rate was 1 ml/min, and elution was accomplished using a linear gradient from 5 to 80% D in 35 min, followed by 80 to 95% D in 7 min, and maintained at 95% D for another 2 min (method B). The column was connected to an on-line radiomonitor to obtain radiochromatograms.

LC-MS/MS Analysis. Analyses were performed using a Sciex API 300 (Applied Biosystems, Foster City, CA) upgraded to API 365 specifications or an API 3000 triple quadrupole tandem mass spectrometer utilizing the ionspray interface. The HPLC system interfaced with the mass spectrometer consisted of two PerkinElmer Series 200 micro LC pumps and a PerkinElmer Series 200 autosampler. Both positive and negative ion detection methods were employed, and the mass spectrometry conditions were optimized using metabolite M-1 in the positive ion mode. The MS/MS experiments were performed by collision-induced dissociation with N_2 as the target gas, and full scan data were acquired. Multiple components were monitored in one LC-MS/MS experiment by separating the total acquisition time (41 min) in segments, during which MS/MS data were acquired for specific ions.

LC-MS/MS Analysis of Plasma, Bile, and Fecal Extracts. For plasma, bile, and fecal extracts from rats or dogs, chromatography was performed on a Zorbax RX-C8 (5 μm; 4.6 × 250 mm) column. A linear gradient from 35 to 80% B in 40 min was used, followed by a step gradient from 80 to 95% B in 0.1 min, and maintained at 95% B for 5 min (method A). The mobile phase consisted of solvent A (10 mM ammonium acetate in water) and solvent B (7.2 mM ammonium acetate in 7.2% methanol/92.8% acetonitrile). Samples (5–200 μl) were injected onto the column via an autosampler. The flow rate was 1 ml/min, of which 0.04 ml/min was diverted into the mass spectrometer, while the remainder was collected in a fraction collector. An aliquot of the latter was subjected to liquid scintillation counting to determine radioactivity. Alterna-

TABLE 1

Excretion of radioactivity in rats after intravenous or oral administration of [^{14}C]aprepitant

Each rat (n = 3) received an i.v. or p.o. dose of [^{14}C]aprepitant (specific activity 10 μCi/mg) at 2 mg/kg. Urine and feces were collected daily for 5 days.

Collection Interval	Percentage of Radioactive Dose[a]		
	Urine	Feces	Total
h		*i.v. at 2 mg/kg*	
0–24	26.1 ± 0.9	35.9 ± 0.7	62.0 ± 1.1
24–48	5.4 ± 0.1	12.8 ± 1.0	18.2 ± 1.1
48–72	1.3 ± 0.2	3.3 ± 0.2	4.6 ± 0.4
72–96	0.6 ± 0.1	1.2 ± 0.3	1.8 ± 0.4
96–120	0.3 ± 0.1	0.56 ± 0.1	0.9 ± 0.1
Subtotal (%)	33.7 ± 0.5	53.8 ± 1.4	88.2 ± 0.7[b]
		p.o. at 2 mg/kg	
0–24	22.7 ± 2.4	39.7 ± 0.6	62.4 ± 2.7
24–48	4.6 ± 0.5	13.2 ± 2.2	17.8 ± 2.5
48–72	1.4 ± 0.3	3.4 ± 0.6	4.8 ± 0.5
72–96	0.5 ± 0.1	1.0 ± 0.1	1.5 ± 0.1
96–120	0.3 ± 0.1	0.5 ± 0.1	0.8 ± 0.1
Subtotal (%)	29.5 ± 1.6	57.8 ± 1.0	88.0 ± 0.9[b]

[a] Each fecal sample was homogenized with ~3 volumes of water. Duplicate aliquots of urine samples were analyzed directly by liquid scintillation counting. Duplicate aliquots of fecal homogenate were air-dried, combusted, and analyzed by liquid scintillation counting to determine the total radioactivity.

[b] Total recovery included the dose recovered in urine, feces, and cage washes. The radioactivity in carcass accounted for ~3% and ~2% of the radioactive dose in rats dosed i.v. and p.o., respectively.

tively, the column was connected to an on-line radiomonitor to obtain radiochromatograms.

LC-MS/MS Analysis of Urine Extracts and Very Polar Metabolites from Plasma Samples. When the positive ion mode was utilized for LC-MS/MS analysis, a Zorbax RX-C8 (5 μm; 4.6 × 250 mm) or a Zorbax SB-C8 (5 μm; 4.6 × 150 mm) column was utilized. The mobile phase consisted of solvent C (0.1% TFA in water) and solvent D (0.1% TFA in acetonitrile). Samples were injected onto the column via an autosampler and analytes were eluted using a linear gradient from 5 to 25% D in 15 min, followed by a step gradient from 25 to 90% D in 0.5 min, and maintained at 90% D for another 2 min. The flow rate was 1 ml/min. A switch valve was set up to divert the highly aqueous solvent to waste, and solvent B was introduced postcolumn and before the mass spectrometer at a flow rate of 0.6 ml/min during this 5-min period. Only 0.04 ml/min was directed into the mass spectrometer; the remainder was collected for liquid scintillation counting.

When the negative ion mode was employed for the LC-MS/MS analysis, a Zorbax RX-C8 (5 μm; 4.6 × 250 mm) or Zorbax SB-C8 (5 μm; 4.6 × 75 mm) column was used. The mobile phase consisted of solvent E (0.1% aqueous formic acid) and solvent F (0.1% aqueous formic acid in acetonitrile). Samples were injected onto the column via an autosampler, and the analytes were eluted using a linear gradient from 5 to 35% F in 5 min, followed by a step gradient from 35 to 90% F in 0.2 min, and maintained at 90% F for another 2 min. The flow rate was 1 ml/min. A switch valve was used to direct the highly aqueous eluate to waste for the first 2 min, during which time solvent B was introduced postcolumn. Only 0.04 ml/min was directed to the mass spectrometer; the remainder was collected for liquid scintillation counting.

NMR Analysis. Purified metabolites were analyzed by proton NMR on a 500 MHz Varian Inovd NMR instrument (Varian, Inc.) using deuterated methanol or acetonitrile as the solvent and a Nalorac 3-mm inverse detection probe. Chemical shifts are reported as ppm downfield from tetramethylsilane, and multiplicities are indicated as singlet (s), doublet (d), or multiplet (m).

TABLE 2

Excretion of radioactivity in dogs after intravenous or oral administration of [^{14}C]aprepitant

Each dog (n = 2) received an i.v. or p.o. dose of [^{14}C]aprepitant (specific activity 5 μCi/mg) at 1 or 2 mg/kg, respectively. Urine and feces were collected daily for 7 days.

Collection Interval	Percentage of Radioactive Dose[a]		
	Urine	Feces	Total
h		*i.v. at 1 mg/kg*	
0–24	13.1,11.2	12.6, NS	25.7,11.2
24–48	8.35,18.0	7.31,27.1	15.7,45.1
48–72	7.94,6.14	12.4,6.15	20.3,12.3
72–96	2.65,2.47	3.17,2.03	5.8,4.5
96–120	1.38,1.53	2.07,1.95	3. 5,3.5
120–144	0.72,0.87	0.98,1.15	1.7,2.0
144–168	0.50,0.60	0.61,0.61	1.1,1.2
Subtotal (%)	34.7,40.8	39.2,39.0	83.7,[b]84.4[b]
		p.o. at 2 mg/kg	
0–24	17.5,13.3	18.4,20.4	35.9,33.7
24–48	12.9,10.5	11.6,9.7	24.5,20.2
48–72	7.4,9.5	7.1,9.2	14.4,18.7
72–96	2.4,2.8	2.2,2.1	4.6,4. 9
96–120	1.4,1.3	1.1,1.8	2.5,3.1
120–144	0.6,0. 9	0.7,1.3	1.3,2.2
144–168	0.4,0.3	0.4,0.2	0.7,0.5
Subtotal (%)	42.5,38.6	41.4,44.7	87.3,[b]86.5[b]

NS, no sample.

[a] Each fecal sample was homogenized with ~3 volumes of water. Duplicate aliquots of urine samples were analyzed directly by liquid scintillation counting. Duplicate aliquots of fecal homogenate were air-dried, combusted, and analyzed by liquid scintillation counting to determine the total radioactivity.

[b] Total recovery included the dose recovered in urine, feces and cage washes.

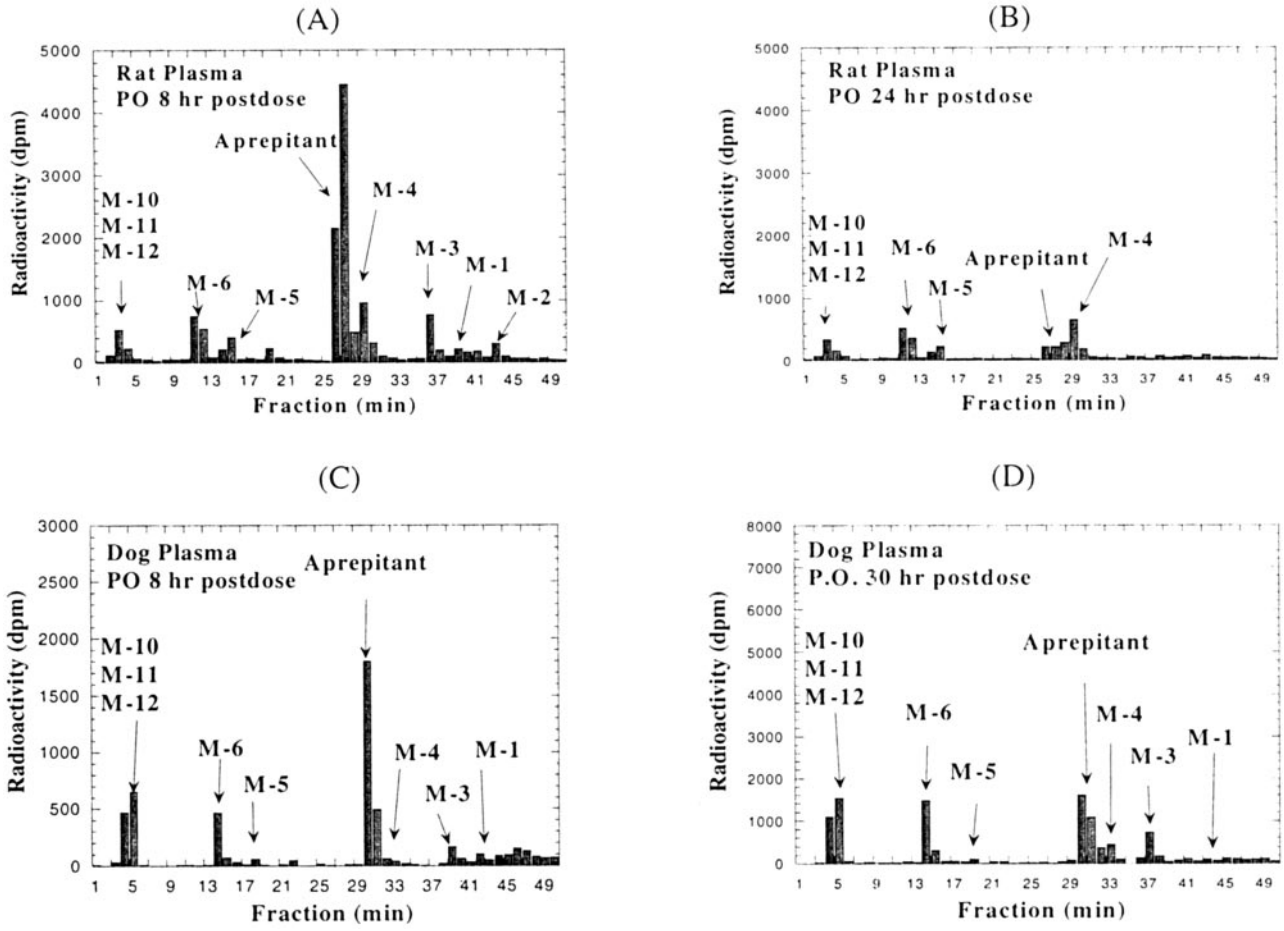

FIG. 2. *Radiochromatograms derived from the analysis of combined plasma specimens from rats (A, B) or dogs (C, D) following oral administration of [^{14}C]aprepitant.*

Blood samples were collected at selected time intervals from rats and dogs dosed at 100 mg/kg and 2 mg/kg, respectively. After centrifugation, plasma samples were extracted and analyzed by LC-MS/MS. The HPLC separation was performed using a Zorbax RX-C8 column, as described under *Materials and Methods*, and radioactivity profiles of plasma samples at 8 h postdose are presented.

TABLE 3

Parent ions and major fragment ions of aprepitant and authentic reference metabolites following LC-MS/MS analyses under positive and negative modes of detection

Compound	$[MH^+]$ *m/z*	$[MH^-]$ *m/z*	Major Fragment Ions[a]
Aprepitant	535		**179**, 277
Aprepitant		533	213, **275**
M-1	438		109, **180**
M-2	436		100,178,**196,** 241
M-3	452		109,137,194,212,**223**,241,452
M-3		450	136,152,165,180,192,208,**241**,255,450
M-4	450(=468−18)		86,109,114,124,136,152,164,210,221, **241**, 450
M-4		466	74,139,196,241**, 391**
M-5	486(=469+17)		**152,**240, 486
M-5		467	75,**167,**241,325,345,391,409
M-6	488(=471+17)		**137,** 155
M-6		469	95,137,139,153,**165**,181,211,285
M-7	711		179,192,**277**, 535
M-8	496		179,210, **238**
M-8		494	177,**192**,213, 236
M-9[b]		455	95,**153**,167,197,213,241,375
M-9[b]	474(=457+17)		80,137,155,**181**,198,412,474
M-10		169	95,123,**125**,141, 169
M-11		167	**95**
M-12	170		97,**125**,153, 170
M-13	267		99,115,125,**153**,170,221,267
M-14	253		91, 98,**115**,119,139,253

[a] The most intense ions are presented in bold for aprepitant and authentic reference metabolites.
[b] The glucuronide of M-9 was detected in bile, but the aglycone was not detected in any biological matrix.

TABLE 4

Occurrence of aprepitant and its metabolites in plasma and excreta after intravenous or oral administration of [^{14}C]aprepitant to rats or dogs

Compound	Rat				Dog			
	Plasma	Feces	Bile	Urine	Plasma	Feces	Bile	Urine
Aprepitant	v	√	√	N.D.	√	√	√	N.D.
M-1	v	√	√	N.D.	√	√	√	N.D.
M-2	v	√	√	N.D.	N.D.	√	√	N.D.
M-3	v	√	√	N.D.	√	√	√	N.D.
M-4	v	√	√	N.D.	√	√	√	N.D.
M-5	v	√	√	N.D.	√	√	√	N.D.
M-6	v	√	√	N.D.	√	√	√	N.D.
M-8	N.S.	√	√	N.D.	√	√	√	N.D.
M-9	N.S.	N.S.	N.D.	N.D.	N.S.	N.S.	N.D.	N.D.
M-10	v	N.S.	√	√	√	N.D.	N.D.	√
M-11	v	N.S.	√	√	N.D.	N.D.	N.D.	√
M-12	v	N.S.	N.D.	N.D.	√	N.D.	N.D.	N.D.
M-13	N.D.	N.S.	N.D.	√	N.D.	N.D.	N.D.	√
M-14	N.D.	N.S.	N.D.	√	N.D.	N.D.	N.D.	√
M-7 (Glu of aprepitant)	N.D.	N.D.	√	N.D.	N.D.	N.D.	√	N.D.
Glu of M-1	N.D.	N.D.	√	N.D.	N.D.	N.D.	√	N.D.
Glu of M-4	N.S.	N.S.	N.S.	N.D.	N.D.	N.D.	√	N.D.
Glu of M-8	N.S.	N.D.	√	N.D.	N.S.	N.S.	√	N.D.
Glu of M-9	N.S.	N.S.	N.D.	N.D.	N.S.	N.S.	√	N.D.

√, detected by either LC-MS/MS or HPLC/radioactivity; N.D., not detected by either LC-MS/MS or HPLC/radioactivity; N.S., not studied because information was not available at the time samples were analyzed.

Results

Total Mass Balance and Biliary Excretion of [^{14}C]Aprepitant in Rats. After i.v. (2 mg/kg) and p.o. (2 mg/kg) administration of [^{14}C]aprepitant to male SD rats, the average recovery of radioactivity in excreta over a 120-h period was 88.2 ± 0.7% and 88.0 ± 0.9%, respectively, with the majority of the radioactivity (>60%) excreted during the first 24 h (Table 1). The excretion pattern of [^{14}C]aprepitant-derived radioactivity was similar for both routes of administration, with recoveries of ~30% in urine and ~55% in feces. After a single i.v. (2 mg/kg) or p.o. (5 mg/kg) dose of [^{14}C]aprepitant to bile duct-cannulated rats, recovery of the radioactive dose in bile over 48 h was 42.3 ± 2.7% and 30.5 ± 10.1%, respectively (data not shown). Absorption of the radioactive oral dose was ~43%, based on combined recovery of radioactivity in the bile (~31%) and urine (~12%) following a single p.o. (5 mg/kg) dose (data not shown).

Total Mass Balance and Biliary Excretion of [^{14}C]Aprepitant in Dogs. After i.v. (1 mg/kg) and p.o. (2 mg/kg) administration of [^{14}C]aprepitant to male beagle dogs, the average recovery of radioactivity in excreta over a 168-h period was ~84% and ~87%, respectively, with the majority of the radioactive dose (~50–65%) recovered in 48 to 72 h, respectively (Table 2). The excretion of radioactivity was divided almost equally between the urine (~35–43%) and the feces (~39–45%). In bile duct-cannulated dogs given [^{14}C]aprepitant, recovery of the radioactivity in bile over a 5-day period was ~49% and ~30% following i.v. (1 mg/kg) and p.o. (2 mg/kg) dosing, respectively (data not shown).

Metabolites of [^{14}C]Aprepitant in Rat and Dog Plasma. The metabolism of aprepitant was evaluated after a single oral dose of [^{14}C]aprepitant to rats (100 mg/kg) and dogs (2 mg/kg). Representative radioactivity profiles derived from plasma samples from rats and dogs at 8, 24, or 30 h postdose are shown in Fig. 2. Plasma metabolite profiles of aprepitant were qualitatively similar in rats and dogs at selected time points. [^{14}C]Aprepitant was the major radioactive component in the plasma for up to 8 h in rats and dogs. The presence in plasma of all metabolites (M-1 to M-6) previously identified in hepatocytes (Huskey et al., 2003) was confirmed by LC-MS/MS analysis, except that M-2 was not detected in dog plasma (Fig. 2; Tables 3 and 4). In addition, the presence of very polar metabolites, designated M-10, M-11, and M-12, were confirmed by LC-MS/MS

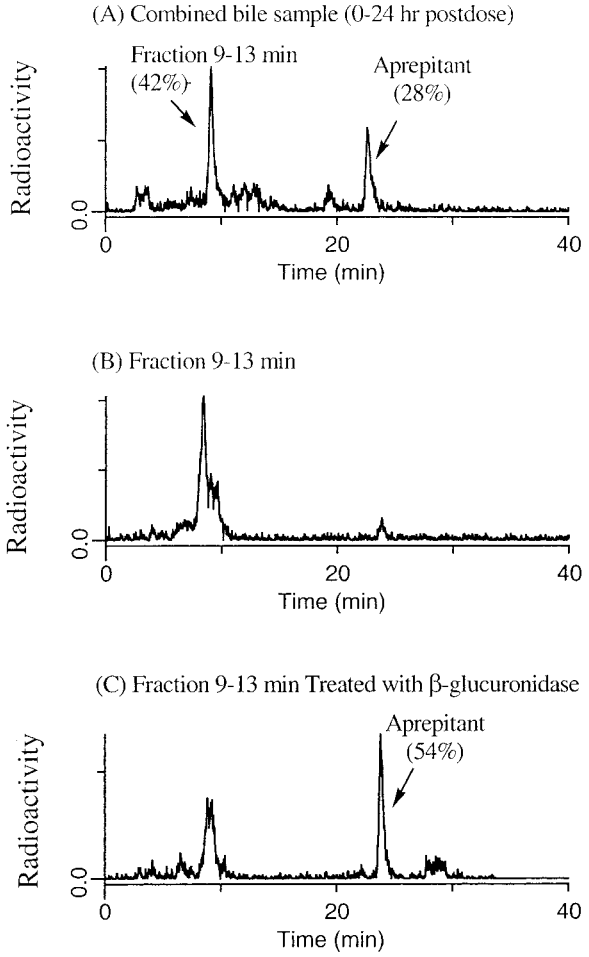

FIG. 3. *Radiochromatograms depicting the analysis of combined bile extracts (with or without β-glucuronidase treatment) from rats following oral administration of [^{14}C]aprepitant at 5 mg/kg.*

At selected time intervals, bile samples from three rats were combined, extracted, and subjected to HPLC analysis (panel A). The HPLC separation was performed using a Zorbax RX-C8 column, as described under *Materials and Methods.* After separation by HPLC, the fractions collected from 9 to 13 min were combined (panel B), and an aliquot was incubated with β-glucuronidase at 37°C for 16 to 24 h before HPLC analysis (panel C).

TABLE 5

Proton NMR data for synthetic triacetyl methyl ester of the glucuronide of aprepitant

Chemical Shift	Assignment	Integral	Multiplicity	Coupling Constants
ppm				*Hz*
7.75	d	1H	s	
7.31	c	2H	s	
7.44	b	2H	m	
7.04	a	2H	m	
5.85	1′	1H	d	J=7.5
5.45	3′	1H	t	J=9.5
5.19	2′	1H	t	
5.18	4′	1H	t	
4.89	e	1H	q	J=6.5
4.35	2	1H	d	J=3.0
4.33	5′	1H	d	J=9.8
4.24	6ax	1H	m	
3.65	OMe	3H	s	
3.58	3	1H	d	J=3.0
3.18	g′	1H	d	J=15.1
3.60	g	1H	d	J=15.1
2.92	5eq	1H	m	
2.57	5ax	1H	m	
1.44	f	3H	d	J=6.6
1.99	OAc	9H	3s	

(Tables 3 and 4). Previously, M-10 and M-11 were identified from rat and dog urine (Dean et al., 2000), and M-12 was determined to be an amino derivative of M-10, based on the LC-MS/MS analysis and comparison with authentic compound. Compounds M-5 and M-6 were the major metabolites in rat plasma at 24 h postdose, whereas M-6 and M-10 predominated in dog plasma at 30 h postdose.

Metabolites of [^{14}C]Aprepitant in Rat Bile. A representative radiochromatogram derived from HPLC analysis of a rat bile sample (0–24 h after an oral dose) revealed two major radioactive components (9–13 and 23–25 min, HPLC method A; Fig. 3, panel A), which accounted for ~42% and ~28% of the total radioactivity in the extract, respectively. The latter component was confirmed by LC-MS/MS to be the parent compound, which accounted for ~5% of the radioactive dose (data not shown).

The effluents from the 9- to 13-min fraction were combined (Fig. 3, panel B) and treated with β-glucuronidase (Fig. 3, panel C). The radiochromatogram of the hydrolysate indicated that the major component (~54%) was [^{14}C]aprepitant, suggesting that the major metabolite in rat bile was a glucuronide of aprepitant, designated as M-7, which accounted for ~18% of the radioactive dose (data not shown). LC-MS/MS analysis also supported the assignment of M-7 as a glucuronide of aprepitant (Table 3). Similar findings were obtained in rats following i.v. administration of [^{14}C]aprepitant when the un-

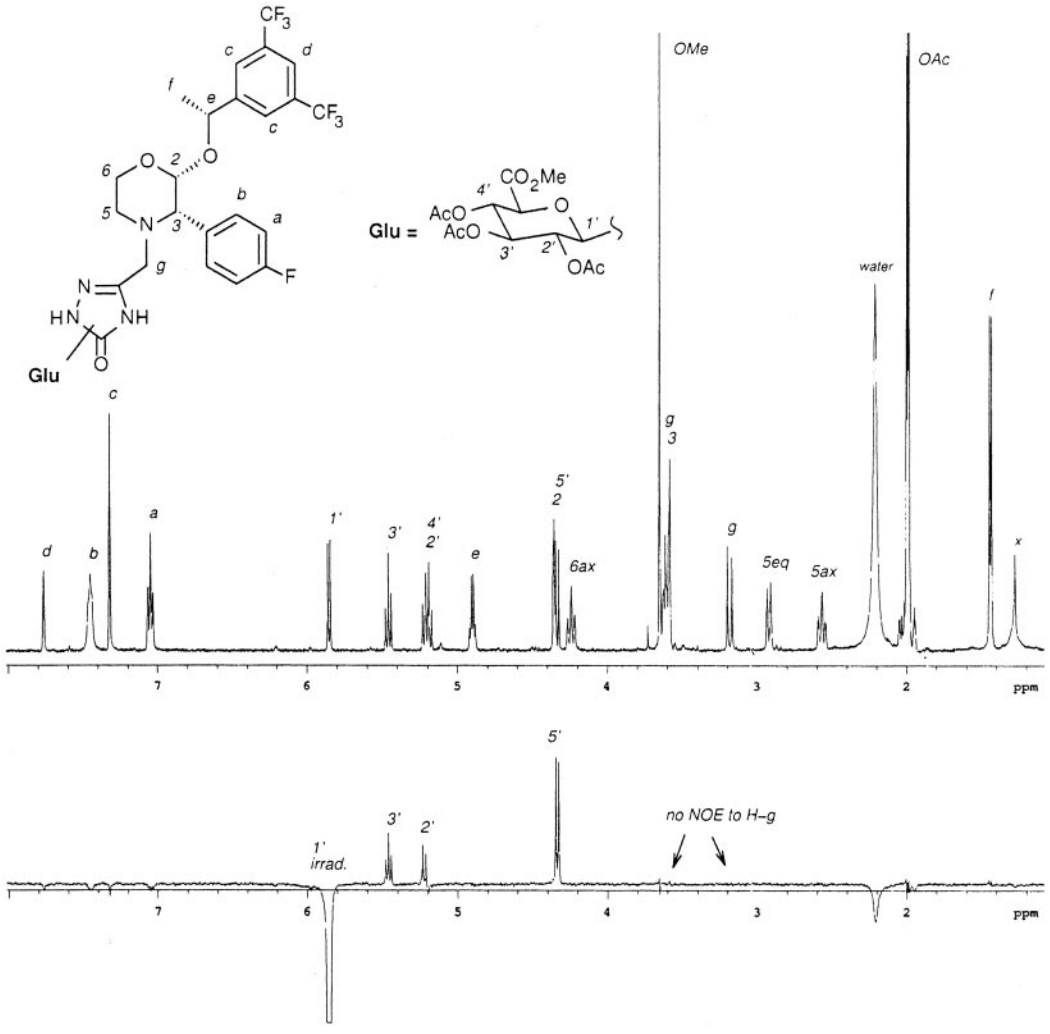

FIG. 4. *Proton NMR spectrum of a synthetic triacetyl methyl ester of the glucuronide of aprepitant.*

Upper panel, proton NMR spectrum of a synthetic protected glucuronide; lower panel, NOE difference spectrum with irradiation of the anomeric proton.

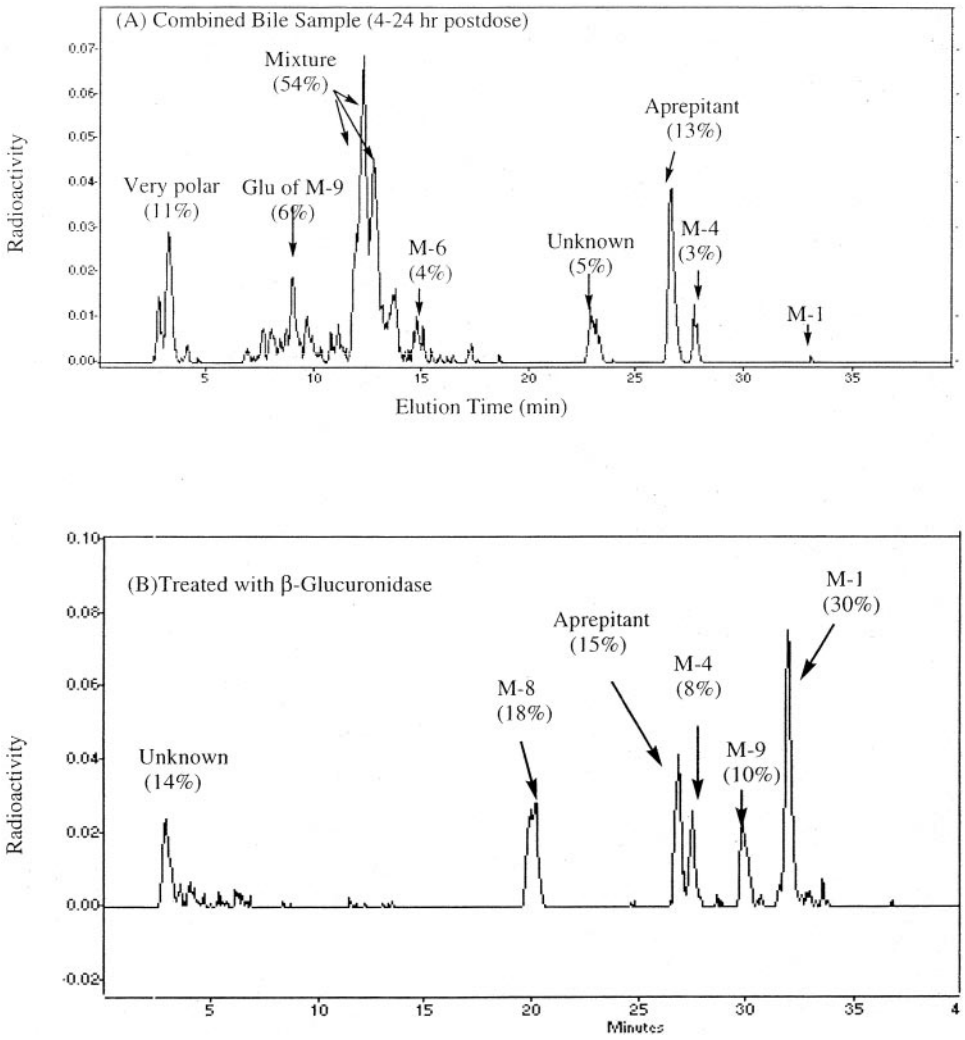

FIG. 5. *Radiochromatograms depicting the analysis of combined bile extracts (with or without β-glucuronidase treatment) from dogs following intravenous administration of [^{14}C]aprepitant at 1 mg/kg.*

At selected time intervals, bile samples from two dogs were combined, extracted, and subjected to HPLC analysis (panel A). The HPLC separation was performed using a Zorbax RX-C8 column, as described under *Materials and Methods*. After separation by HPLC, the fractions collected from 9 to 14 min were combined, and an aliquot was incubated with β-glucuronidase at 37°C for 16 to 24 h before HPLC analysis (panel B).

changed parent drug and glucuronide M-7 accounted for ~7% and ~17% of the radioactive dose (data not shown), respectively.

Stability of an Aprepitant Glucuronide Metabolite, M-7. Based on HPLC analysis, the glucuronide of aprepitant was acid-labile since it was found to revert completely to the parent compound upon mild acid treatment (0.1 N HCl at 37°C for 16 h). However, this conjugate was stable under mildly basic conditions (0.1 N NaOH at 37°C for 16 h) but was hydrolyzed completely to the parent drug upon further treatment with 0.1 N HCl (data not shown). Based on the structure of aprepitant, four regioisomeric structures of glucuronide are possible.

^{1}H NMR Analysis of an Authentic Glucuronide of Aprepitant. An authentic glucuronide of aprepitant was synthesized chemically in an attempt to distinguish between the four possible regioisomeric structures. NMR studies were carried out on a synthetic protected (as triacetyl methyl ester) glucuronide intermediate of aprepitant due to the instability of glucuronide of aprepitant under experimental conditions (Table 5). The possibility that conjugation occurred at the nitrogen of the morpholine ring was considered unlikely since there were no significant downfield shifts in the adjacent protons (Fig. 4, upper panel). In a nuclear Overhauser effect (NOE) experiment, the anomeric proton of the glucuronide was irradiated and a strong NOE was observed within the glucuronic acid moiety, but none was detectable at the exocyclic methylene protons (Fig. 4, lower panel). The results likely exclude structures in which glucuronic acid is attached to either of the nitrogen atoms adjacent to the junction carbon of the triazolone ring. Attempts to distinguish between the two remaining structures were unsuccessful. Collectively, data obtained to date (NMR and LC-MS/MS) do not differentiate between an *O*- and an *N*-glucuronide on the triazolone ring.

After removing the protecting groups with triethylamine, the synthetic glucuronide of aprepitant was subjected to Fourier-transform infrared spectroscopic analysis. The spectrum showed that the isolate was an adduct of the triethylamine salt of a carboxylate glucuronide as exhibited by the carbonyl stretch at 1606 cm^{-1}. This is indicative of an amine salt of a carboxylate. The lack of an additional carbonyl stretch at ~1700 cm^{-1}, as was present in the unsubstituted parent, and the characteristic of an imide carbonyl, suggests that the glucuronide adduct exists as the *O*-substituted regioisomer (data not shown). However, the possibility of an *N*-substituted regioisomer with the resultant carbonyl in the tautomeric enolic form still exists.

Comparison of the Major Biliary Metabolite, M-7, and the Authentic Glucuronide of Aprepitant. The synthetic glucuronide of

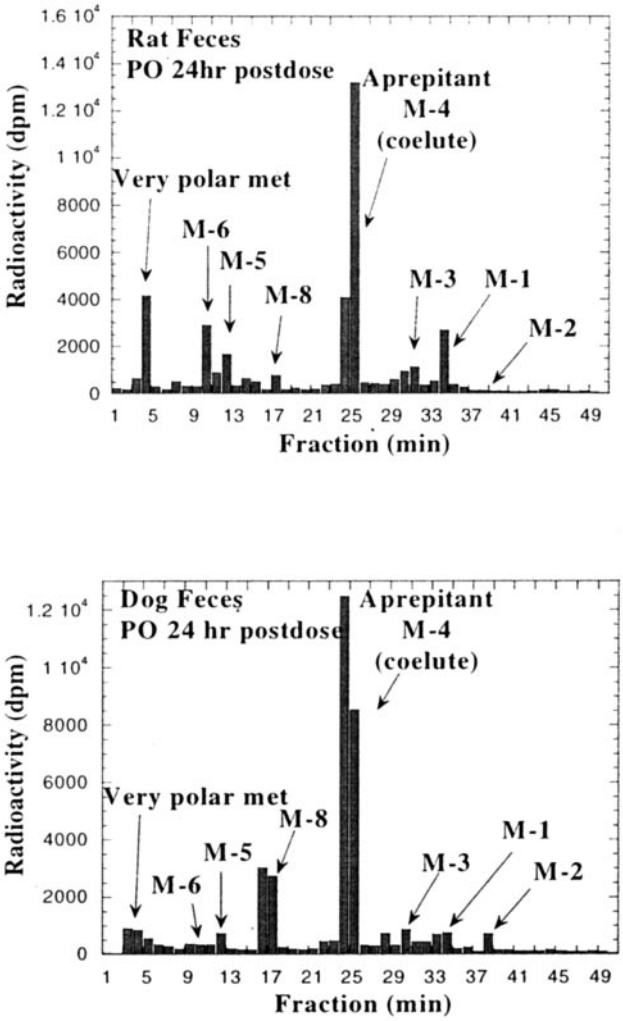

FIG. 6. *Radiochromatograms derived from the analysis of combined fecal extracts from rats or dogs following oral administration of [^{14}C]aprepitant at 2 mg/kg.*

Fecal samples from rats or dogs were homogenized, combined (from three rats or two dogs from either dosing route), extracted, and subjected to LC-MS/MS analysis. The HPLC separation was performed using a Zorbax RX-C8 column, as described under *Materials and Methods*, and radioactivity profiles of fecal extracts from 0 to 24 h postdose are presented.

aprepitant and bile samples were compared in terms of chemical stability and chromatographic properties. Under acidic and basic conditions, the synthetic glucuronide of aprepitant demonstrated similar instability in acid and stability in base, as was observed for the biliary metabolite M-7 (data not shown). In addition, the synthetic glucuronide of aprepitant and M-7 exhibited similar chromatographic properties under two different conditions (method A, Zorbax RX-C8 column, and Hypersil BDS-C18 column). When the two compounds were mixed prior to HPLC, they coeluted as indicated by UV absorbance and radioactivity measurements. Collectively, the data support the contention that M-7 is a glucuronide of aprepitant, but the site of attachment of the sugar moiety remains unknown.

In addition to the glucuronide of aprepitant, which proved to be the major metabolite in rat bile, metabolites M-1 to M-6, M-8 (see below), M-10, and M-11 were confirmed by LC-MS/MS analysis to be minor metabolites of aprepitant (Tables 3 and 4).

Metabolites of [^{14}C]Aprepitant in Dog Bile. A representative radiochromatogram of dog bile (4–24 h after i.v. dose) revealed two major radioactive components (9–14, 27–28 min, HPLC method A; Fig. 5, panel A), which accounted for ~54% and ~13% of the total radioactivity in the extract. The latter component was confirmed by LC-MS/MS to be parent compound, which accounted for ~3% of the radioactive i.v. dose.

The effluents from the 9- to 14-min fractions were combined and treated with β-glucuronidase. The radiochromatogram of the resulting hydrolysate (Fig. 5, panel B) indicated the presence of aprepitant, as well as several metabolites (M-1, M-4, M-8, and M-9). The findings suggested that the major components in dog bile were glucuronides derived from aprepitant and its metabolites, which accounted collectively for ~14% of the radioactive i.v. dose.

Moreover, all of the metabolites observed in plasma also were present in bile, together with M-2. Interestingly, M-9 per se was not detected in dog bile, although the glucuronide conjugate of this metabolite was present (Tables 3 and 4).

Metabolites of [^{14}C]Aprepitant in Rat and Dog Feces. Combined fecal extracts from three rats or two dogs (0–24 h postdose; extraction recovery >70%) were analyzed by HPLC and LC-MS/MS. As shown in Fig. 6, the radioactivity profiles of the fecal extract from rats and dogs revealed a major radioactive component, which was confirmed to be unchanged [^{14}C]aprepitant, as well as several other radioactive components (Tables 3 and 4). These metabolites, including M-1 to M-6, observed previously in rat or dog plasma, also were detected in the fecal samples. In addition, M-8 was found in dog feces, and its

TABLE 6

Proton NMR data for Isolated Metabolite M-8

Basic Condition					Acidic Condition				
Chemical Shift	Assignment	Integral	Multiplicity	Coupling Constants	Chemical Shift	Assignment	Integral	Multiplicity	Coupling Constants
ppm				*Hz*	*ppm*				*Hz*
7.75	d	1H	s		7.75	d	1H	s	
7.39	b	2H	m		7.39	b	2H	m	
7.31	c	2H	s		7.34	c	2H	s	
7.03	a	2H	m		7.04	a	2H	m	
4.92	e	1H	q	J = 6.6	4.90	e	1H	q	J = 6.7
4.30	2	1H	d	J = 2.9	4.34	2	1H	d	J = 2.9
4.26	6ax	1H	dt	J = 2.6, 12.0	4.27	6ax	1H	m	
3.61	6eq	1H	dd	J = 11.3, 2.5	3.74	3	1H	d	J = 2.9
3.53	3	1H	d	J = 2.9	3.12	g	1H	d	J = 16.8
3.13	g	1H	d	J = 15.7	3.01	5eq	1H	m	J = 12
2.97	5eq	1H	m	J = 12	3.63	6eq	1H	m	
2.45	5ax	1H	dt	J = 3.8, 12.0	2.76	g′	1H	d	J = 16.8
2.31	g′	1H	d	J = 15.7	2.75	5ax	1H	m	
1.43	f	3H	d	J = 6.6	1.45	f	3H	d	J = 6.6

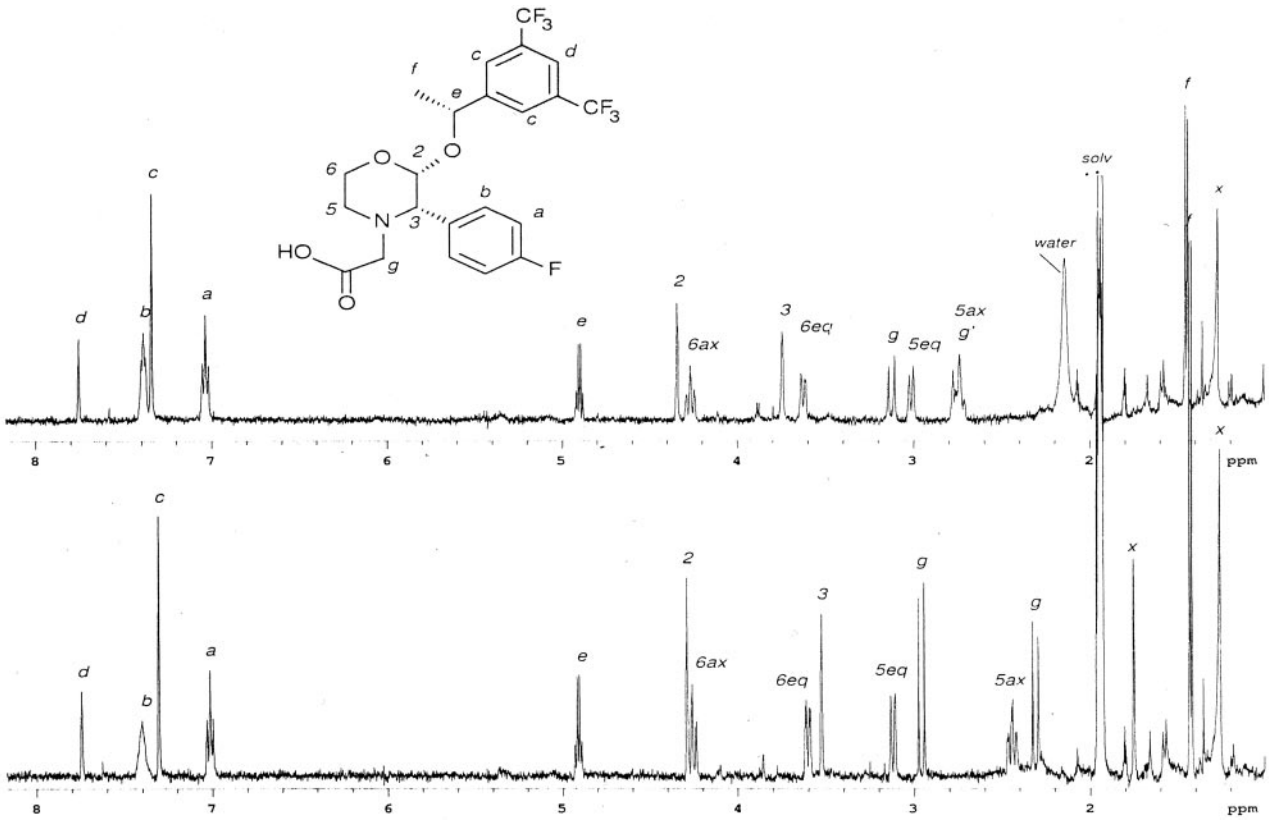

FIG. 7. *Proton NMR spectrum of metabolite M-8.*

Upper panel, under acidic conditions (spiking with TFA); lower panel, under basic conditions (spiking with ND_4OD)

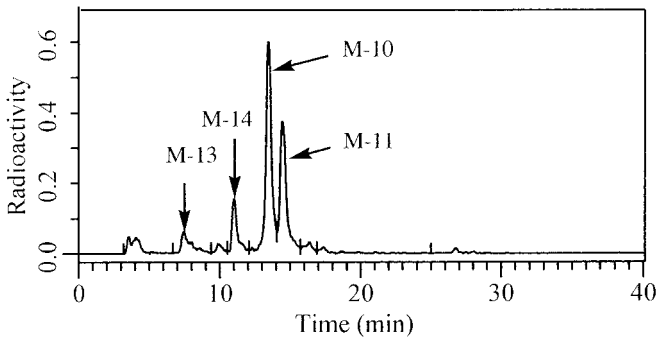

FIG. 8. *Radiochromatogram derived from the analysis of combined urine extracts from rats following oral administration of [^{14}C]aprepitant at 2 mg/kg.*

Urine samples were combined, acidified, extracted and analyzed by LC-MS/MS. The HPLC separation was performed using a Zorbax SB-phenyl column for rat urine, as described under *Materials and Methods*. Radioactivity profiles of urine samples from 0 to 24 h are presented.

identification by ^{1}H NMR is described below. Although low levels of radioactive components were present in the very polar region of the chromatograms, the identities of the responsible components remain to be determined.

Structural Identification of Metabolite M-8. The ^{1}H NMR spectrum of metabolite M-8, isolated from dog fecal extracts, showed that all nonexchangeable protons of aprepitant were present, including those of the methylene bridge. Based on the chemical shift of the exocyclic methylene group (Table 6), the metabolite was proposed to be a triazolone ring-opened carboxylic acid, designated as M-8 (Fig. 7). Addition of deuterated ammonia caused an upfield shift of the methylene bridge signals consistent with the attachment of the carboxylic acid group (Fig. 7, lower panel). LC-MS/MS analysis revealed that M-8 had a molecular weight of 495, suggesting the presence of an odd number of nitrogens in its structure (Table 3). Aprepitant (molecular weight 534) contains four nitrogens, one on the morpholine ring and three on the triazolone ring. Therefore, M-8 was generated by the loss of one or three nitrogens from the parent compound. The presence in the positive ion spectrum of a fragment ion at *m*/*z* 179, indicative of an intact morpholine ring, suggested that M-8 was a product derived from opening of the triazolone ring. Subsequently, an authentic sample was obtained by synthesis, and provided LC-MS/MS and NMR spectra identical to those of M-8 .

Metabolites of [^{14}C]Aprepitant in Rat and Dog Urine. A representative HPLC radiochromatogram derived from rat urine extracts is depicted in Fig. 8. Qualitatively similar profiles were obtained from urine extracts from dogs (data not shown). These chromatograms revealed that no parent compound was excreted unchanged into urine, which contained the very polar metabolites M-10, M-11, M-13, and M-14 (Tables 3 and 4). M-11 and M-12 were identified as 4-fluoro-α-hydroxybenzeneacetic acid and 4-fluoro-α-oxobenzeneacetic acid, respectively, and M-13 and M-14 were triazolone-containing carboxylic acid and the corresponding alcohol. Metabolites M-10 and M-11 were the most predominant metabolites eliminated by the urinary route in rats and dogs.

Figure 9 and Table 4 summarize the metabolites detected in plasma, bile, urine, and feces from rats and dogs following i.v. or p.o. administration of [^{14}C]aprepitant.

Discussion

Previous in vitro studies on the metabolic fate of aprepitant (Huskey et al., 2003) indicated that the drug undergoes *N*-dealkylation,

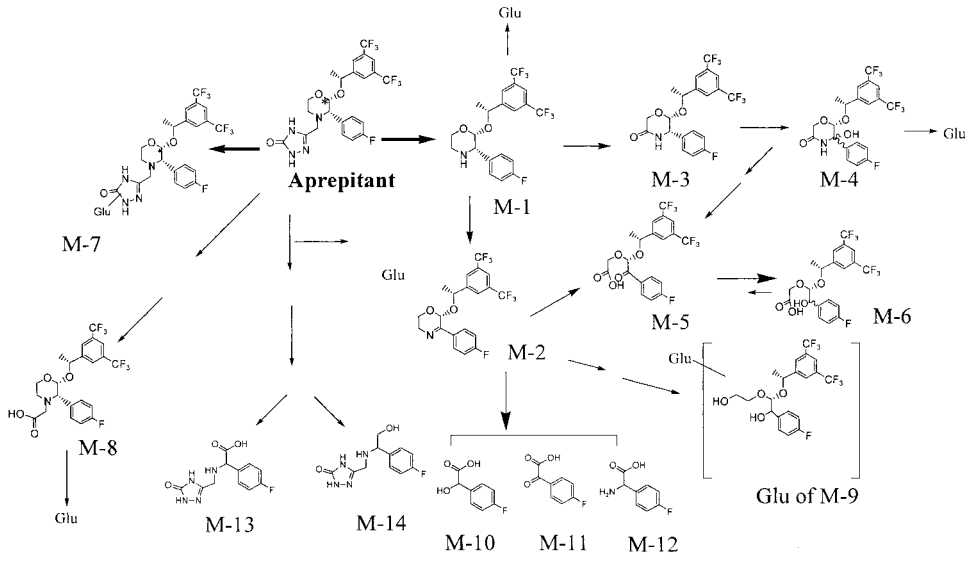

FIG. 9. *Proposed scheme for the metabolism of aprepitant in rats and dogs.*

∗ denotes the position of ^{14}C label; Glu, glucuronic acid. Major metabolic pathways are signified by the bold arrows.

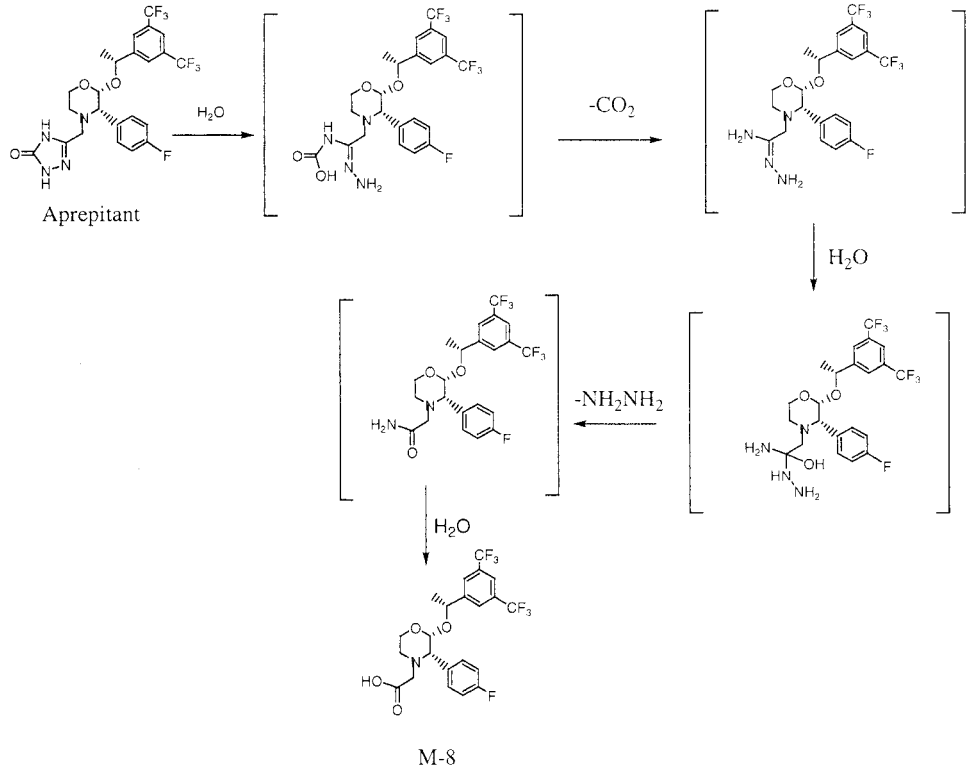

FIG. 10. *Proposed mechanism for the triazolone ring opening of aprepitant.*

leading to the formation of M-1, which, in turn, is subject to extensive metabolism on the morpholine ring. This process leads to several metabolites, namely, M-2, an imine derivative; M-3, a lactam derivative; M-4, two epimers of a hydroxylactam derivative; M-5, a morpholine ring-opened keto acid; and M-6, the corresponding hydroxy-acid. Consistent with the findings from the earlier hepatocyte study, all these metabolites were detected in the plasma, bile, and feces from rats and dogs (Table 4).

In addition to the *N*-dealkylation pathway, glucuronidation was found in the present study to be a quantitatively important pathway in the metabolism and excretion of aprepitant in rats and dogs. Thus, a glucuronide conjugate of aprepitant, M-7, was the major biliary metabolite in rats (Fig. 3). In addition to M-7, several glucuronides derived from the oxidative metabolites, M-1, M-4, M-8, and M-9, were present in the bile of dogs (Fig. 5). To our knowledge, M-7 represents a novel glucuronide with glucuronic acid attached to the triazolone ring, which contains four possible sites (three nitrogens and one carbonyl oxygen) for attachment of the sugar residue (Fig. 4). The instability of M-7 necessitated the structural analysis of a synthetic glucuronide containing protecting groups to stabilize the molecule. Based on proton NMR analyses, the site of glucuronic acid conjugation was narrowed down to either the carbonyl oxygen or the nitrogen

distant from the carbon to which the methylene bridge was attached, but the site of attachment could not be defined unambiguously. After NMR analysis, the protecting groups were removed from this synthetic glucuronide, and the resulting conjugate was compared with M-7 for both chemical stability and chromatographic properties. Metabolite M-7 shared the same chemical and chromatographic properties with the synthetic glucuronide, although the possibility that the two species were regioisomers cannot be excluded completely. Furthermore, the instability of M-7, its abundance in rat and dog bile, and its absence in rat and dog feces suggest that it may play a role in enterohepatic recycling of the parent drug. Finally, it should be noted that whereas M-7 was detected at low levels when aprepitant was incubated with rat hepatocytes (S. Huskey, R. I. Sanchez, G. Doss, B. Arison, B. J. Dean, J. Pang, K. Leung, B. Zhu, M. Braun, P. Finke, D. Luffer-Atlas, T. A. Baillie, and S. H. L. Chiu, unpublished data), it was not detected when aprepitant was incubated with rat liver microsomes fortified with UDP-glucuronic acid and detergent (0.02% Lubrol PX). Low turnover of aprepitant to M-7 in the in vitro system may be attributed to the low intrinsic reactivity of aprepitant and/or the instability of M-7.

Another metabolic event worthy of discussion is related to the phase I metabolism of the triazolone ring of aprepitant. Based on the ^{1}H NMR and LC-MS/MS analyses, M-8 was confirmed to be a carboxylic acid, derived from the opening of the triazolone ring. Presumably, metabolism of aprepitant can proceed by way of two hydrolytic steps and a spontaneous decarboxylation, leading to the formation of an amide derivative, which can be hydrolyzed further to the carboxylic acid (Fig. 10). This carboxylic acid metabolite was detected in plasma from dogs and feces from rats and dogs, as well as in bile from dogs upon treatment with β-glucuronidase, suggesting that the glucuronide of M-8 was present in dog bile. The presence of M-8 and its glucuronide in rat bile was confirmed by LC-MS/MS, albeit at low concentrations; however, their presence in rat plasma was not studied because the information on M-8 was not available at the time of analysis. Similar to the situation with M-7 described above, this conjugate of M-8 was not detected in incubations with rat liver microsomes. However, its presence in rat hepatocyte cultures was confirmed by LC-MS/MS (S. Huskey, R. I. Sanchez, G. Doss, B. Arison, B. J. Dean, J. Pang, K. Leung, B. Zhu, M. Braun, P. Finke, D. Luffer-Atlas, T. A. Baillie, and S. H. L. Chiu, unpublished data), consistent with the in vivo findings. The stability of the acylglucuronide of M-8 in plasma was not studied due to the unavailability of authentic acylglucuronide.

The urinary metabolites of aprepitant consisted of very polar low molecular weight entities (M-10 to M-14; Fig. 8). Notable common features among these metabolites were the absence of the bis-trifluoromethylphenyl moiety and the loss of an intact morpholine ring. The presence of M-13 and M-14, which do retain the intact triazolone ring, suggests that *O*-dealkylation of [^{14}C]aprepitant triggers the metabolic pathway that leads to the formation of this group of very polar metabolites, M-10 to M-14. As reported previously, at least two very polar metabolites were shown to be formed when [^{14}C]aprepitant, M-1, or M-2, was incubated in the presence of Sf21 cells expressing human CYP3A4, CYP1A2, ,or CYP2C19 (S. Huskey, R. I. Sanchez, G. Doss, B. Arison, B. J. Dean, J. Pang, K. Leung, B. Zhu, M. Braun, P. Finke, D. Luffer-Atlas, T. A. Baillie, and S. H. L. Chiu, unpublished data). The identification of these very polar metabolites currently is under investigation in our laboratories.

Aprepitant is a chiral molecule containing three asymmetric carbons. Therefore, stereoisomers may exist among the metabolites as a result of metabolic events occurring at, or adjacent to, these chiral centers. Since the biotransformations leading to six of the metabolites occurred either at a nonchiral part of the molecule (M-1, M-3, M-7, M-8) or by the elimination of one of the three original chiral centers (M-2, M-5), the chirality at the other chiral centers of aprepitant should not be affected and stereoisomers of these metabolites are not expected to be formed. The interconversion of M-4 to its C-3 hydroxy stereoisomer can occur chemically under neutral conditions, presumably through reversible opening of the lactam ring, as described previously (S. Huskey, R. I. Sanchez, G. Doss, B. Arison, B. J. Dean, J. Pang, K. Leung, B. Zhu, M. Braun, P. Finke, D. Luffer-Atlas, T. A. Baillie, and S. H. L. Chiu, unpublished data). M-6 was shown to be formed as a 10:1 mixture of its two hydroxy isomers (the absolute stereochemical assignments were not determined) when incubated with M-1 in rat hepatocytes, presumably via enzymatic reduction of the ketone metabolite (M-5) (S. Huskey, R. I. Sanchez, G. Doss, B. Arison, B. J. Dean, J. Pang, K. Leung, B. Zhu, M. Braun, P. Finke, D. Luffer-Atlas, T. A. Baillie, and S. H. L. Chiu, unpublished data). Since the incubation of either of the pure isomers in hepatocytes afforded the same isomer mixture, in vivo interconversion of this hydroxy via enzymatic oxidation to M-5 and subsequent reduction might also be possible (data not shown). No attempts were made, however, to study the rate of interconversion and the relative stability of all possible stereoisomers in the biological matrices examined.

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References

Ballard TM, Sanger S, and Higgins GA (2001) Inhibition of shock-induced foot tapping behaviour in the gerbil by a tachykinin NK1 receptor antagonist. *Eur J Pharmacol* **412:**255–264.

Beattie DT, Beresford IJ, Connor HE, Marshall FH, Hawcock AB, Hagan RM, Bowers J, Birch PJ, and Ward P (1995) The pharmacology of GR203040, a novel, potent and selective non-peptide tachykinin NK1 receptor antagonist. *Br J Pharmacol* **116:**3149–3157.

Bountra C, Bunce K, Dale T, Gardner C, Jordan C, Twissell D, and Ward P (1993) Anti-emetic profile of a non-peptide neurokinin NK1 receptor antagonist, CP-99,994, in ferrets. *Eur J Pharmacol* **249:**R3–R4.

Campos D, Pereira J, Reinhardt R, Carracedo C, Poli S, Vogel C, Martinez-Cedillo J, Erazo A, Wittreich J, Eriksson L, et al. (2001) Prevention of cisplatin-induced emesis by the oral neurokinin-1 antagonist, MK-869, in combination with granisetron and dexamethasone or with dexamethasone alone. *J Clin Oncol* **19:**1759–1767.

Cheeta S, Tucci S, Sandhu J, Williams AR, Rupniak NM, and File SE (2001) Anxiolytic actions of the substance P (NK1) receptor antagonist L-760735 and the 5-HT1A agonist 8-OH-DPAT in the social interaction test in gerbils. *Brain Res* **915:**170–175.

Cocquyt V, Van Belle S, Reinhardt RR, Decramer ML, O'Brien M, Schellens JH, Borms M, Verbeke L, Van Aelst F, De Smet M, et al. (2001) Comparison of L-758,298, a prodrug for the selective neurokinin-1 antagonist, L-754,030, with ondansetron for the prevention of cisplatin-induced emesis. *Eur J Cancer* **37:**835–842.

Dean B, Rabe M, Doss G, Arison B, Hop C, Chiu S, and Huskey S (2000) Identification of very polar metabolites of MK-0869 in dog urine. *Drug Metab Rev* **32:**261.

Diemunsch P and Grelot L (2000) Potential of substance P antagonists as antiemetics. *Drugs* **60:**533–546.

Gonsalves S, Watson J, and Ashton C (1996) Broad spectrum antiemetic effects of CP-122,721, a tachykinin NK1 receptor antagonist, in ferrets. *Eur J Pharmacol* **305:**181–185.

Gonzalez MI, Field MJ, Holloman EF, Hughes J, Oles RJ, and Singh L (1998) Evaluation of PD 154075, a tachykinin NK1 receptor antagonist, in a rat model of postoperative pain. *Eur J Pharmacol* **344:**115–120.

Griebel G (1999) Is there a future for neuropeptide receptor ligands in the treatment of anxiety disorders? *Pharmacol Ther* **82:**1–61.

Hale JJ, Mills SG, MacCoss M, Dorn CP, Finke PE, Budhu RJ, Reamer RA, Huskey SE, Luffer-Atlas D, Dean BJ, et al. (2000) Phosphorylated morpholine acetal human neurokinin-1 receptor antagonists as water-soluble prodrugs. *J Med Chem* **43:**1234–1241.

Hale JJ, Mills SG, MacCoss M, Finke PE, Cascieri MA, Sadowski S, Ber E, Chicchi GG, Kurtz M, Metzger J, et al. (1998) Structural optimization affording 2-(R)-(1-(R)-3,5-bistrifluoromethyl)phenylethoxy)-3-(S)-(4-fluoro)phenyl-4-(3-oxo-1,2,4-triazol-5-yl) methylmorpholine, a potent, orally active, long-acting morpholine acetal human NK-1 receptor antagonist. *J Med Chem* **41:**4607–4614.

Harrison T, Owens AP, Williams BJ, Swain CJ, Williams A, Carlson EJ, Rycroft W, Tattersall FD, Cascieri MA, Chicchi GG, et al. (2001) An orally active, water-soluble neurokinin-1 receptor antagonist suitable for both intravenous and oral clinical administration. *J Med Chem* **44:**4296–4299.

Kramer MS, Cutler N, Feighner J, Shrivastava R, Carman J, Sramek JJ, Reines SA, Liu G, Snavely D, Wyatt-Knowles E, et al. (1998) Distinct mechanism for antidepressant activity by blockade of central substance P receptors. *Science (Wash DC)* **281:**1640–1645.

Maubach KA, Rupniak NM, Kramer MS, and Hill RG (1999) Novel strategies for pharmacotherapy of depression. *Curr Opin Chem Biol* **3:**481–488.

Navari R-M, Reinhardt R-R, Gralla R-J, Kris M-G, Hesketh P-J, Khojasteh A, Kindler H, Grote T-H, Pendergrass K, Grunberg S-M, et al. (1999) Reduction of cisplatin-induced emesis by a selective neurokinin-1-receptor antagonist. L-754,030 Antiemetic Trials Group. *N Engl J Med* **340:**190–195.

Rudd JA, Jordan CC, and Naylor RJ (1996) The action of the NK1 tachykinin receptor antagonist, CP 99,994, in antagonizing the acute and delayed emesis induced by cisplatin in the ferret. *Br J Pharmacol* **119:**931–936.

Rupniak NM and Kramer MS (1999) Discovery of the antidepressant and anti-emetic efficacy of substance P receptor (NK1) antagonists. *Trends Pharmacol Sci* **20:**485–490.

Rupniak NM, Tattersall FD, Williams AR, Rycroft W, Carlson EJ, Cascieri MA, Sadowski S, Ber E, Hale JJ, Mills SG, et al. (1997) In vitro and in vivo predictors of the anti-emetic activity of tachykinin NK1 receptor antagonists. *Eur J Pharmacol* **326:**201–209.

Saria A (1999) The tachykinin NK1 receptor in the brain: pharmacology and putative functions. *Eur J Pharmacol* **375:**51–60.

Severini C, Improta G, Falconieri-Erspamer G, Salvadori S, and Erspamer V (2002) The tachykinin peptide family. *Pharmacol Rev* **54:**285–322.

Singh L, Field MJ, Hughes J, Kuo BS, Suman-Chauhan N, Tuladhar BR, Wright DS, and Naylor RJ (1997) The tachykinin NK1 receptor antagonist PD 154075 blocks cisplatin-induced delayed emesis in the ferret. *Eur J Pharmacol* **321:**209–216.

Tattersall FD, Rycroft W, Cumberbatch M, Mason G, Tye S, Williamson DJ, Hale JJ, Mills SG, Finke PE, MacCoss M, et al. (2000) The novel NK1 receptor antagonist MK-0869 (L-754,030) and its water soluble phosphoryl prodrug, L-758,298, inhibit acute and delayed cisplatin-induced emesis in ferrets. *Neuropharmacology* **39:**652–663.

Urban LA and Fox AJ (2000) NK1 receptor antagonists—are they really without effect in the pain clinic? *Trends Pharmacol Sci* **21:**462–464.

Watson JW, Gonsalves SF, Fossa AA, McLean S, Seeger T, Obach S, and Andrews PL (1995) The anti-emetic effects of CP-99,994 in the ferret and the dog: role of the NK1 receptor. *Br J Pharmacol* **115:**84–94.

Zaman S, Woods AJ, Watson JW, Reynolds DJ, and Andrews PL (2000) The effect of the NK1 receptor antagonist CP-99,994 on emesis and c-fos protein induction by loperamide in the ferret. *Neuropharmacology* **39:**316–323.