Metabolism, Excretion, and Pharmacokinetics of ((3,3-difluoropyrrolidin-1-yl)((2S,4S)-4-(4-(pyrimidin-2-yl)piperazin-1-yl)pyrrolidin-2-yl)methanone, a DPP-IV Inhibitor, in Rat, Dog and Human

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Running Title: in vivo metabolism of a DPP-IV inhibitor

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Abbreviations: DPP-IV, dipeptidyl peptidase IV; GLP-1 glucogen-like peptide 1; PF-00734200, 3,3-Difluoropyrrolidin-1-yl)((2S,4S)-4-(4-(pyrimidin-2-yl)piperazin-1-yl)pyrrolidin-2-yl)methanone, ESI, electrospray ionization; HFAA, hexafluoroacetylacetone; SD, Sprague Dawley; GLP-1, glucagon like peptide; FAP, fibroblast activation protein; APP, aminopeptidase P; POP, propyl oligopeptidase.
ABSTRACT

The disposition of (3,3-difluoropyrrolidin-1-yl)((2S,4S)-4-(4-(pyrimidin-2-yl)piperazin-1-yl)pyrrolidin-2-yl)methanone (PF-00734200), a DPP-IV inhibitor which progressed to Phase 3 for the treatment of type II diabetes, was examined in rats, dogs and humans after oral administration of a single dose of [14C]PF-00734200. Mean recoveries of administered radioactivity were 97.1, 92.2 and 87.2% in rats, dogs and humans, respectively. The majority of radioactive dose was detected in the urine of dogs and humans and in the feces of rats. Absorption of PF-00734200 was rapid in all species, with maximal plasma concentrations of radioactivity achieved within 1 h post dose. Circulating radioactivity was primarily comprised of parent drug (79.9, 80.2, and 94.4% in rat, dog and human respectively). The major route of metabolism was due to hydroxylation at the 5’ position of the pyrimidine ring (M5) in all species. In vitro experiments with recombinant CYP isoforms suggested that the formation of M5 was catalyzed both by CYP2D6 and CYP3A4. Molecular docking simulations showed that the 5’ position of the pyrimidine moiety of PF-00734200 can access to the heme iron-oxo of both CYP3A4 and CYP2D6 in an energetically favored orientation. Other metabolic pathways included amide hydrolysis (M2), N-dealkylation at the piperazine nitrogen (M3) and an unusual metabolite resulting from scission of the pyrimidine ring (M1). Phase II metabolic pathways included: carbamoyl glucuronidation (M9) glucosidation (M15) on the pyrrolidine nitrogen and conjugation with creatinine to from an unusual metabolite/metabonate (M16). The data from these studies suggest that PF-00734200 is eliminated both by metabolism and renal clearance.
INTRODUCTION

The prevalence of type 2 diabetes worldwide is currently 180 million, and is projected to increase to 308 million by 2025 (King et al., 1998). In addition, it has been estimated that in western societies, up to 25% of the population have impaired glucose tolerance, a prediabetic state (Zimmer, 2003). GLP-1 (glucagon like peptide -1) and its analogues have been shown to be effective treatments for diabetic hyperglycemia. GLP-1 not only stimulates insulin secretion in a glucose-dependent manner (Nathan et al., 1992), but it has also been shown to promote pancreatic β-cell growth (Perfetti et al., 2000), to promote satiety (Flint et al., 1998), and to inhibit glucagon secretion (Ritzel et al., 1995), thereby reducing hepatic glucose output (Hvidberg et al., 1994). These multiple benefits can be enhanced by DPP (dipeptidyl peptidase)-IV inhibitors which have documented beneficial long-term effects on glycemia combined with safety and good toleration (Ahren, 2005; Mentlein, 2005; Nielsen, 2005). The DPP-IV inhibitors which harness the glucose-dependent effects of endogenous GLP-1, therefore, can minimize hypoglycemic risk. The stimulation of insulin secretion combined with inhibition of glucagon secretion provides for euglycemic control under both fed and fasting conditions. In addition, it is hypothesized that beneficial effects on β-cell growth, proliferation or survival would tend to delay progression of the diabetic disease process.

PF-00734200, (3,3-Difluoropyrrolidin-1-yl)((2S,4S)-4-(4-(pyrimidin-2-yl)piperazin-1-yl)pyrrolidin-2-yl)methanone), is a potent, orally active, selective and competitive inhibitor of DPP-IV, the enzyme mainly responsible for the degradation of the incretin peptides GLP-1 and GIP. PF-00734200 demonstrates greater than 200-fold selectivity over other members of the dipeptidyl peptidase family (DPP-2, DPP-3, DPP-8, and DPP-9) and the related serine proteases,
FAP, APP, and POP, enzymes that possess similar catalytic activities. PF-00734200 demonstrates rapid and reversible inhibition of plasma DPP-IV activity when administered orally to rats, dogs, and monkeys. In various nonclinical models, PF-00734200 stimulates insulin secretion and improves glucose tolerance.

Preclinical pharmacokinetic studies in rats and dogs suggested that PF-00734200 was completely absorbed and readily distributed into extravascular tissues. Preliminary in vitro studies suggested that PF-00734200 was stable in rat, monkey and human liver microsomes and hepatocytes. These findings indicated that PF-00734200 was unlikely to undergo rapid oxidative or phase II conjugative metabolism in vivo in the rat, monkey, or human.

The objectives of the present study were to characterize the metabolism, pharmacokinetics and excretion of \[^{14}C\]PF-00734200 in Sprague Dawley rats, beagle dogs and humans. A single dose of \[^{14}C\]PF-00734200 was administered orally to intact Sprague-Dawley rats (5 mg/kg), beagle dogs (5 mg/kg) and humans (20 mg). Profiling and identification of collected urine, bile, plasma and feces was achieved using LC/MS/MS in combination with radioactivity detection. Where possible, structures of proposed metabolites were elucidated using chemical derivatization techniques and/or confirmed by comparison to synthetic standards. Phenotyping studies to determine the CYP isoform(s) primarily responsible for the formation of M5 metabolite were also conducted.

**MATERIALS AND METHODS**

**General Chemicals.** Commercially obtained chemicals and solvents were of HPLC or analytical grade. Hexaflouroacetylacetone was purchased from VWR International (Chester, PA). A C18
Hydro RP column (4.6 mm x 150 mm, 5µM) was purchased from Phenomenex (Torrance, CA). Ultima Gold, Carbosorb and Permafluor E scintillation cocktails were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). HPLC-grade acetonitrile, methanol, and water, and certified American Chemical Society-grade ammonium acetate and acetic acid were obtained from Fisher Scientific Company (Springfield, NJ). HPLC-grade acetonitrile, water, and certified American Chemical Society-grade ammonium acetate were obtained from Fisher Scientific Company (Springfield, NJ). Recombinant CYP isoforms were purchased from PanVera (Madison, WI). Human liver microsomes were purchased from BD Biosciences (Woburn, MA).

**Radiolabeled Drug and Reference Compounds.** \[^{14}\text{C}]PF-00734200, specific activity 4.68 uCi/mg) was synthesized by the radiochemistry group at Pfizer Global Research and Development (Groton, CT). It had a radiochemical purity of >99%, as determined by HPLC using an in-line radioactivity detector. The location of the label on the pyrimidine ring is illustrated in Figure 1.

**Synthesis of M5 [(3,3-difluoropyrrolidin-1-yl)((2S,4S)-4-(4-(5-hydroxypyrimidin-2-yl)piperazin-1-yl)pyrrolidin-2-yl)methanone].**

M5 was synthesized in three steps starting from 4-(5-bromo-pyrimidin-2-yl)-piperazine-1-carboxylic acid tert-butyl ester (1) (Figure 2).

**Step 1. Preparation of 4-(5-hydroxy-pyrimidin-2-yl)-piperazine-1-carboxylic acid tert-butyl ester (2).** Nitrogen was bubbled through a solution of 4-(5-bromo-pyrimidin-2-yl)-piperazine-1-carboxylic acid tert-butyl ester 1 (1.00 g, 2.91 mmol), bis(pinacolato)diboron (0.81 g, 3.20 mmol), palladium acetate (19 mg, 0.08 mmol) and potassium acetate (0.30 g, 2.91 mmol) in 10 mL dry DMF for about 20 min. The mixture was heated at 85 °C. After 6 h, the mixture was
cooled, diluted with water and extracted with 3x EtOAc. The combined organic layers were sequentially washed 2x water and 1x brine, dried over MgSO₄, filtered and concentrated. The crude material was taken up in 12 mL THF and 10 mL water. Sodium perborate tetrahydrate (1.42 g, 8.41 mmol) was added. After stirring for 16 h, saturated NH₄Cl and EtOAc were added. The aqueous layer was extracted 3x EtOAc. The combined organic layers were washed with brine, dried over MgSO₄, filtered and concentrated. Heptane was added and the solvent was removed to give 0.62 g (76%) of 2 as a light yellow solid. H NMR (400 MHz, CDCl₃) δ 8.31 (s, 2H), 3.85-3.75 (m, 4H), 3.57-3.49 (m, 4H), 1.47 (s, 9H), LC/ESI-MS (m/z 181.1.)

Step 2. Preparation of (2S,4S)-2-(3,3-difluoro-pyrrolidine-1-carbonyl)-4-[4-(5-hydroxy-pyrimidin-2-yl)-piperazin-1-yl]-pyrrolidine-1-carboxylic acid tert-butyl ester (4). A solution of 4-(5-hydroxy-pyrimidin-2-yl)-piperazine-1-carboxylic acid tert-butyl ester 2 (240 mg, 0.86 mmol) in 10 mL dichloromethane was treated with excess trifluoroacetic acid (ca. 2 mL). After 2 h, the mixture was concentrated. Heptane was added and the mixture was concentrated again. The residue was dissolved in 10 mL of 1,2-dichloroethane and treated with (S)-2-(3,3-difluoro-pyrrolidine-1-carbonyl)-4-oxo-pyrrolidine-1-carboxylic acid tert-butyl ester 3 (248 mg, 0.78 mmol), sodium triacetoxyborohydride (226 mg, 1.01 mmol), triethylamine (ca. 1 mL) and acetic acid (47 mg, 0.78 mmol). After 5 days, the mixture was diluted with water and dichloromethane and the pH was adjusted to about pH 10 with 1N NaOH. The aqueous layer was extracted 3 x dichloromethane, dried over MgSO₄, filtered and concentrated. The crude solid was re-crystallized from EtOAc to yield 104 mg of 4. A second crop of 55 mg was also isolated giving an overall yield (two steps) of 38 %. mp 235-236 °C (decomp). H NMR (400 MHz, MeOH-d₄):
δ 8.02 (s, 2H), 4.59-4.39 (m, 1H), 4.19-3.59 (m, 9H), 3.30-3.22 (m, 1H), 2.96-2.83 (m, 1H), 2.71-2.33 (m, 7H), 1.81-1.70 (m, 1H), 1.48 and 1.41 (rotomeric s, 9H). LC/ESI-MS (m/z 483.8.)

Step 3. Preparation of (3,3-difluoro-pyrrolidin-1-yl)-{(2S,4S)-4-[4-(5-hydroxy-pyrimidin-2-yl)-piperazin-1-yl]-pyrrolidin-2-yl}-methanone (M5). (2S,4S)-2-(3,3-difluoro-pyrrolidine-1-carbonyl)-4-[4-(5-hydroxy-pyrimidin-2-yl)-piperazin-1-yl]-pyrrolidine-1-carboxylic acid tert-butyl ester (4) (83.6 mg, 0.17 mmol) in 5 mL methanol was briefly warmed with heat gun to give a clear solution; 4.0 M HCl in dioxane (1 mL) was added. After 1 h at room temperature, the mixture was heated under reflux. After 4 h, the mixture was cooled and concentrated. Azetropic removal of the remaining dioxane was effected by addition of solvent followed by concentration (MeOH (1x), heptanes (2x)). An off-white, hygroscopic solid, 75 mg (95 %), was obtained. The solid was recrystallized from MeOH/EtOAc. mp ~245 °C (decomp). 1H NMR (400 MHz, D2O) δ 8.12 (s, 2H), 4.80-4.69 (m, 1H), 4.66-4.59 (m, 1H), 4.24-4.10 (m, 1H), 4.06-3.50 (m, 9H), 3.51-3.33 (m, 4H), 3.17-3.04 (m, 1H), 2.56-2.31 (m, 2H), 2.26-2.13 (m, 1H). LC/ESI-MS (m/z 383.3)

Reaction Phenotyping

Studies to identify the CYP isoform(s) responsible for formation of M5 were conducted using chemical inhibition and incubation in recombinant CYP isozymes. Inhibition studies were performed with each of the following inhibitors: furafylline (10 μM for 1A2), sulfaphenazole (10 μM for 2C9), quinidine (10 μM for 2D6), (+)N-3-benzylnirvanol (10 μM for 2C19), and ketoconazole (1 μM for 3A4). Incubations (1 mL) were performed in duplicate with NADPH (1.3mM) in 1.5mL plastic eppendorf tubes open to air at 37 °C in a shaking water bath. Samples
were preincubated at 37 °C for 5 minutes prior to the addition of NADPH. Each incubation contained microsomes (2 mg/mL protein), 100mM potassium phosphate buffer pH 7.4, MgCl2 (10mM), and 10μM PF-00734200 and one of the above inhibitors. A control sample was also prepared without inhibitor. Additional controls using marker substrates (each at 10 μM), in the presence and absence of P450 specific inhibitors, were used to confirm inhibition results and included phenacitin (CYP1A2), tolbutamide (CYP2C9), (S)-mephenytoin (CYP2C19), dextromethorphan (CYP2D6) and testosterone (CYP3A4). At 0 and 30 minutes incubations were quenched with an equal volume of ice-cold acetonitrile. Samples were then placed on ice for 15 minutes to allow precipitation of protein and subsequently centrifuged at 1800 g for 5 minutes. An aliquot was removed from each sample and injected onto the HPLC/MS system. Studies with recombinant CYP isoforms were conducted using recombinant CYP1A2, CYP2C9 CYP2C19, CYP2D6, CYP3A4 and CYP3A5 enzymes obtained from Pan Vera (Madison, WI). Incubations (1 mL) were performed in duplicate with NADPH (1.3mM) in 1.5mL plastic eppendorf tubes open to air at 37 °C in a shaking water bath. Samples were pre-incubated at 37°C for 5 minutes prior to the addition of NADPH. Each incubation contained recombinant human CYP (50pmol), 100mM potassium phosphate buffer pH 7.4, MgCl2 (10mM), and PF-00734200 (10mM). Protein content was adjusted to 1.0 mg/mL using control rCYP (Pan Vera). At 0 and 30 minutes incubations were quenched with an equal volume of ice-cold acetonitrile. Samples were then placed on ice for 15 minutes to allow precipitation of protein and subsequently centrifuged @ 1900 g for 5 minutes. An aliquot from each sample was removed, transferred to and HPLC vial and injected onto the HPLC/MS system.
Animals, Dosing, and Sample Collection. Bile duct- and/or jugular vein cannulated SD rats (190–270 g) were purchased from Charles River Laboratories (Stoneridge, NY). Beagle dogs (9.2–10.9 kg) were from an in-house colony. Animals were quarantined for a minimum of 3 days before treatment and maintained on a 12-h light/dark cycle. The animals were housed individually in stainless steel metabolism cages. The animals were fasted overnight before administration of the dose and were fed 6 h after the dose. The animals were provided water ad libitum. All studies were conducted in a research facility accredited by the American Association for the Accreditation of Laboratory Animal Care.

Rat study: A group of SD rats ($n = 3$/gender) was administered a single 5-mg/kg oral dose of $^{14}$C PF-00734200 for the mass balance study. For biliary excretion experiments, another group of two male and two female bile duct-cannulated rats was administered a single 5-mg/kg oral dose of $^{14}$C PF-00734200 in similar fashion. The dose was formulated as a suspension in 0.5% methyl cellulose on the day prior to dose administration. Each rat received an approximate dose of $\sim 60 \mu$Ci of radiolabeled material. Urine and feces were collected from intact animals for 7 days at 0 to 8, 8 to 24, 24 to 48, 48 to 72, 72 to 96, 96 to 120, 120 to 144, and 144 to 168 h intervals after the dose. The first feces sample was collected at 0 to 24 h after the dose. Bile and urine samples were collected from bile duct-cannulated animals at 0-8, 8-24 and 24-48 h intervals after the dose. The volumes of urine and bile samples were recorded and all of the biological samples were stored at $-20^\circ$C until analysis. For determination of pharmacokinetic parameters and identification of circulating metabolites, a third group of jugular vein-cannulated rats ($n = 10$/gender) was given an oral dose of 5 mg/kg $^{14}$C PF-00734200. Blood from two animals /gender was collected at 0.5, 1, 2, 4 and 8 h post dose in heparinized (lithium heparin...
anticoagulant) tubes. The blood samples were centrifuged at 1000 g for 10 min to obtain the plasma. Plasma was transferred to clean tubes and stored at -20°C until analysis.

**Dog study.** Two male and two female beagle dogs (9.2–10.9 kg) were administered a single oral 5-mg/kg dose of [14C]PF-00734200. Each dog received approximately 200 μCi of radiolabeled material. Urine and feces and wash/cage wipes were quantitatively collected for at least 18 h before dosing, then at 0 to 6, 6 to 12 to 24 and over 24-h intervals through 168 h post dose. Blood samples were collected by venipuncture of a jugular or cephalic vein before dosing and at 0, 1, 2, 4, 8, 12, 24 and 48 h postdose. The samples were transferred into tubes containing lithium heparin and placed on wet ice until processing.

**Human study.** Four healthy human volunteers were given a single nominal 20 mg oral dose of [14C]PF-00734200 containing a mean of 94.2 μCi of total radioactivity as a solution in water (100 mL). Blood samples (~15 mL to provide approximately of 7.5 mL of serum for pharmacokinetic analysis) were collected into tubes containing no preservatives, anticoagulant or serum separators on Day 1 at 0 h (predose), and at 0.5, 1, 2, 4, 8, 12, 16, 24, 48, 72, 96, 120, 144 and 168 h following dosing for the pharmacokinetic evaluation of PF-00734200 and total radioactivity. Additional blood samples (~20 mL each) to provide approximately 10 mL of serum) were collected at 1, 4, 8, 12, 16, 24 and 48 h post-dose for the identification of circulating metabolites. Within 30 min after collection, the blood samples were centrifuged at approximately 1700 x g for about 10 min at 4°C in a refrigerated centrifuge and serum was separated from whole blood. Urine was collected into containers surrounded by dry ice at predose (-12-0), 0-8, 8-24, 24-48, 48-72, 72-96, 96-120, 120-144, 144-168, 168-192, 192-216 and 216-240 h post-dose. Feces were collected prior to dosing and as passed, from time of
dosing until 240 h post-dose. The total weight of the urine and feces was recorded after each collection.

**Determination of Radioactivity**

The radioactivity in urine, bile, and plasma was determined by liquid scintillation counting. Triplicate aliquots of urine (0.1 - 0.4 g), bile (0.08 g) and serum (100-300 µL) in triplicate for each sampling time point, were mixed with 10-15 mL of scintillation fluid and analyzed directly by liquid scintillation counting using a LS-6000 or LS-0500 liquid scintillation counter (Beckmann Instruments, Inc. Fullerton, CA). Fecal samples were diluted with water to form an approximate 20% (w/w) fecal homogenate. Feces samples were homogenized in water using a probe-type homogenizer. Triplicate aliquots of fecal homogenates (~0.3 to 0.4 g) were placed into cones and pads, dried for at least 8 h at ambient temperature, combusted, and then analyzed by liquid scintillation counting. Sample combustions were performed in a biological sample oxidizer. The resulting $^{14}$CO$_2$ was trapped in 9 mL of Carbo-Sorb E and mixed with 9 mL of Permafluor E+ scintillation fluid. The combustion efficiency was determined for each combustion session. Three levels of radioactivity, corresponding to low, medium, and high carbon-14 standards, were combusted and then counted along with scintillation fluid spiked with the same amount of radioactivity. The average radioactivity recovered from the oxidized standards was compared with the radioactivity in the non-oxidized standards to determine the combustion efficiency value. Amounts of radioactivity analyzed by oxidation were adjusted for the efficiency of the combustion system. When determining the amount of radioactivity excreted in urine and feces at each time point as a proportion of the amount administered, the net radioactivity in the actual dose was considered to be 100%. The amount of radioactivity in
plasma/serum at each time point was calculated using the specific activity of the dose administered and was expressed as nanogram-equiv of parent drug per milliliter.

**Extraction of Metabolites from Biological Samples**

Pooling of plasma/serum from all species was performed for each individual according to the method of Hamilton (Hamilton et al., 1981), such that each sample was representative of total exposures (AUC) to metabolites relative to each other. Pooled samples were treated with 4 volumes of acetonitrile to precipitate plasma proteins, vortexed and subsequently sonicated (~10 min). The samples were subsequently centrifuged (1850 g) for 5 min at ambient temperature and the supernatants were transferred to a clean 50 mL Falcon polypropylene conical tube. The remaining pellet was extracted with 4 mL of acetonitrile as described above. The supernatants were combined and aliquots (0.1-0.2 mL) were analyzed by liquid scintillation counting. The mean recovery of radioactivity after extraction was ≥90%. The supernatants were concentrated at 37 ºC using the Genevac EZ-2 evaporative centrifuge (Genevac Inc., Valley Cottage, NY). The residues were then reconstituted in 200 µL of starting mobile phase, vortexed and centrifuged (1850 g) for 5 min. Aliquots (25 µL) were injected on the HPLC/MS system. Urine samples from all species were pooled such that the pooled sample represented >90% of total urinary radioactivity. Samples were spun in a centrifuge (1850 g) at ambient temperature to remove particulate materials, and aliquots (25 µL) were injected on the HPLC/MS system. Bile samples were pooled from 0-24 h for all rats, the pooled samples represented ~99% of total radioactivity excreted in bile and aliquots (25 µL) were injected on the HPLC/MS system. Fecal homogenates were combined such that at least 80% of the radioactivity excreted in feces was represented, the homogenates were diluted with acetonitrile (3 mL/g homogenate), vortex-mixed
for 5 min, sonicated for 10 min and centrifuged (1850 g) at ambient temperature for 10 min and the resulting supernatants were separated. The remaining fecal pellets were extracted a second time with acetonitrile (10 mL) and treated as described above. All supernatants were combined and aliquots (100 µL) were counted to determine the extraction recovery. The mean recovery of radioactivity after extraction was >80% in all species. The supernatants were concentrated to dryness at 37 ºC using the Genevac EZ-2 evaporative centrifuge (Genevac Inc., Valley Cottage, NY). The residues were reconstituted in 1.0 mL of starting mobile phase and Aliquots (25 µL) were injected on the HPLC/MS system.

**HPLC.** The HPLC system consisted of an Acela quaternary solvent delivery pump, an Acela autoinjector, a Surveyor PDA Plus photodiode array detector (Thermo Electron Corporation, Waltham, MA and state). Chromatography was performed on a Phenomenex Hydro RP column (4.6 mm x 150 mm, 5µM). The mobile phase composed of 5 mM ammonium formate buffer (pH=3) (solvent A) and acetonitrile (solvent B). The mobile phase was initially composed of solvent A/solvent B (95:5), and held for 5 min. The mobile phase composition was then linearly changed to solvent A/solvent B (85:15), over 25 min, solvent A/solvent B (65:35) over 12 min, solvent A/solvent B over 2 min (20:80), held constant for the next 3 min and then returned to starting conditions and equilibrated over the next 7 min.

**Mass Spectrometry.** Identification of metabolites was performed on a Thermo Orbitrap mass spectrometer operating in positive ion electrospray mode. Xcalibur software version 2.0 was used to control the HPLC/MS system. Full scan data were collected at a 15,000 resolution. Data dependent product ion scans of the two most intense ions found in the full scan were obtained at
Quantitative Assessment of Metabolites. The metabolites in serum/plasma, urine, and feces were quantified by measuring the radioactivity fractions collected from HPLC effluent at 0.25 min intervals using an FC 204 fraction collector (Gilson Inc, Middleton, WI). Fractions were collected in a 96 well microbeta plate and dried using the Genevac EZ-2 evaporative centrifuge (Genevac Inc., Valley Cottage, NY). Prior to counting, 175 µL of Ultima Gold liquid scintillant (PerkinElmer Life and Analytical Sciences, Boston, MA) was added to each well. Radioactivity was then counted using a Trilux 1450 microbeta counter (PerkinElmer Wallac, Gaithersburg, MD). Data were imported into Laura program Version 3.1.1.39 (Lab logic System Ltd, UK) to provide an integrated printout of radioactivity and the percentage of the radiolabeled material as well as peak representation.

Quantitation of PF-00734200 in Human Serum

Plasma concentrations of unchanged PF-00734200 were determined at Alta analytical laboratory (El Dorado Hills, CA). Serum concentrations of PF-00734200 were determined using a characterized HPLC-MS/MS assay method. Serum samples (50 µl) were extracted using a Waters Oasis MCX (10 mg) 96-well solid-phase extraction plate. After washing with 200 µl of MeOH followed by 200 µl of 0.1 N HCl, loaded samples were eluted with 200 µl of methanol-ammonium hydroxide (95:5; v/v). The solutions were dried and reconstituted in 150 µl of HPLC mobile phase, and 100 µl of reconstitutes were injected onto a Luna C18 column (3 mm, 50 × 4.6
mm; Phenomenex, Torrance, CA) with a binary mixture of 10 mM ammonium acetate, pH 6.8 (solvent A) and acetonitrile (solvent B). The flow rate was 0.5 ml/min, and the separation was achieved at ambient temperature. A gradient program consisting of 20% B from 0 to 0.7 min, 20 to 90% B from 0.7 to 2 min, 90% B from 2 to 4 min, and 90 to 20% B from 4 to 4.2 min was used for the analysis. A Sciex 4000 mass spectrometer was used in multiple reaction monitoring mode. PF-00734200 and internal standard MS/MS ions were monitored at transitions of \( m/z \) 367 → 122 and 375 → 173, respectively. The dynamic range of the assay was 0.1 to 100 ng/ml.

**Pharmacokinetic Analysis.** Pharmacokinetic parameters were determined using WinNonlin (Ver. 3.2). Maximum observed concentrations (\( C_{\text{max}} \)) of PF-00734200 or total radioactivity (parent drug equivalents) in serum/plasma were estimated directly from the experimental data, with \( t_{\text{max}} \) defined as the time of first occurrence of \( C_{\text{max}} \). Terminal phase rate constants (\( k_{el} \)) were estimated using least squares regression analysis of the serum concentration-time data obtained during the terminal log-linear phase. Half-life (\( t_{1/2} \)) was calculated as 0.693/\( k_{el} \). Area under the serum concentration-time curve from time 0 to the last time (\( t \)) with a measurable concentration (AUC(0-\( t \))) was estimated using linear trapezoidal rule. AUC from time \( t \) to infinity (AUC(\( t-\infty \))) was estimated as \( C_{\text{est}}/k_{el} \) where \( C_{\text{est}} \) represents the estimated concentration at time \( t \) based on the aforementioned regression analysis. AUC from time 0 to infinity (AUC(0-\( \infty \))) was estimated as the sum of (AUC(0-\( t \))) and (AUC(\( t-\infty \))) values.

**Derivatization of M1.** Aliquots (100 μL) of rat bile were separated using a Phenomenex Hydro RP column (4.6 mm x 150 mm, 5μM) and fractions corresponding to M1 (\( m/z \) 331, \( R_t \) = 3.0 min) were collected. The fractions were evaporated to dryness using a Genevac EZ-2 evaporative...
centrifuge (Genevac Inc., Valley Cottage, NY). To one fraction, 100 μL of D2O was added, vortexted and allowed to sit at ambient temperature for 30 min. The sample was then loaded directly on to a syringe for flow infusion analysis using full and daughter ion scan acquisitions of M1 on an Orbitrap mass spectrometer. To a second fraction, 50 μL ethyl acetate and 50 μL of hexaflouroacetylacetone (HFAA) was added, vortexed mixed then heated at 80°C for 3 h. of 1N HCl(5 μL) was then added and the mixture was subsequently heated for another hour in order to promote elimination of water. The solution was then evaporated to dryness and reconstituted in 100 μL of (95:5; v/v) water:acetonitrile and an aliquot (25 μL) was injected on the LC/MS system.

**Molecular Docking studies with PF-00734200 with CYP2D6 and 3A4**

The chemical structure of PF-00734200 was first generated by ChemBio3D (CambridgeSoft Corporation, Cambridge, MA) with geometries optimized using the molecular mechanics method MMFF94. The derived configuration was further structurally optimized using a quantum mechanics-based spin-unrestricted B3LYP (Becke three-parameter Lee-Yang-Parr) density functional theory (DFT) method in Gaussian 09 (Gaussian, Inc., Wallingford, CT). A split valence basis set, 6-31G**, in which polarization functions were added on both heavy atoms and hydrogens, was implemented as previously described (Miao et al., 2012; Sun, 2012; Sun et al., 2012). The energetically minimized structure of PF-00734200 by DFT was then modified by AutoDockTools (The Scripps Research Institute, La Jolla, CA) with Gasteiger atomic charges assigned and flexible torsions defined as the docking input file.

The protein template of CYP2D6 was based on the X-ray crystal structure 3QM4, a prinomastat co-crystal structure of CYP2D6, recently determined by Dr. Eric F. Johnson.
laboratory in the Scripps Research Institute, in collaboration with Pfizer. The active site of 3QM4 differs significantly from the original CYP2D6 structure, 2F9Q, which lacks substrate or inhibitor bound (Wang et al., 2012). This new structure has been extensively evaluated with various CYP2D6 substrates and inhibitors within Pfizer’s drug discovery portfolio. The results demonstrated that 3QM4 can interpret the observed experimental results much better than 2F9Q. Thus, 3QM4 has been applied for structure-based drug design with more accuracy for prediction prospectively. This template was further modified by adding polar hydrogen atoms, Kollman partial atomic charges, and solvation parameters with AutoDockTools, and the substrate binding space within CYP2D6, a 50 Å × 50 Å × 50 Å cubic region covering the active site cavity above the heme porphyrin was defined by the AutoGrid 4.0 (The Scripps Research Institute), which was also used to calculate the grids of van der Waals, hydrogen bonding, electrostatic, torsional, and solvation interaction grids for each ligand atoms.

The protein template of CYP3A4 was re-calculated based on a series of the X-ray crystal structures of CYP3A4 (1TQN, 1W0E, 1W0F, 1W0G, 2J0D, 2V0M, 3NXU, and several others in Pfizer’s Protein Structure Database), as previously described (Sun and Scott, 2010; Sun and Scott, 2011; Sun et al., 2012). Likewise, the selected template of CYP3A4 was further prepared with AutoDockTools, the same procedures as defined for CYP2D6. A 60 Å × 80 Å × 80 Å cubic region was chosen for substrate binding with the grid maps prepared by AutoGrid4.0. Docking was then accomplished on Pfizer’s high performance computing Linux clusters using a Lamarckian genetic algorithm in AutoDock 4.0 (The Scripps Research Institute), which searches the globally energetically optimized conformations and orientations of PF-00734200. A total of 50 million evaluations were performed for each output binding pose, followed by clustering 200 poses by a root-mean-square deviation (RMSD) method. The lowest energy pose clusters that
were also in the proximity of the heme iron were chosen for analysis and visualized by PyMOL (Schrödinger, LLC).

RESULTS:

$^{14}$C Excretion.

**Rats.** After a single oral dose of $[^{14}C]$PF-00734200 to SD rats, an overall mean of 97.1% of the administered radioactivity was recovered in the urine, feces and cage wash over a period of 168 h post-dose (Table 1). The mean cumulative dose recovered in feces was 66.0%. The mean cumulative excretion in the urine was 30.8% (Table 1). Approximately 95% of the excreted radioactivity recovery occurred in the first 48 h. Mean total recoveries of dosed radioactivity from bile duct-cannulated rats were 29.5% in urine, and 62.0% in bile. No gender related differences were observed in the excretion pattern of radioactivity.

**Dogs.** After a single oral dose of $[^{14}C]$PF-00734200 to Beagle dogs, an overall mean of 92.2% of the total dose was recovered in the urine, feces and cage wash through 168 h post-dose (Table 1). The mean cumulative dose recovered in the feces and urine was 16.8 and 66.7%, respectively (Table 1). Approximately 86% of the excreted radioactivity was recovered in the first 48 h. No gender related differences were observed in the excretion pattern of radioactivity.

**Humans.** After a single oral dose of $[^{14}C]$PF-00734200 to human volunteers, an overall mean of 87.2% of the total dose was recovered in the urine and feces (Table 1). The mean cumulative
dose recovered in feces was 10.4%. The mean cumulative excretion in urine was 76.8%. Approximately 74% of the excreted radioactivity recovery occurred the first 48 h.

**Pharmacokinetics of PF-00734200 and Total radioactivity in Humans**

The calculated pharmacokinetic parameters for total radioactivity and PF-00734200 are shown in tables 2 and 3, respectively. Mean serum concentration-time curves for PF-00734200 and total radioactivity are shown in figure 3. Serum concentrations for both PF-00734200 and total radioactivity peaked within 1 h after oral administration. The $C_{\text{max}}$ values for the parent compound ranged from 215 to 273 ng/mL with a mean value of 241 ng/mL. The $C_{\text{max}}$ values for the total radioactivity ranged from 245 to 311 ng-equiv/mL with a mean value of 274 ng equiv/mL. AUC($0-\infty$) values for the parent compound ranged from 2070 to 3280 ng·h/mL with a mean value of 2660 ng·h/mL. AUC($0-\infty$) values for total radioactivity ranged from 2310 to 3430 ng-equiv·h/mL with a mean value of 2850 ng-equiv·h/mL. The mean terminal phase half-life ($t_{1/2}$) for parent compound was 63.5 h. The terminal phase half-life ($t_{1/2}$) for total radioactivity could not be determined since its plasma concentrations could not be determined beyond 24 h.

**Metabolic Profiles.**

**Rat urine.** A representative HPLC-radiochromatogram of urinary metabolites from an intact rat is shown in Figure 4 (panel A). The metabolites were quantified by integration of the radiochromatographic peaks. The mean percentages of urinary metabolites detected in the urine of male and female rats, expressed as percentage of administered dose, are shown in Table 4. A total of 8 metabolites were detected in the urine of male and female rats. Unchanged parent
accounted for 11% of the dose. The major metabolites in urine were M1, M2, M4 and M5 which accounted for 3.1, 3.2, 2.9 and 7.3% of the total administered dose, respectively.

**Rat Feces.** A representative HPLC-radiochromatogram of fecal metabolites from one intact rat is shown in Figure 4 (panel B). The mean percentages of fecal metabolites detected in male and female rats, expressed as percentages of administered dose, are shown in Table 4. A total of 5 metabolites were detected in the feces of male and female rats. Unchanged parent accounted for only 1.1% of the dose. The major metabolites in feces were M1 and M5 which accounted for 17.8 and 32.3% of the total administered dose, respectively.

**Rat Plasma.** A representative HPLC radiochromatogram of circulating metabolites from one rat is shown in Figure 4 (panel C). Mean percentages of total circulating radioactivity are shown in Table 5. A total of 5 metabolites were detected in the circulation of both male and female rats. The majority of circulating radioactivity was attributed to unchanged parent drug (79.9%) followed by M2 (8.4%). The remaining metabolites each represented less than <5% of total circulating radioactivity.

**Rat Bile.** A representative HPLC-radiochromatogram of biliary metabolites from one bile duct cannulated rat is shown in Figure 4 (panel D). No unchanged parent drug was detected in the bile of rat and female rats. The major metabolites detected in bile were M1 and M4, representing 7.3 and 50.4% of the total administered dose in BDC rats, respectively. All remaining biliary metabolites were each present at <2% of the total administered dose.
**Dog urine.** A representative HPLC-radiochromatogram of urinary metabolites from one dog is shown in Figure 5 (panel A). The metabolites were quantified by integration of the radiochromatographic peaks. The mean percentages of urinary metabolites detected in the urine of male and female dogs, expressed as percentages of administered dose, are shown Table 4. A total of 9 metabolites were detected in the urine of male and female dogs. Unchanged parent drug accounted for 25.6% of the dose. M5 represented the major metabolite at 30.1% of the dose. The remaining metabolites each represented < 3% of the dose.

**Dog feces.** A representative HPLC-radiochromatogram of fecal metabolites from one dog is shown in Figure 5 (panel B). The mean percentages of fecal metabolites detected in male and female rats, expressed as percentages of administered dose, are shown in Table 4. A total of 2 metabolites were detected in the feces of male and female dogs. Unchanged drug accounted for 1.8% of the total administered dose, while M1 and M5 represented 4.6 and 9.0 % of the total administered dose, respectively.

**Dog plasma.** A representative HPLC radiochromatogram of circulating metabolites from one dog is shown in Figure 5 (panel C). A total of 7 metabolites were detected. Mean percentages of total circulating radioactivity are shown in Table 5. The majority of circulating radioactivity was attributed to unchanged parent drug (80.1%) followed by M5 (13.7%). The remaining metabolites represented each less than 2% of the total circulating radioactivity.

**Human urine.** A representative HPLC-radiochromatogram of urinary metabolites from male humans is shown in Figure 6. (panel A). The metabolites were quantified by integration of the
radiochromatographic peaks. The mean percentages of metabolite detected in human urine, expressed as percentages of total administered dose, are shown Table 4. A total of 8 metabolites were detected the urine of humans. Unchanged parent drug accounted for 48.5% of the dose while M5 represented the major metabolite at 17.9% of the dose. Metabolites M9, M16 and M18 which could not be chromatographically resolved from each other, collectively represented 6.0% of the radioactive dose. The remaining metabolites each represented less than 2% of the total administered dose.

**Human feces.** A representative HPLC-radiochromatogram of fecal metabolites from male human subjects is shown in Figure 6 (panel B). The mean percentages of fecal metabolites detected in the feces expressed as percentages of total administered dose are shown in Table 5. A total of 3 metabolites were detected, each representing <3% of the total administered dose. Unchanged parent drug accounted for <1% of the dose.

**Human serum.** A representative HPLC radiochromatogram of circulating metabolites in human serum is shown in Figure 6 (panel C). Unchanged PF-00734200 and a total of 2 metabolites were detected in circulation. Mean percentages of total circulating radioactivity are shown in Table 5. The majority of circulating radioactivity was attributed to unchanged parent drug (94.4%) followed by M5 (4.0%) and M9 (1.4%).

**Reaction phenotyping of M5 formation.** Incubation of PF-00734200 in HLM in the presence of selective chemical inhibitors as well as with individual CYP isoforms, revealed that both CYP2D6 and CYP3A4 were responsible for the formation of M5 (data not shown).
Molecular Docking of PF-00734200 with CYP2D6 and 3A4

The energetically favored binding poses of PF-00734200 at the active site of CYP2D6, which is lined with the helix I, B-C loop, K-β loop and helix F above the heme porphyrin, indicate that either end of the molecule can access the heme iron. It binds in a similar position to that found for the prinomastat molecule of the CYP2D6 co-crystal structural template. It appears that specific electrostatic interactions between the middle pyrolidine and E216 of the F-G loop (2.8 Å from the nitrogen atom to one of the carboxylic acid oxygen atoms), together with the hydrophobic π stacking interactions between the terminal pyrimidine ring and F120 of the B-C loop (3.1 Å between two aromatic rings), juxtapose the pyrimidine ring in the proximity of the heme of CYP2D6 (Figure 7A). Indeed, the 5-position carbon atom of the pyrimidine ring is the closest toward the heme iron (4.4 Å), followed by the 4-position carbon atom (4.7 Å). However, the rate of aromatic hydroxylation at the pyrimidine ring would be predicted to be low mainly due to the relative electron deficiency on the ring, as well as the unfavored binding dihedral angle from the plane of the 5-position carbon and hydrogen atoms to that of the heme iron and porphyrin, for the formation of the tetrahedral intermediates prior to aromatic hydroxylation reactions. Another important active site residue, D301, located at the helix I, which in general plays an important role for CYP2D6-catalyzed metabolism of various compounds as internally evaluated, however, is far away from PF-00734200 (the closest distance, from the carboxylic acid to the piperazine ring, is over 6.0 Å).

It is interesting to note that CYP2D6 substrates may interact with either E216 or D301 for electrostatic interactions, or both, which correlates with an empirical “rule” or
pharmacophore of CYP2D6 substrates, that is, approximately 5-7 Å in space between the site of metabolism and the basic center of CYP2D6 substrates. For example, the binding of debrisoquine to CYP2D6, from our evaluation by the same approach, demonstrated that the electrostatic interactions between D301 and the guanidine moiety (3-4 Å in distance) plays a more important role than E216 for the 4-hydroxylation reaction (the distance between the methylene carbon and the heme iron is 3.4 Å). On the other hand, the binding of dextromethorphan showed that E216 plays an essential role instead via electrostatic interactions with the tertiary amine moiety (3.5 Å in distance) for the O-demethylation reaction to form dextrorphan (the distance between the carbon atom of the terminal methyl group and the heme iron is 4.9 Å). In addition, the other binding pose of PF-00734200 (Figure 7B) showed that its terminal difluoropyrrolidine is the closest moiety toward the heme iron (4.7 Å). Due to the steric hindrance and electronic withdrawing effects of two fluorine atoms, no metabolism such as the aliphatic hydroxylation, is expected to happen. It is noticeable that several electrostatic interactions exist for this binding pose including those from either the nitrogen atom of the middle pyrolidine moiety (4.1 Å) or piperazine ring (3.8 Å) to the side chain of D301, and from the nitrogen atom of the piperazine ring (4.7 Å) to the carboxylic acid group of E216. This binding pose is considered as a metabolism-inactive orientation, or it may account for the very weak CYP2D6 inhibition by PF-00734200.

Like CYP2D6, the binding of PF-00734200 to CYP3A4 told a similar story. In general, PF-00734200 binds at the active site of CYP3A4 confined by the helix I, B-C loop, K-β loop and F-G loop, with either end of the molecule accessible to the heme iron. Likewise, the low rate of metabolism of the 5-position pyrimidine is due to the electron deficiency of the ring system. The lack of metabolism at the other end is due to the steric hindrance and electronic
withdrawing effect of two substituted fluorine atoms. The molecule nestles over the heme porphyrin due to several specific electrostatic interactions, as described below, and many other van der Waals interactions. For the pyrimidine-heme binding pose (Figure 7C), the distance between the 5-position carbon atom of the pyrimidine ring and the heme iron, between the piperazine nitrogen atom (pyrolidine side) and carbonyl oxygen atom of I369 backbone, between the nitrogen atom of the terminal difluoropyrrolidine moiety and the carboxylic acid side chain of E374, between the nitrogen atom of the terminal difluoropyrrolidine moiety and the backbone carbonyl group of G481, are measured as 3.8, 4.7, 5.9 and 4.0 Å, respectively. For the other binding pose (Figure 7D), the distance to the corresponding protein atoms/residues, in the order above, is 2.0 Å for the fluorine atom, 3.0 Å for the piperazine ring, 4.5 Å and 4.8 Å for the piperidine nitrogen atoms, respectively.

Identification of Metabolites. The structures of metabolites were elucidated by electro spray LC/MS/MS using combination of Q1 and CID product ion (MS²/MS³) scanning techniques.

Parent (Unchanged Drug)
PF-00734200 had a retention time of ~31.0 min on HPLC and a protonated molecular ion at m/z 367. Its MS² product ion spectrum showed diagnostic fragment ions at m/z 246, 206, 165, 148, 150 and 122 (Figure 8, Panel A). The ions at m/z 246 and 122 formed via cleavage across the piperazine ring with charge retention on both the fragments. Assignments for diagnostic fragment ions are as indicated in Table 6.
Metabolite M1

Metabolite M1 was detected in human, dog and rat feces, rat and dog urine, rat and dog plasma and rat bile and had a retention time of ~3.0 min on HPLC and a protonated molecular ion at \( m/z \) 331, 36 Da lower than that of the PF-00734200. Its MS\(^2\) product ion spectrum showed diagnostic fragment ions at \( m/z \) 314, 289, 272, 246, 229, and 181 (Figure 9, Panel A). The ion at \( m/z \) 314 indicated the loss of an ammonia molecule while the ions at \( m/z \) 246, 272 and 289 all suggested that the (difluoropyrrolidin-1-yl)(-4-(piperazin-1-ylpyrrolidin-2-yl)methanone moiety was intact and that the loss of the 36 Da had occurred from a metabolically modified pyrimidine ring. Treatment of isolated M1 with hexafluoroacetylacetone (HFAA) led to disappearance of the HPLC peak corresponding to M1 (Rt =3.0 min) and appearance of a new peak at 32.9 min corresponding to a \( m/z \) value of 503.1800, 172 amu greater than the underivatized compound, indicating addition of a HFAA moiety and subsequent loss of two molecules of water to form the cyclized bis(trifluoromethyl)pyrimidine derivative. Its MS\(^2\) product ion spectrum showed diagnostic fragment ions at \( m/z \) 368, 342, 325, 301, 258 and 203 (Figure 10, Panel B). The ions at \( m/z \) 301 and 258, 136 da higher than those of parent compound, suggest the addition of hexafluoroacetylacetone to the modified pyrimidine ring. In addition, solution phase H/D exchange of M1 isolated from rat bile using D\(_2\)O, showed a shift of the protonated molecular ion from \( m/z \) 331.2054 to \( m/z \) 336.2370, suggesting the presence 4 exchangeable hydrogen atoms on M1, one from the pyrrolidine nitrogen and the other three from the ‘exposed’ guanidine nitrogens of the proposed ring opened structure (Figure 10, Panel B). M1 was tentatively identified as 4-(3S,5S)-5-(3,3-difluoropyrrolidine-1-carbonyl)pyrrolidin-3-yl)piperazine-1-carboxamidine.
Metabolite M2
Metabolite M2 was observed in human, dog and rat urine, human and rat feces, and rat and dog plasma. It had a retention time of 5.6-6.3 min on HPLC and a protonated molecular ion at m/z 278, 89 Da lower than the parent compound. Its MS\(^2\) product ion spectrum showed diagnostic fragment ions at m/z 260, 206, 165, 150, and 122 (Table 6). The ions at m/z 206, 165, 150, and 122, similar to those of the parent compound, suggested that the 2-(piperazin-1-yl)pyrimidine moiety was unchanged and the modification was due to loss of the 3,3-difluoropyrrolidine moiety of PF-00734200 by amide hydrolysis. Based on these data, M2 was tentatively identified as 4-(4-(pyrimidin-2-yl)piperazin-1-yl)pyrrolidine-2-carboxylic acid.

Metabolite M3
Metabolite M3 was observed in rat and dog urine as well as in rat and dog plasma. It had a retention time of \(\sim\) 6.5 min on HPLC and a protonated molecular ion at m/z 165, 202 amu lower than parent drug. Its MS\(^2\) product ion spectrum showed a diagnostic fragment ion at m/z 122 (Table 6). Based on these data, M3 was tentatively identified as an N-dealkylation product of PF-00734200; 2-(piperazin-1-yl)pyrimidine.

Metabolite M4
Metabolite M4 was observed in rat and dog urine and plasma, and rat bile. It had a retention time of \(\sim\) 14.3-14.8 min on HPLC and a protonated molecular ion at m/z 559, 192 amu greater than parent drug. Its MS\(^2\) product ion spectrum showed diagnostic fragment ions at m/z 383, 357, and 181 (Table 6). The ions at m/z 383, and 181, 16 amu greater than those of the parent compound, suggesting that an oxidation had occurred on the 2-(piperazin-1-yl)pyrimidine moiety.
of PF-00734200. The ions at \( m/z \) 559 and 357, which were 176 amu greater than the fragment ions 383 and 181 respectively, suggested that glucuronidation had occurred on the 2-(piperazin-1-yl)pyrimidine moiety. Based on these data, M4 was tentatively identified as the \( O \)-glucuronide conjugate of the hydroxylated metabolite.

**Metabolite M5**

Metabolite M5 represented the major metabolic pathway in all species and was present in the urine, feces and plasma of rats, dogs and humans. It had a retention time of 25.5-26.3 min on HPLC and a protonated molecular ion at \( m/z \) 383, 16 Da higher than parent compound. Its MS\(^2\) product ion spectrum showed diagnostic fragments at \( m/z \) 246, 222, 181, 166, and 138 (Figure 7, Panel B). The product ions at \( m/z \) 222, 181, 166, and 138, were 16 Da higher than respective product ions at \( m/z \) 206, 165, 150, and 122 for PF-00734200. This suggested that oxidation had occurred on the 2-(piperazin-1-yl) pyrimidine moiety. The structure M5 was further confirmed by comparison of its retention time and CID product ion spectrum with those of a synthetic standard (Figure 2). Based on these data M5 was identified as (3,3-difluoropyrrolidin-1-yl)((2S,4S)-4-(4-(5-hydroxypyrimidin-2-yl)piperazin-1-yl)pyrrolidin-2-yl)methanone.

**Metabolite M6**

Metabolite M6 was observed in rat bile as well as dog urine and plasma. It had a retention time of 24.5-24.8 min on HPLC and a protonated molecular ion at \( m/z \) 463, 96 amu greater than parent drug. Its MS\(^2\) product ion spectrum showed a loss of 80 amu to a diagnostic fragment at \( m/z \) 383 which was similar to M5 (Table 6). This suggested that M5 was further metabolized to a sulfate conjugate. Based on these data, M6 was tentatively identified as the sulfate conjugate of
M5; 2-(4-((3S,5S)-5-(3,3-difluoropyrrolidine-1-carbonyl)pyrrolidin-3-yl)piperazin-1-yl)pyrimidin-5-yl hydrogen sulfate.

**Metabolite M7**

Metabolite M7 was observed in dog urine and plasma. It had a retention time of ≈24.5-24.8 min on HPLC and a protonated molecular ion at $m/z$ 603, 236 amu greater than parent drug. Its MS$^2$ product ion spectrum showed diagnostic fragments at $m/z$ 427, and 383 (Table 6). The fragment ion at $m/z$ 427, 44 amu greater than the M5, suggested an addition of CO$_2$ to M5. Fragment ion at $m/z$ 427, which is also 176 amu less than the molecular ion, suggested M7 was a glucuronide. Based on these data, M7 was tentatively identified as N-carbamoyl glucuronide conjugate of M5.

The fragment ion at $m/z$ 427, 44 amu greater than the M5, suggested an addition of CO$_2$ to M5. Fragment ion at $m/z$ 427, which is also 176 amu less than the molecular ion, suggested M7 was a glucuronide. Based on these data, M7 was tentatively identified as N-carbamoyl glucuronide conjugate of M5.

**Metabolite M8**

Metabolite M8 was a minor metabolite observed in dog urine. It had a retention time of ≈26 min on HPLC and a protonated molecular ion at $m/z$ 426, 59 amu greater than parent drug. Its MS$^2$ product ion spectrum showed diagnostic fragments at $m/z$ 409, 383, 201, 181, and 138 (Table 6). The fragment ion at $m/z$ 409 suggested the loss of ammonia from M8. Based on these data, M8 was tentatively identified as an N-formamide conjugate of M5 [(2S,4S)-2-(3,3-difluoropyrrolidine-1-carbonyl)-4-(4-(5-hydroxypyrimidin-2-yl)piperazin-1-yl)pyrrolidin-1-carboxamide].
Metabolite M9

Metabolite M9 was observed in human and dog urine as well as human and dog plasma. It had a retention time of ~28.2 min on HPLC and a protonated molecular ion at \( m/z \) 587, 220 Da higher than the parent compound. Its MS\(^2\) product ion spectrum showed diagnostic fragments at \( m/z \) 411, and 367 (Table 6). Fragment ion at \( m/z \) 411, 44 Da higher than the parent compound, suggested the addition of carbon dioxide to PF-00734200. The fragment ion at \( m/z \) 411, represented a loss of 176 Da, from the molecular ion, further suggesting that M9 was a glucuronide conjugate. Based on these data, M9 was tentatively identified as the \( N \)-carbamoyl glucuronide conjugate of PF-00734200. 6-(((2S,4S)-2-(3,3-difluoropyrrolidine-1-carbonyl)-4-(4-(pyrimidin-2-yl)piperazin-1-yl)pyrrolidine-1-carbonyl)oxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid.

Metabolite M10

Metabolite M10 was a minor metabolite observed rat feces. It had a retention time of ~20.1 min on HPLC and a protonated molecular ion at \( m/z \) 359, 8 Da lower than the parent compound. Its MS\(^2\) product ion spectrum showed a diagnostic fragment at \( m/z \) 331 (Table 6) suggesting a loss of carbon monoxide from M1. M10 was tentatively identified as the \( N \)-formyl conjugate of M1. 4-((3S,5S)-5-(3,3-difluoropyrrolidine-1-carbonyl)-1-formylpyrrolidin-3-yl)piperazine-1-carboximidamide.
Metabolite M11

Metabolite M11 was observed in rat feces. It had a retention time of ~27.4 min on HPLC and a protonated molecular ion at $m/z$ 411, 44 amu greater than parent drug. Its MS$^2$ product ion spectrum showed diagnostic fragments at $m/z$ 383, 276, 274, 181, and 138 (Table 6). Fragment ions at $m/z$ 383, 181, and 138, which were similar to those of M5, suggested the hydroxylation of the 2-(piperazin-1-yl)pyrimidine moiety. Fragment ion at $m/z$ 276, suggested the hydroxylation of the 2-(piperazin-1-yl)pyrimidine moiety and an $N$-formylation of the pyrrolidine ring. Based on these data, M11 was tentatively identified as the $N$-formyl conjugate of M5 (2S,4S)-2-(3,3-difluoropyrrolidine-1-carbonyl)-4-(4-(5-hydroxypyrimidin-2-yl)piperazin-1-yl)pyrrolidine-1-carbaldehyde.

Metabolite M12

Metabolite M12 was a minor metabolite observed in rat urine. It had a retention time of ~22.0 min on HPLC and a protonated molecular ion at $m/z$ 399, 32 amu greater than the parent drug. Its MS$^2$ product ion spectrum showed diagnostic fragments at $m/z$ 122, 165, 206, 278 (Table 6). Fragment ions at $m/z$ 122, 165, 206 were similar to those of the parent drug indicating that the 2-(piperazin-1-yl)pyrimidine moiety remained unchanged. The fragment ion at $m/z$ 278, which was 32 mass units greater than the fragment $m/z$ 246 from the parent drug suggested the dihydroxylation of the (3,3-difluoropyrrolidin-1-yl)(pyrrolidin-2-yl)methanone moiety.

Metabolite M13

Metabolite M13 was a minor metabolite observed in rat urine. It had a retention time of ~27.0 min on HPLC and a protonated molecular ion at $m/z$ 399, 32 amu greater than the parent drug. Its
MS\(^2\) product ion spectrum showed diagnostic fragments at \(m/z\) 138, 181 and 246 (Table 6). Fragment ions at \(m/z\) 138 and 181 were similar to those of M5 suggesting that the hydroxylation had occurred at the 2-(piperazin-1-yl)pyrimidine moiety. The fragment ion at \(m/z\) 262, which was 16 amu greater than the fragment \(m/z\) 246 from the parent drug suggested an additional hydroxylation of the (3,3-difluoropyrrolidin-1-yl)(pyrrolidin-2-yl)methanone moiety. Based on these data, M13 was identified as a hydroxy metabolite of M5.

**Metabolite M14**

Metabolite M14 was a minor metabolite observed in rat urine. It had a retention time of \(~30.0\) min on HPLC and a protonated molecular ion at \(m/z\) 383, 16 amu greater than parent drug. Its MS\(^2\) product ion spectrum showed diagnostic fragments at \(m/z\) 122, 165 and 262 (Table 6). Fragment ions at \(m/z\) 122 and 165 were similar to those of parent drug, suggesting that the 2-(piperazin-1-yl)pyrimidine moiety remained unmodified. Fragment \(m/z\) 262, 16 amu higher that the \(m/z\) 246 fragment of parent drug, suggested hydroxylation of the (3,3-difluoropyrrolidin-1-yl)(pyrrolidin-2-yl)methanone moiety.

**Metabolite M15**

Metabolite M15 was a minor metabolite observed in human urine and had a retention time of \(~29.2\) min on HPLC and a protonated molecular ion at \(m/z\) 529, 162 Da higher than the parent compound. Its MS\(^2\) product ion spectrum showed diagnostic fragments at \(m/z\) 511, 493, 409 and 367 (Table 6). The fragment ions at \(m/z\) 511 and 493 were formed by the loss of one and two molecules of water, respectively. The fragment at \(m/z\) 367 indicated a neutral loss of the conjugated moiety to intact PF-00734200. The fragmentation pattern and exact mass

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measurements suggested the addition of a molecule of glucose to parent drug. Treatment of PF-00734200 (10 μM) with D (+) glucose (5 mM) for 12 hours in phosphate buffer (pH 7.4), yielded a product of m/z 529 with retention time and MS² product ion spectrum identical to those of M15. Based on this data, M15 was tentatively identified as the N-glucoside conjugate of PF-00734200, (3,3-difluoropyrrolidin-1-yl)((2S,4S)-4-(4-(pyrimidin-2-yl)piperazin-1-yl)-1-(3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)pyrrolidin-2-yl)methanone.

**Metabolite M16**

Metabolite M16 was observed in human urine and had a retention time of ~28.2 min on HPLC and a protonated molecular ion at m/z 480, 113 Da greater than the parent compound. Its MS² product ion spectrum showed diagnostic fragments at m/z 462 and 367 (Table 6). The exact mass of M16 indicated the addition of a molecule of creatinine to the PF-00734200. Treatment of PF-00734200 (10 μM) with creatinine (1 mM) for 12 hours in phosphate buffer (pH 7.4) yielded a product at m/z 480 with retention time and CID product ion spectrum identical to that of M16. While the conjugation of creatinine is believed to occur on the pyrrolidine nitrogen; the precise location of the creatinine conjugate and mechanism of conjugation could not be discerned based on mass spectral data.
Metabolite M17

Metabolite M17 was observed in human urine and had a retention time of \(~30.2\) min on HPLC and a protonated molecular ion at \(m/z\) 410, 43 Da greater than parent drug. Its MS\(^2\) product ion spectrum showed diagnostic fragments at \(m/z\) 393, 367, 286, 275, 165, and 122 (Table 6). Fragment ion at \(m/z\) 393 suggested the loss of ammonia from M17. Based on these data, M17 was tentatively identified as the \(N\)-formamide conjugate of PF-00734200, \((2S,4S)-2-(3,3\text{-difluoropyrrolidine-1-carbonyl})-4-(4\text{-pyrimidin-2-yl)piperazin-1-yl})\text{pyrroolidine-1-carboxamide}.

Metabolite M18

Metabolite M18 was observed in human urine and had a retention time of \(~28.2\) min on HPLC and a protonated molecular ion at \(m/z\) 545, 178 Da greater than parent drug. Its MS\(^2\) product ion spectrum showed diagnostic fragments at \(m/z\) 383, 425, 455, 509, 527 (Table 6), which were 16 Da higher than those found for M15. Fragment ion at \(m/z\) 383 was similar to that of protonated molecular ion of M5. This fragmentation pattern suggested the addition of one molecule of glucose to M5. Based on these data M18 was tentatively identified as the \(N\)-glucoside conjugate of M5, \((3,3\text{-difluoropyrrolidin-1-yl})((2S,4S)-4-(4\text{-5-hydroxypyrimidin-2-yl)piperazin-1-yl})-1-(3,4,5\text{-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl})\text{pyrroolidine-2-yl})\text{methanone}.

Discussion

The following study reports the comparative disposition and \textit{in-vivo} metabolic profiles of PF-00734200 in rats, dogs and humans. \(^{14}\text{C}\) PF-00734200 labeled at the C-2 position of the pyrimidine ring was administered orally to SD rats, beagle dogs and healthy human volunteers.
The administered radioactive dose was quantitatively recovered in all species (97.1, 92.2 and 87.2% in rats, dogs and humans respectively). Excretion of radioactivity was rapid and nearly complete within 48 h after dosing in all species. The majority of dose was recovered in urine for dogs and humans and in feces for rats (66.7 and 76.8%, and 66.0% respectively). In a separate study with bile-duct cannulated rats, 62.1% of the radioactivity was recovered in the bile, suggesting that a major portion of the dose excreted in the feces of rats was due to biliary excretion rather than excretion of unabsorbed dose. The O-glucuronide of M5 (M4) metabolite represented the major biliary metabolite in rat (~82% of radioactivity in bile) and appears to undergo \( \beta \)-glucuronidase mediated cleavage after secretion into the intestine to yield M5 which was the major metabolite detected in feces. Across species the majority of circulating radioactivity was attributed to unchanged parent drug (79.9, 80.2, 94.4% in rat, dog and human respectively).

The urine and fecal radiochromatograms for rats, dogs and humans indicated that PF-00734200 undergoes significant metabolism in rats and dogs and moderate metabolism in human. Unchanged drug accounted for 12.1, 27.4 and 48.9% in excreta of rat, dog and human respectively. A total of 12 metabolites in rats, 8 metabolites in dogs and 8 metabolites in human were identified by LC/MS/MS and, the proposed structure of a major metabolite (M5) was supported by comparison of its HPLC retention time and \( MS^2 \) spectrum with those of a synthetic standard. There were no notable gender related differences in the circulating and excreted parent/metabolite profiles in any of the species examined in this study. A proposed scheme for the biotransformation pathways of PF-00734200 in rats, dogs and humans is shown in Figure 11.
The primary oxidative metabolic pathways of PF-00734200 involved hydroxylation at the 5’ position of the pyrimidine ring (M5), hydrolysis of the amide bond to yield the corresponding acid (M2), N-dealkylation at the piperazine nitrogen (M3) and an unusual metabolite deriving from scission of the pyrimidine ring to yield M1. A number of phase II conjugative pathways were detected and included O-glucuronidation (M4), O-sulfation (M6) on the phenol of M5 and N-carbamoyl glucuronidation (M7 and M9) on the pyrrolidine nitrogen. A number of metabolites/metabonates, were detected in the urine of dogs and humans and included conjugations of parent drug with formamide (M8, M17), glucose (M15, M18) and creatinine (M16). The structures for all metabolite/metabonates are proposed to involve conjugation directly to the nucleophilic nitrogen of the pyrrolidine ring.

The major components of drug related material in rat excreta were identified as M5 and M1 which collectively represented the majority of excreted radioactivity at 39.6 and 20.9% respectively. M3 and M11 each represent 5.5% of excreted radioactivity in rat. Only 12.1% of the drug related material in excreta was attributable to parent drug, suggesting that PF-00734200 underwent extensive metabolism. In rat bile, M1 and M4 collectively accounted for >90% of radioactivity in bile. The majority of circulating radioactivity in rat was attributable to parent drug (79.9%), the most abundant circulating metabolites were M2, M4, M5 and M1 representing 8.4, 4.7, 2.7 and 2.6% of total radioactivity in plasma.

The major components of drug related material in dog excreta were identified as M5, M1 and M2 which represented 39.1, 5.8 and 2.5% of total administered radioactivity respectively. Parent drug accounted for 27.4% of excreted radioactivity, suggesting that PF-00734200 had undergone extensive metabolism. The major circulating metabolite in dog was identified as M5 which
represented 13.7% of the total radioactivity while parent represented the majority of radioactivity at 80.1%.

In humans PF-00734200 underwent moderate metabolism, with unchanged drug representing roughly half of the total excreted radioactivity (48.9%). M5 was the major excreted metabolite accounting for 20.6% of the dose, followed by a number of metabolites each representing less than 6% of dose. In circulation, parent drug was the predominant component, representing 94.5% of circulating radioactivity followed by M5 and M9 which accounted for 4.0 and 1.2% of radioactivity, respectively.

In-vitro studies using recombinant CYP isoforms demonstrated that the formation of M5 is mediated both by CYP3A4 and CYP2D6 (no evidence of involvement of aldehyde oxidase). Considering the relatively low electron density of the pyrimidine ring, the C5 position would not appear to be a favorable site for p450 hydroxylation, yet 20-40% of the radioactive dose is excreted as M5 across species. A possible explanation for this result comes from molecular docking studies which demonstrated that PF-00734200 could interact in an energetically favorable position with both the 2D6 and 3A4 active sites. The proximity of the C5 position and the favorable steric interactions of the side chain residues appear to facilitate hydroxylation of the pyrimidine ring.

A number of unusual metabolites were identified in this study: Firstly, the M1 metabolite which was observed predominantly in the excreta of rats, dogs and humans (20.9, 5.8 and 3.0% of total radioactive dose, respectively) is proposed to derive via oxidative scission of the pyrimidine ring resulting in a net loss of three carbons and formation of a guanidino group. The guanidino group of M1 was successfully derivatized using a highly selective and diagnostic nucleophilic addition-elimination reaction with HFAA to yield the corresponding cyclized
bis(trifluoromethyl)pyrimidine derivative. Furthermore, treatment of isolated M1 with D2O suggested the presence of four exchangeable hydrogens, consistent with the proposed ring opened structure. (Figure 10, Panel A). Since the opening of the ring is believed to proceed through an oxidative mechanism, possible metabolic intermediates of this reaction were rationalized and the predicted masses were extracted from the bile/feces of rats and dogs. Of the potential intermediates, one (MH\(^+\) 419, Rt= 2.8 min) appeared to be present at minor levels in rat bile of both dogs and rats. The identification of this peak as an intermediate was supported by its CID spectra which showed a major fragment ion at MH\(^+\) 331 (Figure 9, panel B), corresponding to the exact mass of M1. Examples of in-vivo enzymatic cleavage of heteraromatic substructures in xenobiotics, are not unprecedented but are rare in the literature. (Prakash et al., 1997; 1998) The M7 and M9 metabolites, which represented N-carbamoyl glucuronides of M5 and parent respectively, were detected at low levels in the urine and plasma of both dogs and humans. Carbamoyl glucuronides are known to form on highly nucleophilic primary and secondary amines. The mechanism of conjugation is still not entirely understood (whether carbamic acid adds to the glucuronide or substrate first prior to conjugation). Despite the inherent instability of carbamic acids, their formation in solution has been demonstrated (Caplow et al., 1968) (Morrow et al., 1974) (Masuda et al., 2005). The position of the conjugation is therefore believed to be on the alicyclic nitrogen of the pyrrolidine ring.

A number of metabolites or “metabonates” were observed primarily in the urine of human subjects which are also believed to be conjugated directly to the pyrrolidine nitrogen atom. Metabolites M18 and M15 which represented N-glucoside conjugates of parent and M5, respectively, and were found at minor levels in human urine. Whether the formation of the N-glucosidation reaction was an enzymatic or an ex-vivo process is not clear, however incubation
of parent drug in the presence of glucose in buffer at pH 7.4 yielded sufficient levels of M18, suggesting ex-vivo formation of this metabolite/metabolonate was possible. Metabolites M8 and M17 represented N-formamide conjugates of parent and M5 respectively, and were found in minor levels in human (M17) and dog urine (M8). M16 represented an unusual and to our knowledge unprecedented metabolite/metabolonate involving conjugation of parent drug with endogenous creatinine. Incubation of parent drug with creatinine in phosphate buffer yielded a product with retention time and CID spectra identical to M16. We believe formation of M16 likely represents an ex-vivo process owing the high concentrations of creatinine in human urine, a breakdown product of creatine phosphate in, particularly in males.

In conclusion, the results of this study provide the first analysis of formation and excretion of metabolites of PF-00734200 in rats, dogs and humans. The predominant component in circulation in all species is parent drug; however, PF-00734200 undergoes moderate to extensive metabolism prior to excretion. The data from these studies suggest that PF-00734200 is eliminated almost equally both by metabolism and renal clearance thus mitigating the potential for drug-drug interactions in the clinic. The dispositional profile and the identification of the metabolic pathways of PF-00734200 should aid in the use of this agent in clinical practice.
Acknowledgements
We are grateful to Dr. Eric F. Johnson for providing the coordinates of CYP2D6 prinomastat co-crystal structure, and his continuous advice on the structure biology, molecular modeling and application of cytochrome P450s in drug discovery. The authors would also like to thank Dr. Douglas Spracklin for his review of this manuscript.

Authorship Contributions

Participated in research design: Sharma, Sun, Ryder, Doran, Dai, Prakash.

Conducted experiments: Sharma, Sun, Piotrowski, Ryder

Performed data analysis: Sharma, Sun, Ryder, Prakash.

Wrote or contributed to the writing of the manuscript: Sharma, Sun, Piotrowski, Prakash.
References


Miao Z, Sun H, Liras J, and Prakash C (2011) Excretion, Metabolism and Pharmacokinetics of 1-(8-(2-chlorophenyl)-9-(4-chlorophenyl)-9H-purin-6-YL)-4-(ethylamino)piperidine-4-carboxamide, CP-945,598, a Selective Cannabinoid Receptor Antagonist, in Healthy Male Volunteers. Drug Metab Dispos, in press


Footnote

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Figure Legends

FIG. 1. Structure of $[^{14}\text{C}]$ PF-00734200 and position of radiolabel.

FIG 2. Synthesis scheme for M5 metabolite. Conditions: (a) bis(pinacolato)diboron 3 % Pd(OAc)$_2$, KOAc, DMF, 85 °C, (b) NaBO$_3$, aq. THF, (c) TFA, CH$_2$Cl$_2$, (d) Na(OAc)$_3$BH, Et$_3$N, HOAc, 1,2-dichloroethane, (e) 4N HCl in dioxane, methanol.

FIG. 3. Mean serum concentration-time curves of PF-00734200 and total radioactivity in healthy male volunteers following oral administration of $[^{14}\text{C}]$PF-00734200.

FIG. 4. Representative radiochromatograms of rat urine (panel A), feces (panel B), plasma (panel C) and bile (panel D).

FIG. 5. Representative radiochromatograms of dog urine (panel A), feces (panel B), plasma (panel C).

FIG. 6. Representative radiochromatograms of human urine (panel A), feces (panel B), plasma (panel C).

FIG. 7. Molecular Docking of PF-00734200 in CYP2D6 pose 1 (panel A) pose 2 (panel B) and CYP3A4 pose 1 (panel C), pose 2 (panel D) active sites.

FIG. 8. CID spectra for PF-00734200 (panel A) and M5 metabolite (panel B).

FIG. 9. CID spectra of M1 metabolite (panel A) and proposed M1 intermediate (panel B).

FIG. 10. Treatment of M1 with D$_2$O (panel A) showing four exchangeable hydrogens and hexafluoracetylacetone (panel B) showing CID spectra of cyclized bis(trifluoromethyl)pyrimidine derivative. The origins of diagnostic fragments are as indicated.

Table 1. Material balance and routes of excretion of $[^{14}C]$ PF-00734200 drug-related material in rats, dogs and humans

Values represent mean ± SD

<table>
<thead>
<tr>
<th>Species</th>
<th>Rat</th>
<th>Dog</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of Dose Urine</td>
<td>30.8 ± 1.9</td>
<td>66.7 ± 5.0</td>
<td>76.8 ± 3.5</td>
</tr>
<tr>
<td>Percentage of Dose Feces</td>
<td>66.0 ± 1.8</td>
<td>16.8 ± 6.8</td>
<td>10.4 ± 4.9</td>
</tr>
<tr>
<td>Percentage of Dose Recovereda</td>
<td>97.1 ± 1.7</td>
<td>92.2 ± 0.7</td>
<td>87.2 ± 5.3</td>
</tr>
</tbody>
</table>

aPercentage of dose also includes wipes and cagewashes
Table 2. Individual and mean pharmacokinetics parameters of total circulating radioactivity in healthy male volunteers following oral administration of \([^{14}\text{C}]\text{PF-00734200}\)

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>(T_{\text{max}}) (h)</th>
<th>(C_{\text{max}}) (ng equiv/mL)</th>
<th>(\text{AUC}_{(0-168)}) (ng equiv.h/mL)</th>
<th>(\text{AUC}_{(0-\infty)}) (ng equiv.h/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>283</td>
<td>2860</td>
<td>3320</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>311</td>
<td>2870</td>
<td>3430</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>245</td>
<td>2130</td>
<td>2310</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>257</td>
<td>2100</td>
<td>2330</td>
</tr>
<tr>
<td>Mean</td>
<td>1</td>
<td>274</td>
<td>2490</td>
<td>2850</td>
</tr>
<tr>
<td>SD</td>
<td>0</td>
<td>29</td>
<td>434</td>
<td>610</td>
</tr>
</tbody>
</table>
Table 3. Individual and mean pharmacokinetics parameters of PF-00734200 in healthy male volunteers following oral administration of $[^{14}\text{C}]$PF-00734200

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>$T_{\text{max}}$ (h)</th>
<th>$C_{\text{max}}$ (ng/mL)</th>
<th>AUC (0-168) (ng.h/mL)</th>
<th>AUC (0-$\infty$) (ng.h/mL)</th>
<th>$t_{1/2}$ (h)</th>
<th>CL/F (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>255</td>
<td>3100</td>
<td>3280</td>
<td>69.8</td>
<td>101.6</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>273</td>
<td>3090</td>
<td>3130</td>
<td>44.3</td>
<td>106.5</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>222</td>
<td>2090</td>
<td>2140</td>
<td>54.3</td>
<td>155.8</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>215</td>
<td>1980</td>
<td>2070</td>
<td>85.7</td>
<td>161.0</td>
</tr>
<tr>
<td>Mean</td>
<td>1</td>
<td>241.3</td>
<td>2560</td>
<td>2660</td>
<td>63.5</td>
<td>125.3</td>
</tr>
<tr>
<td>SD</td>
<td>0</td>
<td>27</td>
<td>613</td>
<td>638</td>
<td>11</td>
<td>31.5</td>
</tr>
</tbody>
</table>
**Table 4.** Relative abundance of urinary and fecal metabolites of PF-00734200 in rat, dog and human following oral administration of [14C]PF-00734200 (% of total administered dose)

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Rat</th>
<th>Dog</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urine</td>
<td>Feces</td>
<td>Total</td>
</tr>
<tr>
<td>Parent</td>
<td>11.0</td>
<td>1.1</td>
<td>12.1</td>
</tr>
<tr>
<td>M1</td>
<td>3.1</td>
<td>17.8(11.7)</td>
<td>20.9</td>
</tr>
<tr>
<td>M2</td>
<td>3.2</td>
<td>2.3</td>
<td>5.5</td>
</tr>
<tr>
<td>M3</td>
<td>1.4</td>
<td>1.4</td>
<td>0.9</td>
</tr>
<tr>
<td>M4</td>
<td>2.9</td>
<td>(81.6)</td>
<td>2.9</td>
</tr>
<tr>
<td>M5</td>
<td>7.3</td>
<td>32.3</td>
<td>39.6</td>
</tr>
<tr>
<td>M6</td>
<td>(2.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M10</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>M11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M12</td>
<td>0.2</td>
<td>(1.3)</td>
<td>0.2</td>
</tr>
<tr>
<td>M13</td>
<td>0.2</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>M14</td>
<td>0.2</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>M15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M16</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>M17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M18</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* metabolites M9, M16 and M18 could not be chromatographically resolved in human urine. Metabolites M6 and M7 could not be resolved in dog urine. () indicates biliary metabolites.
Table 5. Relative abundance of circulating metabolites of PF-00734200 in rats, dogs and human following oral administration of [14C]PF-00734200

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Rat</th>
<th>Dog</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>79.9</td>
<td>80.1</td>
<td>94.5</td>
</tr>
<tr>
<td>M1</td>
<td>2.6</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td>8.4</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td>1.7</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>M4</td>
<td>4.7</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>M5</td>
<td>2.7</td>
<td>13.7</td>
<td>4.0</td>
</tr>
<tr>
<td>M6/M7</td>
<td></td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>M9</td>
<td></td>
<td>1.5</td>
<td>1.2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
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Table 6. MS² ions of metabolites

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>m/z</th>
<th>Species</th>
<th>Structure</th>
<th>LC/MS²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>367</td>
<td>Rat, Dog, Human</td>
<td><img src="image1" alt="Structure" /></td>
<td>122.0713 165.1135 206.1400 246.1412</td>
</tr>
<tr>
<td>M1</td>
<td>331</td>
<td>Rat, Dog, Human</td>
<td><img src="image2" alt="Structure" /></td>
<td>112.0869 181.1084 246.1412 272.1569 289.1834 314.1787</td>
</tr>
<tr>
<td>M2</td>
<td>278</td>
<td>Rat, Dog, Human</td>
<td><img src="image3" alt="Structure" /></td>
<td>122.0713 165.1135 206.1400</td>
</tr>
</tbody>
</table>
M3  165  Rat, Dog  122.0713

M4  559  Rat, Dog  181.1084  357.1405  383.2002

M5  383  Rat, Dog, Human  138.0662  181.1084  222.1352  246.1412

M6  463  Dog  181.1084  383.2002
DMD #47316

M7  603  Dog

M8  426  Dog

M9  587  Dog, Human
M10 359  Rat  

M11 411  Rat  

M12 399  Rat
Figure 1.
Figure 2.

1 \[\text{Br} \quad \text{N} \quad \text{N}\]
2 \[\text{OH} \quad \text{N} \quad \text{N}\]
3 \[\text{O} \quad \text{N} \quad \text{F} \quad \text{F}\]
4 \[\text{R} = \text{BOC}\]

\[\text{R} = \text{H} \quad \text{M1}\]

\[\text{c, d}\]

\[\text{a, b}\]
Figure 3.

- Mean PF-00734,200 (ng/mL)
- Mean Total Radioactivity (ng-equiv/mL)
Figure 4.
Figure 5.

(A) Dog Urine

(B) Dog Feces

(C) Dog Plasma

Legend:
- M5
- PF-00734200
- M1

Y-axis: DPM
X-axis: Time (min)
Figure 6.
Figure 7.
Figure 8.

A

122.0712
150.1025
165.1135
206.1403
246.1416
324.1640
350.1798

PF-00734200

M5

138.0662
166.0976
181.1084
222.1354
246.1412
246.1417
366.1750

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Figure 9.
Figure 10.

A

Exact Mass: 336.2366

B
Figure 11.