

DMD #13110

**DISPOSITION OF THE DIPEPTIDYL PEPTIDASE-4 INHIBITOR
SITAGLIPTIN IN RATS AND DOGS**

Maria G. Beconi, James R. Reed, Yohannes Teffera, Yuan-Qing Xia, Christopher J.
Kochansky, David Q. Liu, Shiyao Xu, Charles S. Elmore, Suzanne Ciccotto, Donald
F. Hora, Ralph A. Stearns, and Stella H. Vincent

*Departments of Drug Metabolism (MGB, JRR, YT, YQX, CJK, DQL, SX, CE, SC,
RAS, SHV) and Comparative Medicine (DH), Merck Research Laboratories,
Rahway, NJ*

DMD #13110

Running title: Disposition of sitagliptin in rats and dogs

Corresponding author:

Dr. Stella Vincent

RY 80-141

Merck Research Laboratories

P.O. Box 2000

Rahway, NJ 07065

Phone: (732) 594-6694

Fax: (732) 594-2382

E-mail: stella_vincent@merck.com

Number of Text pages: 31

Number of Tables: 3

Number of Figures: 7

Number of References: 35

Words in Abstract: 210

Words in introduction: 274

Words in Discussion: 779

Abbreviations: BDC, bile duct-cannulated; DPP-4, dipeptidyl-peptidase-4; GLP-1, glucagon-like peptide-1; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MK-0431, 2R)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-*a*]pyrazin-7(8H)-yl]-1-(2,4,5-trifluorophenyl)butan-2-amine); MRL, Merck Research Laboratories.

DMD #13110

ABSTRACT

The pharmacokinetics, metabolism and excretion of sitagliptin (MK-0431), a potent dipeptidyl peptidase 4 inhibitor, were evaluated in male Sprague-Dawley rats and beagle dogs. The plasma clearance and volume of distribution of sitagliptin were higher in rats (40-48 mL/min/kg, 7-9 L/kg) than dogs (~9 mL/min/kg, ~3 L/kg), and its half-life was shorter in rats, ~2 hr compared to ~4 hr in dogs. Sitagliptin was absorbed rapidly after oral administration of a solution of the phosphate salt. The absolute oral bioavailability was high, and the pharmacokinetics were fairly dose-proportional. Following administration of [¹⁴C]sitagliptin, parent drug was the major radioactive component in rat and dog plasma, urine, bile and feces. Sitagliptin was eliminated primarily by renal excretion of parent drug; biliary excretion was an important pathway in rats, while metabolism was minimal in both species in vitro and in vivo. Approximately 10 to 16% of the radiolabeled dose was recovered in the rat and dog excreta as Phase I and II metabolites, which were formed by *N*-sulfation, *N*-carbamoyl glucuronidation, hydroxylation of the triazolopiperazine ring, and by oxidative desaturation of the piperazine ring followed by cyclization via the primary amine. The renal clearance of unbound drug in rats, 32-39 ml/min/kg, far exceeded the glomerular filtration rate, indicative of active renal elimination of parent drug.

DMD #13110

INTRODUCTION

Dipeptidyl-peptidase 4 (DPP-4) is a ubiquitous proline-specific serine protease responsible for the rapid inactivation of the incretin glucagon-like peptide 1 (GLP-1) (Mentlein et al., 1993, Gorrell, 2005). GLP-1 and GLP-1 analogs have been shown to decrease fasting and postprandial glucose in diabetic patients when given as a continuous intravenous infusion or subcutaneous administration (Zander et al., 2002; Gautier, 2005). The effectiveness of orally administered DPP-4 inhibitors has been demonstrated in obese Zucker rats (Balkan et al., 1999; Pospisilik et al., 2002), mice (Mu et al. 2006), and humans (Ahren et al., 2004; Herman et al., 2004, 2005; Scott et al., 2005, Cornell 2006). Thus, stabilization of GLP-1 via DPP-4 inhibition represents a new therapeutic approach for type 2 diabetes (Drucker, 2006; Green et al., 2006).

Sitagliptin (Januvia™), also known as MK-0431, (2R)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-A]pyrazin-7(8H)-yl]-1-(2,4,5-trifluorophenyl)butan-2-amine (Fig. 1), is a potent and selective, reversible inhibitor of DPP-4 with an IC₅₀ value of 18 nM (Kim et al., 2005). Sitagliptin has been shown to inhibit plasma DPP-4 activity in normal volunteers (Bergman 2005, 2006; Herman et al., 2005a) and in patients with type 2 diabetes (Herman 2004), and to significantly reduce HbA_{1c} and fasting plasma glucose in patients with type 2 diabetes (Herman 2005b; Scott et al., 2005). In support of the suitability of the rat and dog as toxicology species, the disposition of sitagliptin was evaluated in these species and compared to humans. Results from in vitro (rat, dog, human) and in vivo studies in rats and dogs are discussed herein. The absorption, metabolism and excretion of sitagliptin in humans are reported in the accompanying manuscript (Vincent et al.).

MATERIALS AND METHODS

Chemicals and Reagents. Sitagliptin was prepared as the hemi-fumarate and phosphate salts by the Departments of Medicinal Chemistry and Process Research, respectively, Merck Research Laboratories (MRL, Rahway, NJ). [Trifluorophenyl-3-³H]sitagliptin (specific activity 9.44 mCi/mg, radioactivity purity 98%), [2-amine-3-¹⁴C]butyl]sitagliptin (specific activity 132 μ Ci/mg, radioactivity purity ~99%) and the *N*-sulfate of sitagliptin (M1, Fig. 1) were prepared by the Labeled Compound Synthesis Group, MRL (Rahway, NJ). [³H]- or [¹⁴C]-Labeled sitagliptin was diluted with unlabeled sitagliptin to achieve the final specific activities used in the studies described below. The 2,5-difluoro analog of sitagliptin, used as an internal standard in the quantitative LC-MS/MS assay, was provided by Medicinal Chemistry, MRL, Rahway. Synthetic standards of metabolites were prepared by the Labeled Compound Synthesis Group, MRL, Rahway, NJ (M1), and Medicinal Chemistry, MRL, Rahway, NJ (M2 and M5). Human, rat, dog and monkey liver microsomes were prepared following procedures described in literature (Raucy and Lasker, 1991). Cryopreserved human, dog and monkey hepatocytes were obtained from In Vitro Technologies.

In Vitro Incubations. [¹⁴C]Sitagliptin (10 μ M) was incubated at 37°C with liver microsomes (2 mg protein) in 0.5 ml of 0.1 M KH₂PO₄ (pH 7.4) containing 1 mM MgCl₂ and an NADPH-regenerating system (10 mM glucose-6-phosphate, 1.4 units/ml glucose-6-phosphate dehydrogenase, and 1 mM NADP). Control incubations (without NADPH) were conducted for each species under the same conditions. Reactions were started by adding NADP (or buffer for controls). In separate experiments designed to measure the formation of the carbamoyl glucuronide, incubations were carried out in 0.5 M NaHCO₃

DMD #13110

buffer, containing liver microsomes (2 mg/ml), 50 µg/ml alamethicin, 5 mM saccharolactone, and 5 mM UDPGA. The incubation mixtures were saturated with CO₂ before starting the reactions with substrate. Incubations were carried out for 1 hr at 37°C, and the reaction was quenched with 12.5 µl of perchloric acid and 25 µl of acetonitrile. Samples were centrifuged at 14000 rpm for 10 min, transferred to HPLC vials, and 50 µl aliquots were analyzed by LC-MS and radiometric detection for metabolite profiling.

Incubations of [¹⁴C]sitagliptin (10 µM) with hepatocytes (2 ml suspension, 1x10⁶ cells/ml) were conducted for 0 (control), 1, 2 and 4 hr. The samples were mixed with 4 ml acetonitrile and aliquots of the supernatant (200 µl) were analyzed by LC-MS and radiometric detection.

Animal studies. All animal studies were conducted with approval from the Institutional Animal Care and Use Committee of MRL, Rahway, NJ. Sitagliptin was dissolved in normal saline for both intravenous (i.v.) and oral (p.o.) administration

Pharmacokinetic Studies. Male Sprague-Dawley rats (360 to 450 g) were surgically implanted with a cannula in the femoral vein (i.v. dosing) and/or femoral artery (blood collection). Rats were dosed intravenously at 0.5, 2 and 5 mg/kg and orally at 2, 20, 60 and 80 mg/kg. Male beagle dogs (10 to 14 kg) were dosed intravenously via a cephalic vein catheter at 0.5 and 1.5 mg/kg and orally at 0.4, 1.6, and 30 mg/kg. Doses are expressed in free base equivalents/kg body weight. Animals were fasted overnight before and for 4 hr after dosing. Blood (0.3 ml from rats and 2 ml from dogs) was collected at 0, 0.083 (i.v. only), 0.25, 0.5, 1, 2, 4, 6, 8 and 24 hr post-dose into EDTA-containing tubes. Plasma was obtained by centrifugation at 4°C and stored at -70°C.

DMD #13110

Metabolism and Excretion in Rats. Male Sprague-Dawley rats were surgically prepared two days before dosing as described by Krieter et al. Briefly, cannulas were inserted into the bile duct and duodenum of rats under deep anesthesia. The cannulas were tunneled under the skin, externalized, and threaded through a Covance® harness and tethering system. The biliary and duodenal cannulas were connected to permit recirculation of bile, and the rats were housed individually in metabolism cages, and observed frequently during the recovery period. On the day of dosing, the biliary and duodenal cannulas were disconnected and a solution of 13.4% sodium taurocholate/0/.9% saline/0.05% potassium chloride was infused through the duodenal cannula, while the bile was collected. Groups of three bile duct-cannulated (BDC) rats (~400 g) received a single dose of [¹⁴C]sitagliptin either intravenously (2 mg/kg, via a tail vein) or by oral gavage (5 and 20 mg/kg). The specific activity was ~21.5 μCi/mg for the i.v. and 5-mg/kg p.o. dose, and ~5 μCi/mg for the 20-mg/kg p.o. dose. Bile and urine were collected into pre-tared bottles containing a known volume of 0.5 M formate buffer, pH 3.0, at specified time intervals as follows: 0 to 2, 2 to 4, 4 to 6, 6 to 8, 8 to 24, 24 to 48, and 48 to 72 hr post-dose for bile; and 0 to 8, 8 to 24, 24 to 48, and 48 to 72 hr post-dose for urine. In a separate study, intact rats were dosed with [¹⁴C]sitagliptin (9.7 μCi/mg) at 2 mg/kg i.v. and 5 mg/kg p.o. (n=3/route) and urine and feces were collected at 24-hr intervals for 5 days.

Plasma for metabolite profiling was collected from intact male Sprague-Dawley rats (350-450 g) dosed orally with [¹⁴C]sitagliptin (26 μCi/mg) at 20 mg/kg. Blood was collected at 1, 4 and 8 hr post-dose from 2 rats at each time point.

DMD #13110

Metabolism and Excretion in Dogs. The in-life phase of the study was conducted at Charles River (Worcester, MA, USA) in BDC male beagle dogs (11 to 14 kg) fitted a priori with cannulas in the common bile duct, duodenal lumen and iliac vein. The surgical procedures were conducted according to a protocol approved by the institution's Animal Care and Use Committee. Briefly, the dogs were anesthetized and their gall bladder removed and replaced with a balloon catheter. An outflow catheter was inserted in the proximal portion of the common bile duct so that its tip was immediately proximal to the sphincter of Oddi. The operation of the system was tested several times to insure proper inflation of the balloon, collection of bile, and bidirectional flushing. Using a trocar, the catheters were brought individually through the skin and externalized. The animals were assessed 14 days following surgery based on several clinical parameters such as bilirubin and ALT levels, catheter patency and bile flow rate. The BDC dogs were dosed intravenously (0.5 mg/kg) or orally (2 mg/kg) with [¹⁴C]sitagliptin (n = 3 per route). Each dog in the i.v. and oral groups received approximately 115 and 450 μCi of [¹⁴C]sitagliptin, respectively. Bile and urine were collected in pre-tared bottles containing a known volume of formate buffer, pH 3.0, as described above for the rat study, except that samples were collected for 5 days. Feces were collected at 24-hr intervals for 5 days. The cages used to house the dogs were washed with a mixture of ethanol and water and the washes and debris were analyzed for radioactivity by combustion and/or liquid scintillation counting. Blood was collected from the same dogs at several time points between 0.25 and 72 hr post dose.

Plasma Protein Binding. [³H]Sitagliptin (~1x10⁶ dpm) was mixed with known amounts of unlabeled sitagliptin to achieve final concentrations of 0.1 to 200 μM and

DMD #13110

evaporated to dryness. Fresh plasma was added to the dry extracts, and the samples were vortexed and incubated at 37°C for 30 min. Following incubation, one aliquot was obtained from each sample for total radioactivity determination, and triplicate 1-ml aliquots were subjected to ultracentrifugation at 100,000 rpm (Beckman ultracentrifuge model TL-100, rotor TLA 120.2) at 37°C for 3 hr. From each test tube, four 200-ml aliquots were removed sequentially from the top and assayed for radioactivity. A blank plasma sample was processed in the same way and used to determine the protein concentration of these fractions. The unbound fraction was calculated as the ratio of concentration of radioactivity in the fraction with the lowest protein concentration and the concentration of total radioactivity in plasma.

Analytical Methods. *LC-MS/MS Quantitation Assay.* Concentrations of sitagliptin in rat and dog plasma were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) following on-line extraction. Plasma samples (30 µl) including study samples, standards, quality controls, double blanks, and single blanks were dispensed into 96-well plates. An equal volume of the internal standard solution (2,5-difluoro analog of sitagliptin) at 1 µg/ml in 0.5 M formic acid in water/acetonitrile [80/20, v/v]) was added into each well. The samples were vortexed for 5 min, centrifuged at 2000 g for 10 min at 10°C, and extracted on-line. An in-house assembled parallel on-line extraction system, described in detail by Xia et al., 2001, was used. The system consisted of a Leap HTS PAL autosampler coupled with two Shimadzu LC-AD pumps, two Perkin-Elmer series 200 micro pumps, two ten-port switching valves, a FluoroSep-RP phenyl analytical column (2 x 50 mm, 5 µm) eluted at 0.8 mL/min, two Oasis HLB extraction columns, washed at 4 mL/min, and two in-line pre-column filters.

DMD #13110

The total analysis time per sample, including loading, elution and equilibration steps, was 2.5 min (0.5 min for on-line extraction and 2.0 min for the analytical separation). Sitagliptin and the internal standard eluted at 1.3 min.

Analyte detection was achieved with positive turbo ion spray tandem mass spectrometry using multiple reaction monitoring (MRM) of transitions unique to each compound, m/z 408.3 \rightarrow m/z 235.2 and m/z 390.2 \rightarrow m/z 235.1 for sitagliptin and internal standard, respectively. The PE Sciex Analyst 1.1 software was used for data acquisition and peak integration. The peak area ratios of sitagliptin to the internal standard were plotted as a function of the nominal concentrations of sitagliptin. The standard calibration curves were constructed using weighted ($1/x^2$) quadratic regression of the data points. The lower limit of quantification was 1 ng/ml (2.5 nM) for rat and 5 ng/ml (12 nM) for dog using 30 μ l plasma. The performance of the calibration standard samples was better than 9.5% CV, and accuracy was between -7.5 to 5.3%. The within-run precision of the QC samples was 11% or better, (excluding the LLOQ) and between run precision was better than 5%. The accuracy of the QC samples was between 87 to 111%. At the lower limit of quantification, the precision was \geq 14% and accuracy \geq 11%. Matrix ion suppression was evaluated using post-column infusion of sitagliptin and injection of the blank plasma to the on-line LC-MS/MS system, and determined to be minimal. Sitagliptin and the internal standard were shown to be stable in rat and dog plasma for 4 hr at room temperature and for 24 hr at 10°C.

Pharmacokinetic Calculations. Pharmacokinetic parameters were calculated by established non-compartmental methods using the Watson software (version 6.4.0.04). The area under the plasma concentration versus time curve from 0 to t ($AUC_{(0-t)}$), where

DMD #13110

t =last time point with concentrations above the lower limit of quantitation, was calculated using linear trapezoidal interpolation in the ascending slope and logarithmic trapezoidal interpolation in the descending slope. The portion of the AUC from the last measurable concentration to infinity ($AUC_{(t-\text{inf})}$) was estimated by C_t/k_{el} , where C_t represents the last measurable concentration and k_{el} is the elimination rate constant. The latter was determined from the concentration versus time curve at the terminal phase by linear regression of the semi-logarithmic plot. The renal clearance of sitagliptin in rats was estimated by multiplying the plasma clearance of unbound drug (total clearance divided by unbound fraction in plasma) with the fraction of dose excreted unchanged into urine.

Determination of Total Radioactivity. Concentrations of total radioactivity in plasma, bile, urine, cage washes and samples from in vitro experiments were determined by direct counting in a Packard 1900TR or a Beckman LS-6500 Liquid Scintillation Analyzer, after mixing 50-100 μ l with 6 ml of ScintiSafe Gel scintillation cocktail (Fisher Scientific). Total radioactivity levels in feces and cage debris were determined by combusting triplicate aliquots of homogenates (1:3 specimen/water, w/w) in a Packard Model 307 Oxidizer, followed by determination of radioactivity in a Beckman LS-6500 Liquid Scintillation Analyzer.

Processing of Samples for Metabolite Profiling. Urine and bile from rats (0 to 24 hr) and dogs (0 to 72 hr) were pooled according to volume and across animals (3 per sample), mixed with acetonitrile (~0.3-1 ml/ml), vortexed and centrifuged at ~14000 rpm. The supernatants were analyzed using LC-MS coupled with radiometric monitoring. Plasma samples were pooled across animals, mixed with an equal volume of acetonitrile, vortexed (10 min) and centrifuged at 4000 rpm for 15 min. The resulting supernatants

DMD #13110

were analyzed directly or after evaporation under nitrogen and reconstitution in water:acetonitrile:acetic acid (90:10:0.1).

LC-MS Methods for Metabolite Identification. Initial identification of metabolites was achieved by analysis of samples from the in vitro and in vivo studies using a Perkin-Elmer Sciex API 3000 triple quadrupole mass spectrometer interfaced to a Perkin-Elmer HPLC system equipped with two Series 200 pumps and a Perkin-Elmer Series 200 autosampler (Norwalk, CT). LC-MS and LC-MS/MS experiments were conducted using a Turbo-ionspray interface scanning in the positive ion mode.

Radiolabeled components in samples from in vivo studies (rat and dog bile, urine and plasma) were separated using HPLC, detected by an on line radioflow detector, and identified by MSⁿ. The chromatography system consisted of a Perkin-Elmer Series 200 autosampler (Norwalk, CT), a Shimadzu SCL-10Avp system controller and two Shimadzu LC-10ADvp pumps (Columbia, MD) coupled in parallel to a Packard radioactivity flow detector (Downers Grove, IL) and to a ThermoFinnigan LCQ Deca XP ion trap mass spectrometer (San Jose, CA) The electrospray source of the LCQ Deca was operated in positive ion mode with a voltage of 5 kV. Data acquisition and reduction were carried out using Xcalibur software (version 1.2). Metabolites were identified based on characteristic [M+H]⁺ and fragment ions: M1, 488 to 408 and 193; M2 and M5, 406 to 174 and 191; M3, 600 to 424 and 406; M4, 628 to 408 and 452; and M6, 424 to 406 and 191.

Analyte separation was achieved on a ThermoHypersil Fluophase PFP (3.0 x 150 mm; 5 μm) column eluted at 0.6 ml/min with a 33-min linear gradient from 82% A (5 mM ammonium acetate in water plus 0.05% acetic acid) to 80% B (5 mM ammonium acetate

DMD #13110

and 0.05% acetic acid (by volume) in methanol), followed by a 5-min gradient to 95% B and a hold at 95% B for 5 min. The effluent from the HPLC column was split (3:1 ratio) between the radiometric flow detector and the mass spectrometer. Scintillation cocktail (Packard Ultima Flo-M) was pumped at 1.2 ml/min in the radiometric detector.

The proposed structures for metabolites M1, M2, and M5 were confirmed by comparing their HPLC retention times and mass spectral fragmentation patterns with those of the corresponding synthetic standards.

RESULTS

Pharmacokinetics. The i.v. and p.o. pharmacokinetic parameters of sitagliptin in rats and dogs are summarized in Tables 1 (i.v.) and 2 (p.o.). The i.v. pharmacokinetics were dose-proportional between 0.5 and 5 mg/kg in rats and 0.5 to 1.5 mg/kg in dogs. The mean plasma clearance and volume of distribution values were higher in rats (40-48 ml/min/kg and ~7-9 liters/kg) than in dogs (~9 ml/min/kg and ~3 liters/kg), and the half-life was shorter, ~2 hr in rats compared to ~4 hr in dogs. Following p.o. administration, absorption was rapid in both species (T_{\max} 0.5 to 2 hr in most animals). The increase in plasma $AUC_{0-\infty}$ was approximately dose-proportional between 2 and 180 mg/kg in rats, and between 0.4 and 30 mg/kg in dogs. The mean bioavailability was ~59% in rats and 89 to 97% in dogs (Table 2).

Plasma Protein Binding. The fraction of [^3H]sitagliptin (0.1 to 200 μM) bound to plasma proteins in vitro, as determined by ultracentrifugation was 28 to 36% in rats, 31 to 37% in dogs, and 34 to 46% in humans.

Identification of Metabolites. The structures of the metabolites of sitagliptin shown in Fig. 1 were elucidated by LC-MS (Sciex API 3000 triple quadrupole mass spectrometer) using a combination of full scan and product ion scan (MS/MS) analyses of samples from hepatocyte incubations, as well as rat and dog bile. Full scan analysis of sitagliptin gave $[\text{M}+\text{H}]^+$ at m/z 408. The product ion mass spectrum of m/z 408 showed predominant fragment ions at m/z 193, 235 and 174 (Fig. 2). The ions at m/z 193 and 235 were assigned to the triazolopiperazine moiety while the ion at m/z 174 was assigned to the trifluorophenyl-containing portion of the molecule. All product ions from portions

DMD #13110

of the molecule containing the primary amino group had a characteristic loss of a 17 amu fragment corresponding to the mass of ammonia.

Metabolite M1 displayed an $[M+H]^+$ of m/z 488 consistent with sulfate conjugation. The product ion mass spectrum of m/z 488 showed product ions identical to those of parent drug following the loss of 80 amu from m/z 488 (spectrum not shown). The structure of the sulfate conjugate was confirmed by comparison with a synthetic standard (HPLC retention time and mass spectrum).

Metabolite M4 displayed an $[M+H]^+$ of m/z 628 consistent with formation of a carbamoyl glucuronide (+220 amu). The product ion mass spectrum (Fig. 3) of m/z 628 produced sequential loss of glucuronic acid (176 amu) and carbon dioxide (44 amu) to give m/z of 452 and 408, respectively. This is a typical fragmentation pattern for a carbamoyl glucuronic acid conjugate (Liu et al., 2002; Beconi et al., 2003).

Metabolites M2 and M5 displayed a $[M+H]^+$ of m/z 406, 2 amu lower than parent. The product ion mass spectra of m/z 406 were identical for both metabolites and showed two major fragment ions: m/z 174 and 191 indicating a loss of 2 amu from the triazolopiperazine (spectra not shown). The elucidation of the structures of these metabolites is described in detail by Liu et al. (accepted).

Metabolite M3 displayed an $[M+H]^+$ of m/z 600 corresponding to addition of one oxygen and glucuronic acid. The product ion mass spectrum of m/z 600 produced ions of m/z 424 and 406 corresponding to a sequential loss of glucuronic acid and water (Fig. 4). The product ions of m/z 174 and 191 indicated an intact trifluorophenyl moiety and loss of water from an oxygenated triazolopiperazine moiety, respectively. This suggested that M3 could be a glucuronic acid conjugate of a hydroxylated metabolite with the hydroxyl

DMD #13110

group on the triazolopiperazine moiety. The exact site of hydroxylation, however, could not be determined based on the mass spectral data, and identification by NMR was not possible due to the low amounts of M3 in all in vitro and in vivo samples.

Metabolite M6 gave an $[M+H]^+$ at m/z 424, 16 mass units higher than sitagliptin. Upon collision-induced dissociation, it gave a prominent ion at m/z 251 which was also 16 mass units higher than the corresponding ion in the parent compound. Further fragmentation of m/z 251 gave m/z 233 (loss of water). This suggested that M6 could be a hydroxylated metabolite. The exact site of hydroxylation could not be determined based on the mass spectral data.

The glutathione conjugates detected in rat hepatocytes and rat bile gave an $[M+H]^+$ at m/z 711, and a major fragment at m/z 582 formed by loss of 129 amu (pyroglutamate) (Fig. 5), which is characteristic of glutathione conjugates (Baillie and Davis, 1993). The exact structures of the glutathione conjugates could not be determined from the mass spectral data. The conjugates were postulated to be formed by oxidative defluorination of the trifluoro-phenyl ring and addition of reduced glutathione, as described in the literature (Rietjens et al., 1997; Park et al., 2001).

Excretion of Radioactivity. Following administration of [^{14}C]sitagliptin to rats at 2 mg/kg i.v. and 5 mg/kg p.o., ~54 and 40% of the i.v. dose and 37 and 59% of the oral dose were recovered in urine and feces, respectively (Table 3). Most of the excretion occurred within 24 hr, 89% of the i.v. dose and 90% of the oral dose. In bile duct-cannulated rats, ~55% of the i.v. dose (2 mg/kg) and 53 and 54% of the p.o. doses (5 and 20 mg/kg) were recovered in urine (Table 3). Excretion into bile (21% of the i.v. and 20-29% of the p.o. doses) was lower than expected based on the recovery of radioactivity

DMD #13110

in feces after i.v. administration to intact rats (40% of the dose). Feces were not collected in the BDC rat study. The high recovery of radioactivity from the oral doses in bile and urine ($\geq 74\%$ of the dose), indicated that absorption was high.

In male BDC beagle dogs dosed with [^{14}C]sitagliptin at 0.5 mg/kg i.v. and 2 mg/kg p.o., most of the dose was excreted into urine, ~ 74 and 77% of the i.v. and p.o. doses, respectively (Table 3). Approximately 8% of the dose was excreted into bile after both routes of administration.

As described below, most of the radioactivity in the rat and dog excreta was comprised of sitagliptin, indicating that the drug was eliminated primarily unchanged.

In Vivo Metabolism in Rats and Dogs. Radiochromatograms of extracts of rat and dog urine and bile following oral administration of [^{14}C]sitagliptin at 5 mg/kg in the rat and 2 mg/kg in the dog are presented in Fig. 6, which were acquired using electrospray LC-MS (LCQ Deca XP ion trap mass spectrometer) coupled with on-line radiometric detection. The mass spectra of the metabolites were comparable to those obtained on the triple quadrupole instrument and thus not shown. Similar profiles were obtained after i.v. administration in both species, and at 20 mg/kg p.o. in the rat. Parent drug accounted for the majority of the radioactivity in rat urine and bile ($\sim 99\%$ and 78% , respectively) and dog urine (95%). Most of the radioactivity in dog bile was comprised of M4, the carbamoyl glucuronide (53%), and parent drug (38%). Minor metabolites in rat and dog bile and urine were M1 (*N*-sulfate conjugate of parent drug; rat bile only), M2 and M5 (*cis* and *trans* cyclized derivatives, respectively; rat and dog urine, dog bile), M3 (ether-linked glucuronide of a hydroxylated product; rat urine and bile), and M6 (hydroxylated metabolite; rat urine, rat and dog bile). Rat bile also contained low amounts of a

DMD #13110

glutathione adduct (Fig. 1), comprising approximately 4-5% of the biliary radioactivity (<1% of the total dose). The metabolite profiles in urine and feces from intact rats (not shown) were similar to the profiles in urine and bile, respectively.

Parent drug was the major radioactive component of rat plasma, comprising 80-85% of the circulating radioactivity in orally dosed rats and dogs. Representative profiles are shown in Figure 7. All of the metabolites excreted into rat urine and bile were observed at low levels in rat plasma, each representing ~2-7% of circulating radioactivity; the only exception was M4, which was not detected in rat plasma. The only metabolites detected in dog plasma were M2 and M5. M2 was found at trace levels at the early time points, and contributed up to 8% of the circulating radioactivity between 7 and 24 hr, while the contribution of M5 to circulating radioactivity increased steadily from 4% at 1 hr to 14% at 5 hr, 25% at 7 hr and 48% at 24 hr. It was estimated that M2 and M5 contributed to ~5 and 19%, respectively, of the AUC of total radioactivity in dog plasma after oral administration of [¹⁴C]sitagliptin (data not shown).

In Vitro Metabolism in Rats, Dogs, Monkeys and Humans. The metabolism of [¹⁴C]sitagliptin (10 μM) in vitro was minimal in rat, dog, monkey and human liver microsomes and hepatocytes (2-13% turnover in liver microsomes after 1 hr, and 1-15% in hepatocytes after 4 hr, data not shown). LC-MS/MS analysis of microsomal and hepatocyte incubation extracts revealed the presence of M2 and M5 in male rat, dog, monkey, and human liver microsomes, and human hepatocytes, M3 in male rat hepatocytes, and M6 in male monkey liver microsomes, human liver microsomes and rat and human hepatocytes. Also, two glutathione conjugates were observed in rat hepatocytes but not in hepatocytes from the other species. M4 was not detected in the

DMD #13110

hepatocyte incubations, but was observed in UDPGA-enriched dog liver microsomal incubations under CO₂ enriched environment. M1 was not detected in any of the in vitro incubations.

DISCUSSION

The present studies indicated that the disposition of sitagliptin in rats and dogs is similar to that in humans (Vincent et al., accompanying manuscript) in that it is eliminated primarily unchanged into the urine. Biliary excretion was an important elimination pathway in rats but not dogs, while metabolism was minimal in both species. As reported in humans (Bergman et al., 2006), results from the present studies indicate that sitagliptin is subject to active renal secretion in rats, as well. The renal clearance of unbound drug, estimated from the total plasma clearance (~40-48 ml/min/kg in rats, ~9 ml/min/kg in dogs), the unbound fraction in plasma (~0.65 in both species) and the fraction of dose eliminated unchanged into urine (~0.54 in rats and 0.75 in dogs) was ~33 to 39 ml/min/kg in rats and ~10 ml/min/kg in dogs. Thus, the renal clearance in the rat but not dog was much higher than the glomerular filtration rate, ~5-15 ml/min/kg (Caron and Kramp, 1999; Haller et al., 1998). Results from studies described elsewhere indicate that sitagliptin is a substrate of the human renal organic anion transporter, hOAT3, and P-glycoprotein, which may be involved in its uptake into the kidney and efflux into urine, respectively (Chu et al., submitted). P-glycoprotein does not appear to limit the oral absorption of sitagliptin, as $\geq 74\%$ of radioactivity was recovered in bile and urine, primarily as parent drug, following administration of [^{14}C]sitagliptin. The lower recovery of radioactivity in bile from BDC rats than in feces from intact animals (21 ± 1.2 vs. $40 \pm 3.2\%$ of an i.v. dose) is suggestive of intestinal secretion, possibly mediated by p-glycoprotein. As well, the small increase in the dose-normalized AUC of sitagliptin with oral dose (0.6 ± 0.1 at 2 mg/kg increasing to 1.2 ± 0.1 at 180 mg/kg) is consistent with

DMD #13110

this speculation, although this could be caused also by saturation of metabolism and renal secretion at the high oral doses in rats.

Although metabolism of [¹⁴C]sitagliptin was minimal in rats and dogs, with ~10 and 16% of the total dose recovered as metabolites in the excreta, respectively, a variety of metabolites (Fig. 1) were observed which were formed by Phase I (M6, hydroxylation; M2 and M5, oxidative desaturation) and Phase II pathways (M1, sulfation; M3, glucuronidation following hydroxylation; M4, carbamoyl glucuronidation). All six metabolites were observed in rats, while dog generated 4 of these metabolites, namely, M2, M4, M5 and M6. A mixture of glutathione conjugates (Fig. 1) speculated to have been formed by epoxidation of the trifluorophenyl ring, followed by addition of reduced glutathione and loss of fluoride (Rietjens et al., 1997; Park et al., 2001) appeared to be rat-specific, in that they were not detected in humans (Vincent et al.) or dogs. Another interesting species difference was the relative abundance of M2 and M5 in dog plasma especially at the later time points (~33 and 56% of the plasma radioactivity at 7 and 24 hr, respectively), even though a similar fraction of the dose, less than 5%, was eliminated as M2 and M5 in all species. This observation suggested that these metabolites were cleared at a slower rate than sitagliptin in dogs.

Metabolism to a carbamoyl glucuronide was a minor elimination pathway, accounting for <1, ~4 and ~2% of the dose in rats, dogs, and humans, respectively. Carbamoyl glucuronic acid conjugates are formed by reaction of the primary amine with carbon dioxide to form a carbamic acid with subsequent conjugation with glucuronic acid. In the presence of carbon dioxide, amino acids (Mirrow et al., 1974) and other amines (Greenaway and Whatley, 1987; Delbressine et al., 1990) can undergo non-enzymatic,

DMD #13110

reversible reactions to form carbamic acids. Formation of a carbamoyl glucuronide of a primary amine was reported for the dipeptidyl-peptidase inhibitors *L-threo* isoleucine thiazolidide and its *allo* stereoisomer (Beconi et al., 2003) and seems to be common for a group of low molecular weight amino amides.

Another interesting biotransformation pathway of sitagliptin in rats and humans, also minor, is sulfation of the primary amine. To our knowledge, *N*-sulfation of aliphatic amine groups has not been reported in the literature for xenobiotics, although it is a critical step in heparan sulfate/heparin biosynthesis. The latter is catalyzed by heparan sulfate/heparin *N*-deacetylase/*N*-sulfotransferase-1, a bifunctional enzyme which removes *N*-acetyl groups from selected *N*-acetyl-d-glucosamine units followed by *N*-sulfation of the generated free amino groups (Sugahara and Kitigawa, 2002).

In conclusion, the systemic clearance of sitagliptin in rats and dogs is driven primarily by renal elimination of intact parent drug, with contribution from biliary excretion (mostly in rats) and metabolism (minor in both species). Our data also suggest that active transport mechanisms are probably involved in the renal elimination of sitagliptin in rats but not dogs.

Acknowledgements

We would like to thank the following individuals, all of MRL: Drs. Ashok Chaudhary, David Schenk, Dennis Dean, Conrad Raab, Allen Jones, and Adria Colletti, Ms. Ann Mao, Mr. Randy Miller, Ms. Courtney Nugent, Ms. Yolanda Jakubowski, Mr. Chris Freeden, and Mr. John Strauss for their contribution in these studies, and Drs. Eugene Tan and Jiunn Lin for helpful discussions. Also, we acknowledge the contribution of Paul Zavorskas and other Charles River Laboratories personnel in the BDC dog study.

REFERENCES

- Ahren B, Landin-Olsson M, Jansson PA, Svenson M, Holmes D, Schweizer A (2004) Inhibition of dipeptidyl peptidase-4 reduces glycemia, sustains insulin levels, and reduces glucagon levels in type 2 diabetes. *J Clin Endocrinol Metab* **89** (5): 2078-2084.
- Baillie TA, Davis MR (1993) Mass spectrometry in the analysis of glutathione conjugates. *Biol Mass Spectr* **22**:319-325.
- Balkan B, Kwasnik L, Miserendino R, Holst JJ and Li X (1999) Inhibition of dipeptidyl peptidase IV with NVP DPP728 increases plasma GPL-1 (7-36 amide) concentrations and improves oral glucose tolerance in obese Zucker rats. *Diabetologia* **42**:1324-1331.
- Beconi MG, Mao A, Liu DQ, Kochansky C, Pereira T, Raab C, Pearson P and Lee Chiu S-H (2003) Metabolism of a dipeptidyl peptidase IV (DP-IV) inhibitor in rats, dogs, and monkeys with selective carbamoyl glucuronidation of the primary amine in dogs. *Drug Metab Dispos* **31**:1269-1277.
- Bergman AJ, Stevens C, Zhou YY, Yi B, Laethem M, De Smet M, Snyder K, Hilliard D, Tanaka W, Zeng W, Tanen M, Wang AQ, Chen L, Winchell G, Davies, MJ, Ramael S, Wagner JA, Herman GA (2006) Pharmacokinetic and pharmacodynamic properties of multiple oral doses of sitagliptin, a dipeptidyl peptidase-IV inhibitor: A double-blind, randomized, placebo-controlled study in healthy male volunteers. *Clin Ther* **28**:55-72.
- Caron N and Kramp R (1999) Measurement of changes in glomerular filtration rate induced by atrial natriuretic peptide in the rat kidney. *Exp Phys* **84**: 689-696.

DMD #13110

Chu XY, Bleasby K, Yabut J, Cai X, Xu S, Bergman AJ, Braun MP, Dean DC, and Evers

R Transport of the dipeptidyl peptidase 4 inhibitor sitagliptin by human organic anion transporter hOAT3 and MDR1 p-glycoprotein (submitted).

Cornell S (2006) Vildagliptin (LAF237): A dipeptidyl peptidase IV inhibitor for the treatment of type 2 diabetes mellitus. *J Pharm Technol* **22**:105-109.

Delbressine LPC, Funke CW, van Tilborg M and Kaspersen FM (1990) On the formation of carbamate glucuronides. *Xenobiotica* **20**:133-134.

Drucker DJ (2006) The biology of incretin hormones. *Cell Metab* **3**:153-165.

Gautier JF, Fetita S, Sobngwi E, Salaun-Martin C (2005) Biological actions of the incretins GIP and GLP-1 and therapeutic perspectives in patients with type 2 diabetes. *Diabetes & Metab* **31**:233-242.

Gorrell MD (2005) Dipeptidyl peptidase IV and related enzymes in cell biology and liver disorders. *Clin Sci (Lond)* **108**(4): 277-292.

Green BD, Flatt PR, Bailey CJ (2006) Inhibition of dipeptidyl peptidase IV activity as a therapy of type 2 diabetes. *Expert Opin Emerging Drugs* **11**(3):525-539.

Greenaway W and Whatley FR (1987) Formation of N-carboxy compounds during trimethyl-silanization of amino acids in the presence of carbon dioxide. *J Chrom* **409**:383-389.

Haller M, Muller W, Binder H, Estelberger W, Arnold P (1998) Single injection inulin clearance – a simple method for measuring glomerular filtration rate in dogs. *Res Vet Sci* **64**(2):151-156.

Herman GA, Zhao PL, Dietrich B, Golor G, Schrodter A, Keymeulen B, Lasseter KC, Kipnes MS, Hilliard D, Tanen M, De Lepeleire I, Cilissen C, Stevens C, Tanaka W,

DMD #13110

Gottesdiener KM, Wagner JA (2004) The DP-IV inhibitor MK-0431 enhances active GLP-1 and reduces glucose following an OGTT in type 2 diabetics. *Diabetes* **53**(Suppl. 2): A82.

Herman G, Hanefeld M, Wu M, Chen X, Zhao P, Stein P (2005a) Effect of MK-0431, a dipeptidyl peptidase IV (DPP-IV) inhibitor, on glycemic control after 12 weeks in patients with type 2 diabetes. *Diabetes* **54**(Suppl. 1):A134.

Herman GA, Stevens C, Van Dyck K, Bergman A, Yi B, De Smet M, Snyder K, Hilliard D, Tanen M, Tanaka W, Wang AQ, Zeng W, Musson D, Winchell G, Davies MJ, Ramael S, Gottesdiener KM, Wagner JA (2005b) Pharmacokinetics and pharmacodynamics of single doses of sitagliptin, an inhibitor of dipeptidyl peptidase-IV, in healthy subjects. *Clin Pharm Ther* **78**:675-688.

Kim D, Wang L, Beconi M, Eiermann GJ, Fisher MH, He H, Hickey G-J, Kowalchick JE, Leiting B, Lyons K, Marsilio F, McCann ME, Patel RA, Petrov A, Scapin G, Patel SB, Roy RS, Wu JK, Wyvratt MJ, Zhang BB, Zhu L, Thornberry NA, Weber AE (2005) (2R)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl]-1-(2,4,5-trifluorophenyl)butan-2-amine: a potent, orally active dipeptidyl peptidase IV inhibitor for the treatment of type 2 diabetes. *J Med Chem* **48**(1):141-151.

Krieter PA, Colletti AE, Doss GA, and Miller RR (1994) Disposition and metabolism of the hypoglycemic agent pioglitazone in rats. *Drug Metab Dispos* **22**(4):625-630.

Liu DQ and Pereira T (2002) Interference of a carbamoyl glucuronide metabolite in quantitative mass spectrometry. *Rapid Commun Mass Spectrom* **16**: 142-146.

DMD #13110

Liu DQ, Arison BH, Stearns RA, Kim D and Vincent SH Characterization of two cyclic metabolites of sitagliptin *Drug Metab Dispos* (accepted).

Mentlein R, Gallwitz B, Schmidt WE (1993) Dipeptidyl peptidase IV hydrolyzes gastric inhibitory polypeptide, glucagon-like peptide-1 (7-36) amide, peptide histidine methionine and is responsible for their degradation in human serum. *Eur J Biochem* **214**:829-835.

Mirrow JS and Keim P and Gurd FRN (1974) CO₂ adducts of certain amino acids, peptides and whale myoglobin studied by carbon 13 and proton nuclear magnetic resonance. *J Biol Chem* **249**:7484-7494.

Mu J, Woods J, Zhou YP, Roy RS, Li Z, Zycband E, Feng Y, Zhu L, Li C, Howard AD, Moller DE, Thornberry NA and Zhang BB (2006) Chronic inhibition of dipeptidyl peptidase-4 with a sitagliptin analog preserves pancreatic β -cell mass and function in a rodent model of type 2 diabetes. *Diabetes* **55**:1695-1704.

Park B, Kitteringham N, O'Neill P (2001) Metabolism of fluorine-containing drugs. *Ann Rev Pharmacol Toxicol* **41**:443-470.

Pospisilik JA, Stafford SG, Demuth H-U, Brownsey R, Parkhouse W, Finegood DT, McIntosh CHS and Pederson RA (2002) Long-term treatment with the dipeptidyl peptidase IV inhibitor P32/98 causes sustained improvements in glucose tolerance, insulin sensitivity, hyperinsulinemia, and β -cell glucose responsiveness in VDF (fa/fa) Zucker rats. *Diabetes* **51**:943-950.

Raucy JL and Lasker JM (1991) Isolation of P450 enzymes from human liver. *Methods Enzymol* **206**:557-587.

DMD #13110

Rietjens IM, den-Besten C, Hanzlik RP, van-Bladeren PJ (1997) Cytochrome P450-catalyzed oxidation of halobenzene derivatives. *Chem Res Toxicol* **10**(6):629-635.

Schaefer WH (1992) Formation of a carbamoyl glucuronide conjugate of carvedilol in vitro using dog and rat liver microsomes. *Drug Metab Dispos* **20**:130-133.

Scott R, Herman G, Zhao P, Chen X, Wu M, Stein P (2005) Twelve-week efficacy and tolerability of MK-0431, a dipeptidyl peptidase IV (DPP-IV) inhibitor, in the treatment of type 2 diabetes (T2D). *Diabetes* **54**(Suppl. 1):A10.

Sugahara K and Kitagawa H (2002) Heparin and heparan sulfate biosynthesis. *IUBMB-Life* **54**(4):163-75.

Vincent SH, Reed JRR, Bergman A, Elmore CS, Zhu B, Xu S, Ebel D, Larson P, Zeng W, Chen L, Dilzer S, Lasseter K, Gottesdiener K, Wagner JA and Herman GA (submitted) Metabolism and excretion of the DPP-4 inhibitor [¹⁴C]sitagliptin in humans.

Xia YQ, Hop CECA, Liu DQ, Vincent SH, Chiu SHL (2001) Parallel extraction columns coupled with liquid chromatography tandem mass spectrometry for on-line simultaneous quantification of a drug candidate and its six metabolites in dog plasma. *Rapid Commun Mass Spectrom* **15**:2135-2144.

Zander M, Madsbad S, Lysgaard J and Holst JJ (2002) Effect of 6-week course of glucagon-like peptide 1 on glycaemic control, insulin sensitivity, and β -cell function in type 2 diabetes: a parallel-group study. *The Lancet* **359**:824-830.

DMD #13110

List of Figure Legends

FIG. 1. Biotransformation pathways of sitagliptin in the rat and dog.

FIG. 2. Product ion mass spectrum and proposed fragmentation of the MH^+ ion of sitagliptin

FIG. 3. Product ion spectrum and proposed fragmentation of the sitagliptin carbamoyl glucuronic acid conjugate (M4)

FIG. 4. Product ion spectrum and proposed fragmentation of the sitagliptin hydroxy glucuronic acid conjugate (M3)

FIG. 5. Product ion spectrum and proposed fragmentation of the sitagliptin glutathione conjugates

FIG. 6. HPLC radiochromatograms of bile and urine following oral dosing of [^{14}C]sitagliptin to rats at 5 mg/kg and dogs at 2 mg/kg. The numbers in parentheses represent the percentage of radioactivity in the HPLC chromatogram associated with each peak.

FIG. 7. Representative HPLC radiochromatograms of rat and dog plasma extracts following oral administration of [^{14}C]sitagliptin at 2 mg/kg. The numbers in parentheses represent the percentage of radioactivity in the HPLC chromatogram associated with each peak.

DMD #13110

TABLE 1

Pharmacokinetic parameters of sitagliptin in rats and dogs following intravenous administration

Species	Dose (mg/kg)	AUC _{0-∞} (μM•hr)	Cl _p (ml/min/kg)	Vd _{ss} (liters/kg)	t _{1/2} (hr)
Rat	0.5	0.459 ± 0.100	46 ± 8.2	8.8 ± 3.4	1.7 ± 0.3
	2	2.05 ± 0.051	40 ± 0.96	8.1 ± 2.0	1.9 ± 0.3
	5	4.28 ± 0.318	48 ± 3.6	7.4 ± 1.2	1.8 ± 0.2
Dog	0.5	2.20 ± 0.36	9.3 ± 1.3	3.2 ± 0.1	4.0 ± 0.4
	1.5	7.11 ± 1.35	8.8 ± 1.5	3.4 ± 0.5	4.2 ± 0.2

Male Sprague-Dawley rats and beagle dogs were dosed with a solution of the phosphate salt in saline.

Mean ± standard deviation values are listed (n=3 or 4). Dose is expressed in mg of free base/kg body weight.

DMD #13110

TABLE 2

Pharmacokinetic parameters of sitagliptin in rats and dogs following oral administration

Species	Dose (mg/kg)	AUC _{0-∞} (μM•hr)	C _{max} (μM)	T _{max} (hr)	F (%)
Rat	2	1.20 ± 0.178	0.210 ± 0.132	0.5 to 4	59
	20	15.2 ± 2.04	4.00 ± 0.384	0.5 to 1	n.d.
	60	60.9 ± 6.78	17.1 ± 6.57	0.5 to 1	n.d.
	180	217 ± 26.9	27.8 ± 6.73	0.5 to 2	n.d.
Dog	0.4	1.62 ± 0.503	0.247 ± 0.080	1.0 to 2.0	89 ± 20
	1.6	7.18 ± 1.56	1.32 ± 0.240	0.5 to 1.0	97 ± 29
	10	54.2 ± 13.4	10.3 ± 2.05	0.5 to 1.0	n.d.
	30	156 ± 29.2	26.5 ± 6.23	0.5 to 1.0	n.d.

Male Sprague-Dawley rats and beagle dogs were dosed with a solution of the phosphate salt in saline. Mean ± standard deviation values are listed (n=3 or 4). Dose is expressed in mg of free base/kg body weight. n.d. =not determined.

TABLE 3

*Dose recovery (%) in urine and feces following single dose administration of
¹⁴C]sitagliptin to rats ^a*

Species	Dose	Recovery of radioactivity (% dose)			
		Urine	Bile	Feces	Total
Rat	2 mg/kg i.v.	54 ± 5.8	n.d. ^b	40 ± 3.2	94 ± 9.0
	5 mg/kg p.o.	37 ± 2.6	n.d.	59 ± 2.5	96 ± 5.1
BDC Rat	2 mg/kg i.v.	55 ± 1.4	21 ± 1.2	n.d.	n.d.
	5 mg/kg p.o.	54 ± 7.3	20 ± 6.7	n.d.	n.d.
	20 mg/kg p.o.	53 ± 7.7	29 ± 5.8	n.d.	n.d.
BDC Dog	0.5 mg/kg i.v.	75.0 ± 4.5	8.1 ± 4.2	9.3 ± 2.0	95 ± 4.9 ^c
	2 mg/kg p.o.	77.5 ± 1.7	7.8 ± 5.3	4.2 ± 2.1	94 ± 6.0 ^c

^a BDC = bile duct-cannulated; excreta were collected for 3 days from BDC rats and 5 days from intact rats and BDC dogs; Mean ± standard deviation (N = 3); ^b n.d. = not determined; ^c total recovery in dogs includes radioactivity in cage washes and debris.

Fig. 1

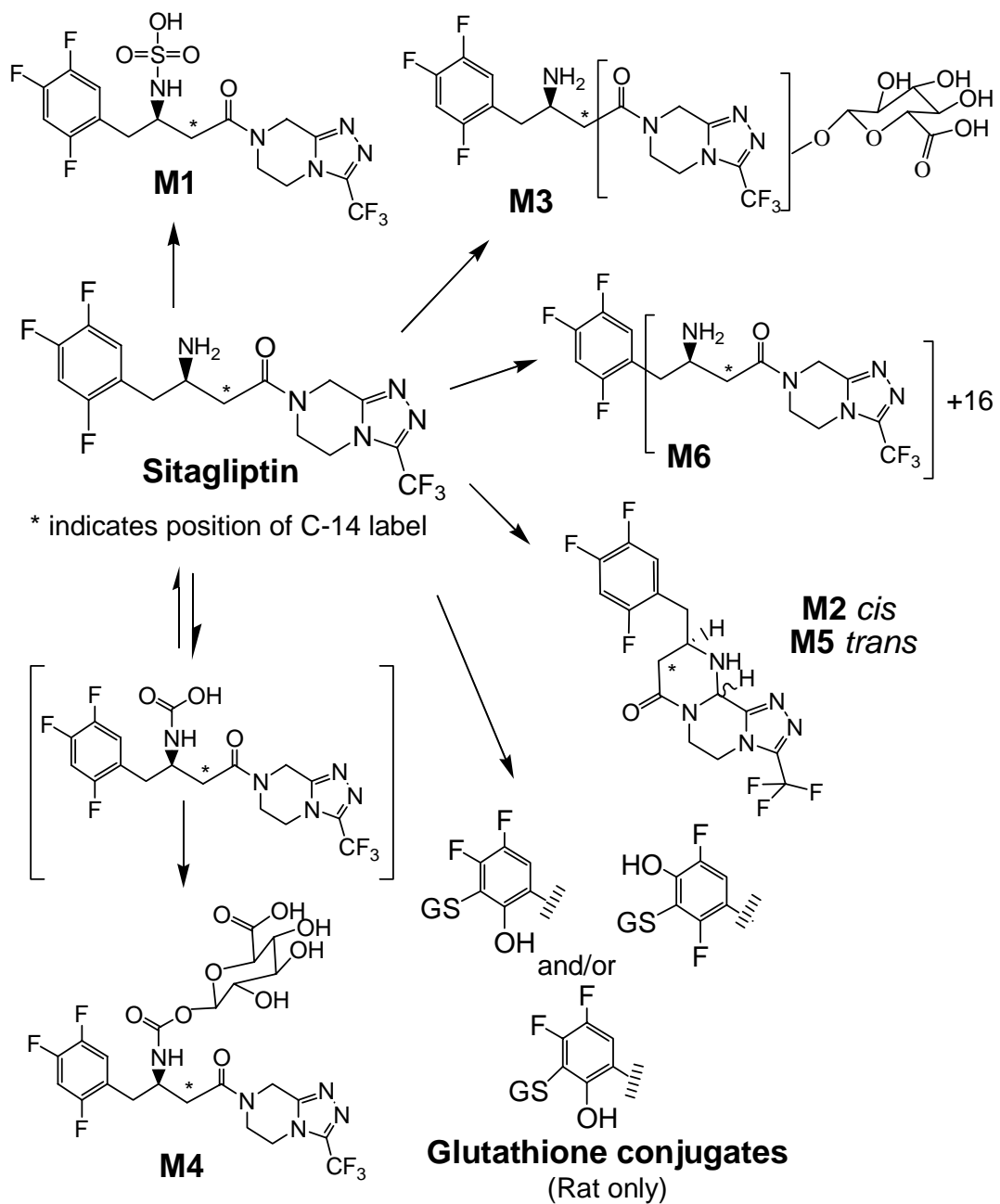


Fig. 2

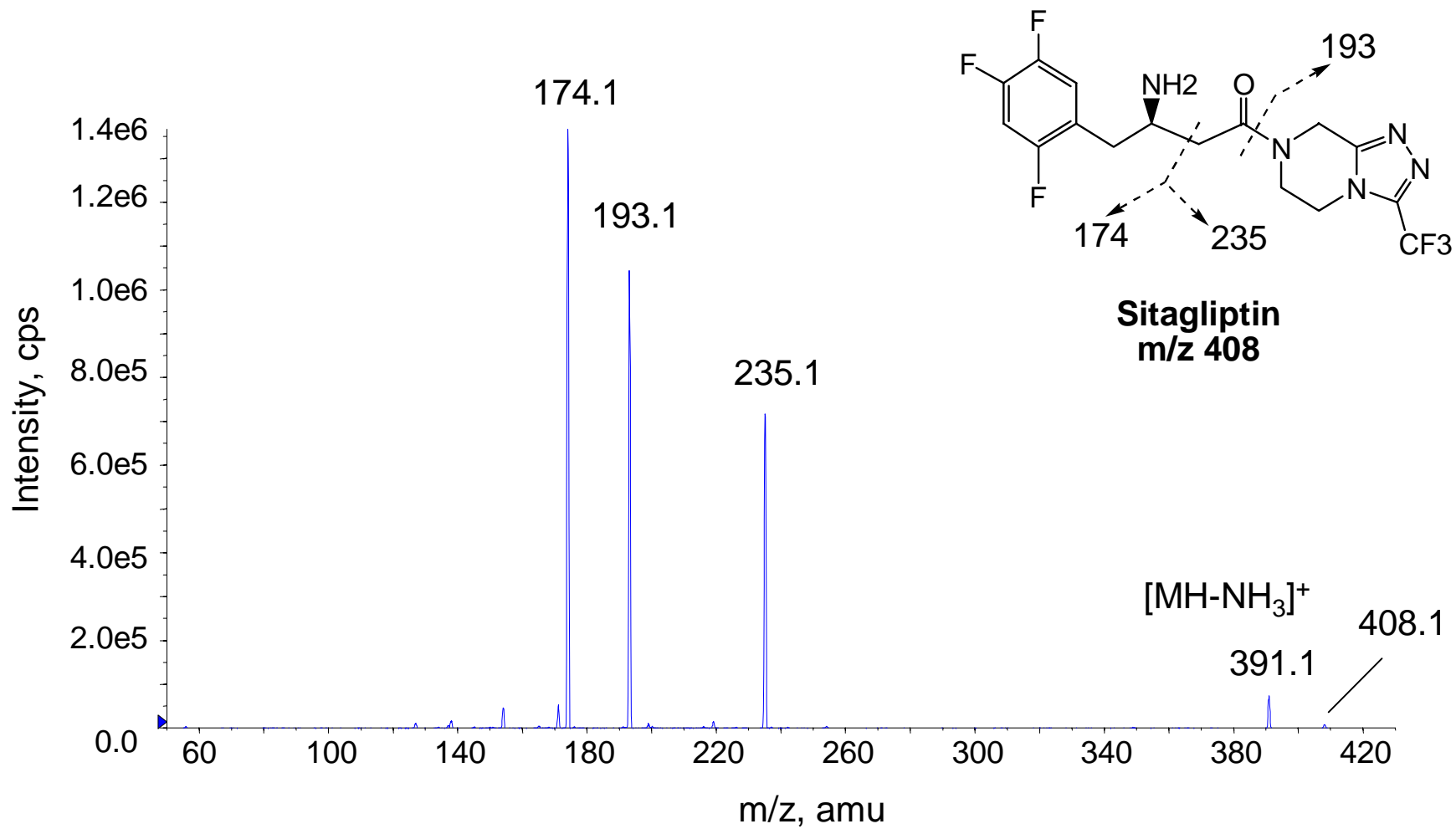


Fig. 3

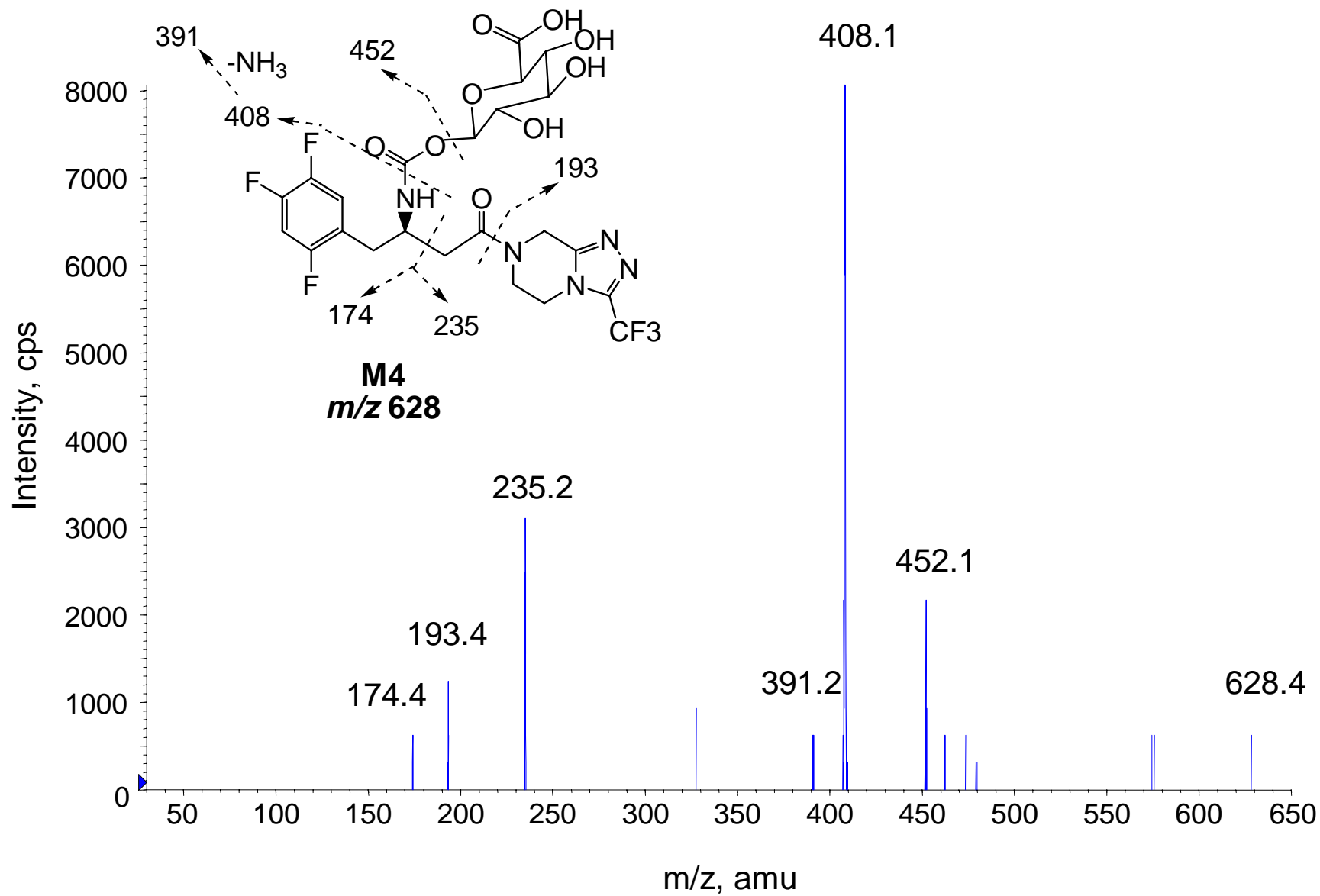


Fig. 4

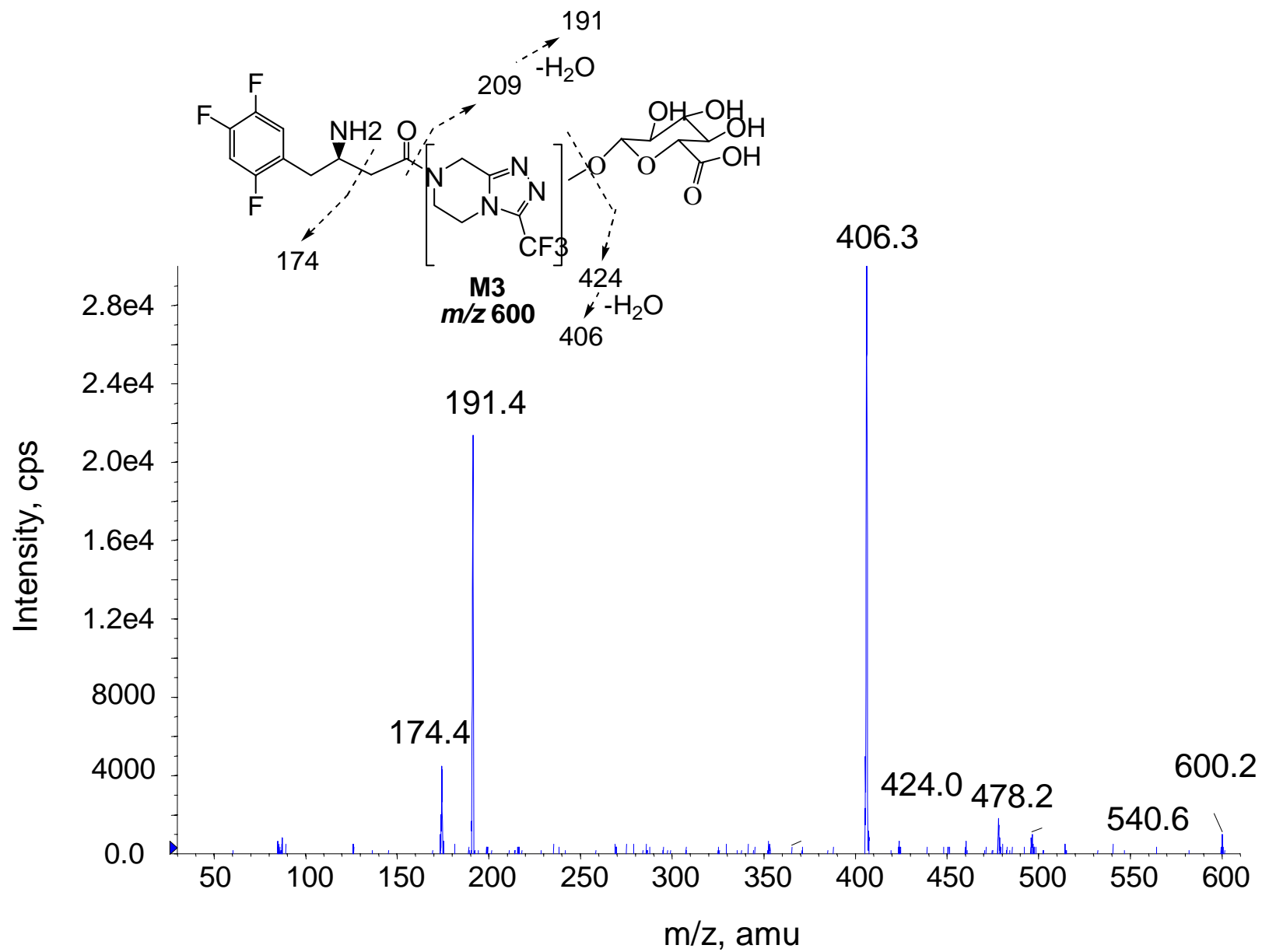


Fig. 6

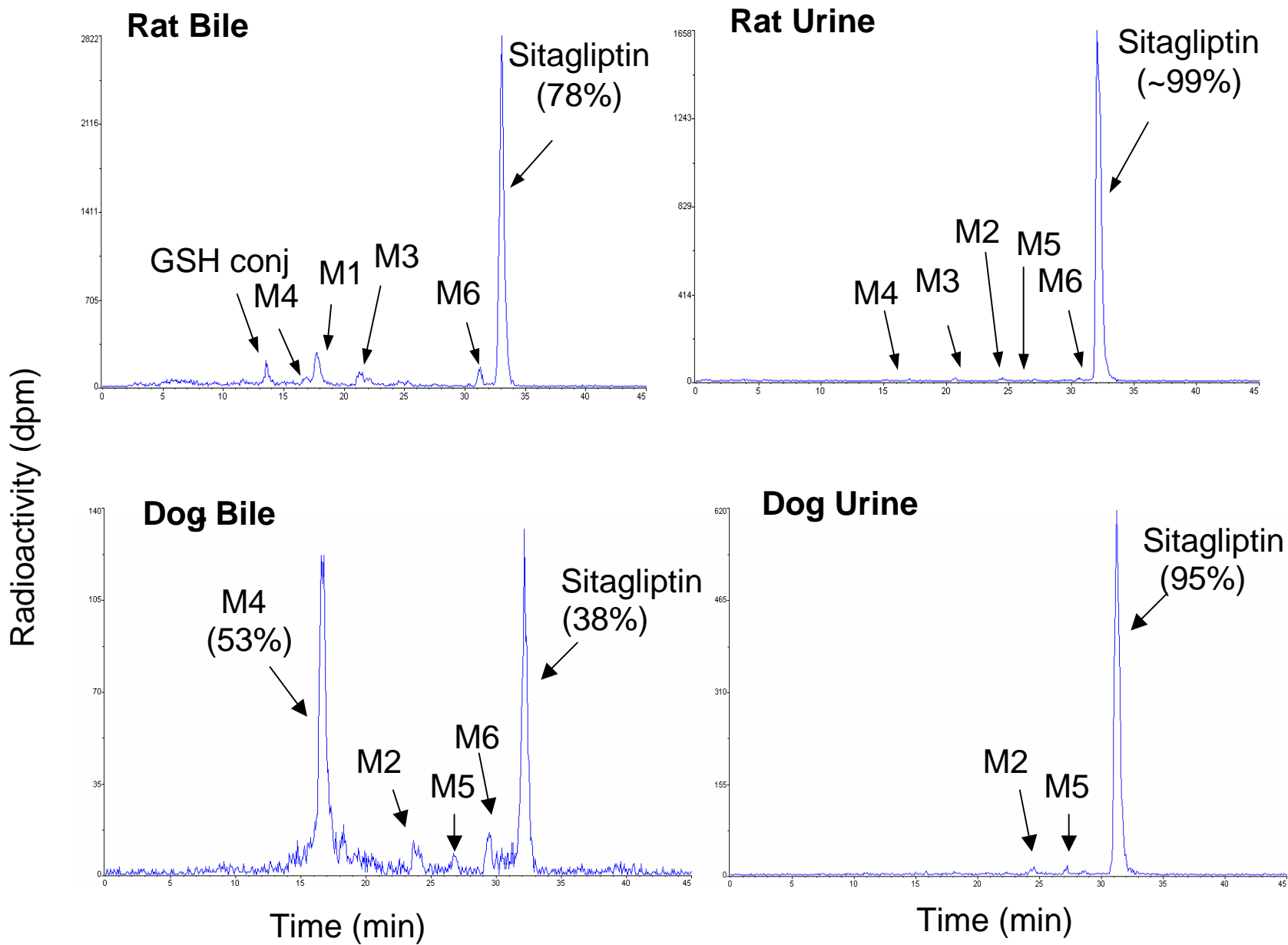


Fig. 7

