Comparative Metabolism of \(^{14}C\)-Labeled Apixaban in Mice, Rats, Rabbits, Dogs, and Humans\(^{\text{S}}\)

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ABSTRACT:
The metabolism and disposition of \(^{14}C\)apixaban, a potent, reversible, and direct inhibitor of coagulation factor Xa, were investigated in mice, rats, rabbits, dogs, and humans after a single oral administration and in incubations with hepatocytes. In plasma, the parent compound was the major circulating component in mice, rats, dogs, and humans. \(\text{O-}\)Demethyl apixaban sulfate (M1) represented approximately 25% of the parent area under the time curve in human plasma. This sulfate metabolite was present, but in lower amounts relative to the parent, in plasma from mice, rats, and dogs. Rabbits showed a plasma metabolite profile distinct from that of other species with apixaban as a minor component and M2 (\(\text{O-}\)demethyl apixaban) and M14 (\(\text{O-}\)demethyl apixaban glucuronide) as prominent components. The fecal route was a major elimination pathway, accounting for >54% of the dose in animals and >46% in humans. The urinary route accounted for <15% of the dose in animals and 25 to 28% in humans. Apixaban was the major component in feces of every species and in urine of all species except rabbit. M1 and M2 were common prominent metabolites in urine and feces of all species as well as in bile of rats and humans. In vivo metabolite profiles showed quantitative differences between species and from in vitro metabolite profiles, but all human metabolites were found in animal species. After intravenous administration of \(^{14}C\)apixaban to bile duct-cannulated rats, the significant portion (approximately 22%) of the dose was recovered as parent drug in the feces, suggesting direct excretion of the drug from gastrointestinal tracts of rats. Overall, apixaban was effectively eliminated via multiple elimination pathways in animals and humans, including oxidative metabolism, and direct renal and intestinal excretion.

Thromboembolic events, including acute myocardial infarction, unstable angina, deep vein thrombosis, pulmonary embolism, and ischemic stroke, continue to be the leading cause of morbidity and mortality in the United States and other Western countries (Heit et al., 2005; Rosamond et al., 2007). Current therapies for the treatment and prevention of thromboembolic events, such as vitamin K antagonists (e.g., warfarin), heparin, and low-molecular-weight heparin (e.g., enoxaparin), are suboptimal (O’Donnell and Weitz, 2004; Wittkowsky, 2004; Campbell, 2006). The requirement for intravenous or subcutaneous injection and/or the need for careful monitoring because of the risk of excessive bleeding or unpredictable/inconsistent pharmacokinetics can complicate administration and present barriers to the use of these agents (O’Brien and Caro, 2002; Wittkowsky, 2004; Campbell, 2006). Therefore, new orally active anticoagulants with predictable pharmacokinetic profiles that can be administered with a reduced need for monitoring are needed.

Factor Xa is a key serine protease in the coagulation cascade and is a promising target enzyme for new therapeutic agents for the treatment and prevention of arterial and venous thrombosis (Kaiser, 2002; Samama, 2002; Walenga et al., 2003). In particular, factor Xa plays a critical role in blood coagulation, serving as the juncture between the extrinsic (tissue factor initiated) and intrinsic (surface activation and amplification) systems (Mann et al., 2003). Factor Xa forms the prothrombinase complex with phospholipids, calcium ions, and a cofactor, factor Va, which is responsible for the generation of thrombin from prothrombin. Although factor Xa inhibition attenuates the generation of thrombin, it does not affect thrombin activity, thereby preserving hemostasis, which, in clinical terms, may translate to efficacy with lower bleeding risk (Comp, 2003; Kubitz and Haas, 2006). Indeed, studies have shown that factor Xa inhibitors interrupt thrombus growth in large blood vessels under low shear (venous thrombosis) and high shear (arterial thrombosis) conditions, with minimal disruption of normal hemostasis, suggesting the suitability of such agents in the management of both venous and arterial thromboembolic disorders (Orvim et al., 1995; Shimbo et al., 2002). Clinical

ABBREVIATIONS: BDC, bile duct cannulation; BMS-562247, 1-\{4-(methoxyphenyl)-7-oxo-6-\{4-[2-oxopiperidin-1-yl]phenyl\}-4,5-dihydro pyrazolo[5,4-c]pyridine-3-carboxamide; HPLC, high-performance liquid chromatography; GI, gastrointestinal; MS, mass spectrometry; LC, liquid chromatography; MS/MS, mass spectrometry; amu, atomic mass units.
Apixaban is a promising oral anticoagulant in late-stage clinical development for the prevention and treatment of venous thromboembolism, stroke prevention in atrial fibrillation, and secondary prevention in acute coronary syndrome. It is a potent, oral, reversible, selective, and direct factor Xa inhibitor, which inhibits both free and prothrombinase-bound factor Xa activity and shows considerable efficacy in the prevention of arterial and venous thrombosis at doses that preserved hemostasis in rabbits (Pinto et al., 2007; Wong et al., 2008).

It is also effective and safe for the prevention and treatment of venous thrombosis in humans (Lassen et al., 2007; Buller et al., 2008). Positive preliminary results from a phase II clinical trial evaluating the prevention of venous thromboembolic events (asymptomatic and symptomatic deep vein thrombosis and nonfatal symptomatic pulmonary embolism) demonstrate the potential benefit of this agent versus standard therapy in anticoagulation monotherapy treatment in humans (Lassen et al., 2007).

Apixaban was well absorbed in rats and dogs (mean bioavailability of 34–80%, respectively). The mean volume distribution was 0.31 and 0.2 to 0.29 l/kg in rats and dogs, respectively. The systemic clearance of apixaban was less, <10 and <3% of hepatic blood flow in rats and dogs, respectively (Shantsila and Lip, 2008). Metabolic pathways of apixaban in humans included O-demethylation, hydroxylation, and O-demethylation followed by sulfation (Raghavan et al., 2009). This study was conducted to evaluate the comparative metabolic disposition of apixaban in different species.

The study included an in vitro component, in which hepatocytes of mice, rats, dogs, monkeys, and humans were incubated with [14C]apixaban, and an in vivo component, in which mice, rats, rabbits, dogs, and humans were dosed orally with [14C]apixaban and samples of plasma, urine, feces, and bile were collected and analyzed for radioactive components. In addition, the elimination properties of [14C]apixaban were studied in bile duct-cannulated (BDC) rats after intravenous administration.

Materials and Methods

Materials. Apixaban (BMS-562247, purity >99%) and [14C]apixaban were synthesized at Bristol-Myers Squibb (Princeton, NJ). The radiochemical purity was >98.6% for [14C]apixaban. The metabolite standard M2 (O-demethyl apixaban) and its sulfate conjugate M1 were also prepared at Bristol-Myers Squibb. Formic acid and ammonium hydroxide were purchased from Thermo Fisher Scientific (Waltham, MA). Deepwell LumaPlate-96 well plates were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Ecolite liquid scintillation cocktail was purchased from MP Biomedicals (Irving, CA). All other organic solvents and reagents were of high-performance liquid chromatography (HPLC) grade.

Fresh hepatocytes from mice (male ICR/CD-1) and rats (male Sprague-Dawley) were prepared. Cryopreserved hepatocytes from dogs (male beagle) were prepared by Tissue Transformation Technologies (Franklin Lakes, NJ). Cryopreserved hepatocytes from monkeys (male cynomolgus) and humans (male and female) were obtained from Celsis In Vitro Technologies, Inc. (Baltimore, MD). Hepatocytes were stored in liquid nitrogen before use. Pooled human liver microsomes (22 donors) and Supersomes (membranes from insect cells transfected with baculovirus containing cDNA of human CYP3A4) were purchased from BD Gentest (Woburn, MA).

Study Subjects, Dosing, and Sample Collection. All animal housing and care conformed to the standards recommended by the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996). Animal rooms were maintained on a 12-h light/dark cycle. The human study was performed in accordance with the principles of the Declaration of Helsinki and its amendments, and the study protocol was approved by the institutional review board and radiation safety committee at the investigational site. All subjects were in good health and gave written, informed consent to participate in the study.

All blood samples were collected into tubes containing K$_2$EDTA. These samples were placed on ice immediately after collection and centrifuged within 30 min of collection to harvest plasma.

The [14C]apixaban oral dose formulations were prepared in 0.5% (v/v) Tween 80 in Labrafil at 6 mg/ml for mouse and rat, at 6 mg/ml (9 µCi/ml) for rabbit, at 2.5 mg/ml (12.5 µCi/ml) for dog, and at 2 mg/ml (10 µCi/ml) in PEG-400-ethanol (85:15, v/v) for humans. The intravenous dosing solution was prepared by dissolving 30 mg of [14C]apixaban in 7 ml of vehicle containing 10% dimethyl acetamide and 30% propylene glycol in water.

Mouse. Male CD-1 mice (25–35 g) were obtained from Charles River Laboratories (St. Constant, QC, Canada), and provided fresh filtered tap water ad libitum. Mice were fasted for approximately 2.5 to 3.5 h before dosing. Each animal received an oral gavage dose of 30 mg/kg (150 µCi/kg) of [14C]apixaban. Food was returned to the animals approximately 2 to 4.5 h after dosing. Urine and feces were collected from five mice at 12-h intervals over 0 to 48 h after dosing. Terminal blood samples (five mice per time point) were collected via cardiac puncture under carbon dioxide anesthesia from mice at 1, 4, 12, 24, and 48 h.

Rat. Male BDC and intact Sprague-Dawley rats (250–280 g) were obtained from Harlan (Indianapolis, IN), fed with Certified High Fiber Rodent Diet 5002 (LabDiet; PMI Nutrition International, Purina Mills, St. Louis, MO), and provided fresh filtered tap water ad libitum. Rats were fasted overnight before dosing. Intact animals and those previously prepared with bile duct cannulas each received an oral gavage dose of 30 mg/kg (150 µCi/kg) of [14C]apixaban. Urine, bile, and feces were collected at 12-h intervals over 0 to 48 h after dosing from three BDC rats. Urine and feces samples were collected at 24-h intervals over 0 to 168 h after dosing from three intact rats. Terminal blood samples were collected via cardiac puncture under carbon dioxide anesthesia from three intact rats per time point at 1, 4, 12, 24, and 48 h.

Three BDC and three intact rats were each given an intravenous infusion of [14C]apixaban in dimethyl acetamide and propylene glycol solution at 0.1 ml/min for 10 min (15 mg/kg, 30–45 µCi/rat, specific activity 7.6 µCi/mg). Bile, urine, feces, and gastrointestinal (GI) tract samples were collected from BDC rats from 0- to 24-h intervals after dosing. A bile salt solution (18 mg/ml cholic acid and 1.1 mg/ml sodium bicitarone in saline, pH 7.2) was infused via the duodenal cannula at 1 ml/h for BDC rats. Urine and fecal samples were collected from intact rats over 24-h periods. The plasma samples were collected at 24 h postdose.

Rabbit. Three female New Zealand rabbits (2.5 kg) each received a single oral gavage dose of [14C]apixaban at a dose level of 30 mg/kg (45 µCi/kg). The animals were fasted for 11 h before dosing, fed daily with Certified High Fiber Rodent Diet 5002 (LabDiet; PMI Nutrition International), and provided fresh filtered tap water ad libitum. The actual amount of dosing solution administered to each rabbit was determined by weighing the loaded dosing syringe before dose administration and the emptied syringe after dose administration. Urine and feces samples were collected over 0 to 48 h at 12-h intervals after dosing. Blood samples (4 ml) were collected at 1, 4, 12, 24, and 48 h into tubes containing potassium EDTA.

Dog. Non-naive male beagle dogs (7–11 kg) were taken from the Charles River Laboratories test facility. The animals were fed with Canine Diet 5006 (LabDiet; PMI Nutrition International) and provided water ad libitum. Dogs were fasted overnight before dosing. Each animal received an oral dose of 5 mg/kg (25 µCi/kg) of [14C]apixaban followed by a 5- to 10-ml water rinse through the gavage tube. Urine and feces were collected at 24-h intervals from three animals over 0 to 168 h after dosing. Blood samples were collected via the cephalic vein at 1, 4, 12, 24, and 48 h.

Human. Ten healthy male subjects, aged 18 to 45 years, participated in the study (Raghavan et al., 2009). After at least an 8-h overnight fast, each subject received a single oral 20-mg (108.8 µCi) dose of [14C]apixaban as a solution in PEG-400-ethanol (85:15, v/v). Blood samples (10 ml each) for biotransformation analysis were drawn at 1, 4, 12, 24, and 48 h postdose. Urine and feces were collected over 24-h intervals from six subjects for 10 days. Bile samples were collected during 3 to 8 h postdose under suction using a weighted
oral-gastroduodenal tube in the second group of four subjects as described previously (Wang et al., 2006). At 7 h postdose a 20 ng/kg i.v. dose of cholecystokinin carboxyl-terminal octapeptide [sinalcine (Kinevac)] was infused over 5 min to stimulate gallbladder contraction and enhance bile flow.

**Incubation of [14C]Apixaban with Hepatocytes.** [14C]Apixaban, at concentrations of 1 and 10 μM in incubation buffer media, was separately incubated with mouse, rat, dog, monkey, and human hepatocytes in duplicate. Incubations were carried out in Costar 24-well cell culture plates (Corning, NY) with each well containing 0.5 or 1.0 ml of the incubation mixture. The cell concentration in the final incubation mixture was approximately 1 × 10^6 viable cells. All incubations were conducted in an incubator maintained at 37°C, in an atmosphere consisting of 95% air/5% CO₂ and humidity was maintained at 95%. Additional incubations at a concentration of 100 μM apixaban with dog and rat hepatocytes for 4 h were conducted for metabolite characterization and identification.

Incubations at 100 μM coumarin and 7-ethoxy-coumarin were used as positive controls. At the end of the 4-h incubations, the [14C]apixaban incubation mixtures at the same concentration were pooled and mixed with 2 volumes of ice-cold acetoneitrile, followed by centrifugation at 2000g for 10 min. The supernatants were individually transferred to another set of prelabeled 15-ml conical centrifuge tubes. Each well was rinsed with 1.0 ml of acetoneitrile-buffer (2:1). The rinse solution was used to wash each respective protein precipitate, followed by centrifugation. The supernatants were combined, and duplicate aliquots (20 μl) of each sample were assayed by liquid scintillation counting. A portion of the acetoneitrile extracts from each hepatocyte incubation was evaporated to dryness by a rotary evaporator or pretreated by solid-phase extraction (C18 column). The remaining residues were reconstituted in an appropriate volume of methanol-water (2:1, v/v) for HPLC analysis.

**Radioactivity Analysis.** The levels of total radioactivity in plasma, urine, bile, and fecal homogenate were determined by liquid scintillation counting after an aliquot of each sample was collected. Plasma samples were thawed and a water-ethanol (50:50, v/v) solution was added to an appropriate volume (w/w) of plasma sample, and the mixture was vortexed and centrifuged for 5 min at 2000g. The supernatants were reconstituted in methanol-water (2:1, v/v) for HPLC analysis.

**Sample Preparation for Biotransformation Analysis.** Pooled plasma samples (at 1, 4, 12, 24, and 48 h) were prepared separately by mixing an equal volume (0.5 ml) of plasma sample from each subject. For rabbits, only 1- and 4-h plasma samples contained appreciable levels of radioactivity for extraction and profiling. Each pooled plasma sample was extracted in duplicate at each time point by addition of 4 ml of acetoneitrile-methanol (1:1, v/v) to 1 ml of plasma, and the mixture was sampled on a vortex mixer. After centrifugation at 2000g for 1 h, each supernatant fraction was removed and saved, and the precipitate was resuspended in 2 ml of acetoneitrile and 1 ml of methanol. After centrifugation of the precipitate mixture for 30 min at 2000g, the supernatant fraction was removed and combined with the first supernatant; the precipitate was resuspended in 2 ml of acetoneitrile. The combined supernatant fractions were evaporated to dryness under nitrogen and reconstituted in 0.15 ml of acetoneitrile and 0.05 ml of methanol. After centrifugation at 2000g for 5 min, a 0.06- to 0.1-ml portion of supernatant was subjected to HPLC analysis. Each pooled urine and bile sample was thawed and a water-ethanol (50:50, v/v) solution was added to an appropriate volume (w/w) of plasma sample, and the mixture was sampled on a vortex mixer. After centrifugation at 2000g for 5 min, a 0.1-ml portion of each urine sample was subjected to HPLC analysis. Bile samples were diluted with water (1:1, v/v) and a 0.03- to 0.1-ml portion was subjected to HPLC analysis followed by ion trap analysis or quadruple mode of flight mass spectrometry (MS) analysis (Waters, Milford, MA).

Each pooled fecal homogenate sample (0.5 ml) was extracted in replicate by addition of 1.5 ml of methanol-acetoneitrile (1:2, v/v), and the sample was mixed on a vortex mixer. After centrifugation at 2000g for 30 min, the supernatant fraction was removed and saved; the precipitate was resuspended, and the extraction was repeated a second time. The combined supernatant was evaporated to dryness under nitrogen and reconstituted in 0.15 ml of methanol. After centrifugation at 2000g for 5 min, a 0.04-ml portion of supernatant was injected into the high-performance liquid chromatograph. The average column recovery from the replicate (n = 2) samples was calculated relative to the total radioactivity injected.

**Metabolite Profiles, Identification, and Quantification.** Metabolite profiles. Sample analysis by HPLC was performed on a LC-10AT system (Shimadzu, Kyoto, Japan) equipped with a photodiode array UV detector. An Ace 3 C18 (3 μm), 150 × 4.6 mm column (MacMod Analytical, Inc., Chadds Ford, PA) was used, and the mobile phase flow rate was 0.7 ml/min. The retention times of reference standards were confirmed by their UV spectral analysis. The HPLC solvent system was a gradient of two solvents: 0.4% formic acid in water, pH 3.2 (A), and 100% acetoneitrile (B). The gradient for elution was 0% B for 3 min, 0 to 10% B in 2 min, 10 to 25% B in 15 min, hold 25% B for 30 min, 25 to 50% B in 10 min, 50 to 100% B in 5 min, and 100% B for 5 min. The relative retention times of apixaban and its metabolites were sensitive to minor variations in buffer preparations, between individual columns, and in sample preparations. However, a portion of HPLC elute was always diverted to liquid chromatography (LC)-MS for confirmation of metabolites. For quantitation of radioactivity, HPLC effluent was collected into Deepwell Luma-Plate-96 96-well plates in 0.26-mm intervals using a Gilson model 204 fraction collector (Gilson Inc., Middleton, WI). The plates were dried in an Automatic Environmental Speed Vac (Thermo Fisher Scientific) and counted for radioactivity for 20 min (counts per minute) using a Packard TopCount NXT microplate scintillation and luminescence counter (PerkinElmer Life and Analytical Sciences). Radiochromatograms were reconstructed from the Top-Count data using Microsoft Excel software. For each injection, the average counts per minute value from a baseline section of 2 to 3 min in the chromatogram was subtracted from the counts per minute value of each fraction. Biotransformation profiles were prepared by plotting the counts per minute of radioactivity against time after injection. Radioactivity peaks in the biotransformation profiles were reported as a percentage of the total radioactivity collected during the entire HPLC run.

**Incubation, Isolation, and NMR Analysis of M7.** [14C]Apixaban at a concentration of 250 μM (1 μCi/μg) was incubated with CDNA-expressed CYP3A4 Supremes at a final enzyme concentration of 200 pmol of cytochrome P450/ml in 0.05 M phosphate buffer (pH 7.4). NADPH was added to the incubation mixture at a final concentration of 1.2 mM. The total volume of the incubation mixture was 37.5 ml. Aliquots of 2.5 ml were transferred into 20-ml glass vials. The mixture was incubated in a water bath at 37°C for 1 h. At the end of the incubation period, acetoneitrile (1 volume) was added to each sample, and the mixture was vortexed and centrifuged for 5 min at 2000g. The supernatant from each vial was transferred to a 50-ml polypropylene centrifuge tube and concentrated to approximately 2.5 ml. Portions of 250-μl aliquots of samples were injected, and the metabolite M7 peak eluate was collected into a tube. The eluate was then evaporated under a stream of nitrogen and reconstituted with 0.5 ml of deuterated acetoneitrile and vortexed. A 20-μl sample was mixed with 15 ml of scintillation cocktail and counted on a scintillation counter for 10 min; the amount of metabolite M7 was calculated based on the amount of radioactivity and specific radioactivity. The remaining sample was evaporated under nitrogen and reconstituted again with 300 μl of deuterated acetoneitrile. After evaporation, approximately 80 μg of the metabolite was dissolved in 200 μl of CD3CN and transferred into a 3-mm NMR tube for analysis.

Proton NMR was performed on a Bruker DRX-600 MHz NMR spectrometer equipped with an indirect detection 5-mm TCI Bruker Cryo-Probe for one-dimensional and two-dimensional 1H NMR. The temperature was controlled at 30°C. The WET solvent suppression system was used to remove residual hydrogen/oxygen/deuterium and CH3CN signals. The free induction
Excretion of Radioactive Dose. After oral administration, the recovery values of radioactive dose in urine and feces (mice, rats, rabbits, dogs, and humans) and in bile (rats and humans) are shown in Table 1. In each species, total recovery of the radioactive dose was >77%, indicating good mass balance (Roffey et al., 2007), except in rabbits in which approximately 1.8% and 54.3% of dose was recovered in the 0 to 48 h collection of urine and feces, respectively. In all species including BDC rats and humans with bile collections, the majority of the recovered radioactive dose was present in feces. Recovery of radioactivity in urine ranged from 1.8 to 29% across species and was similar for rats and humans with and without bile collection. Humans appeared to show a higher percentage of dose excreted in feces within 96 h postdose. Urinary dose was excreted in urine within 48 h of dosing, and approximately 45% of the dose was excreted in urine. In humans, approximately 23% of the dose was excreted in feces within 96 h postdose. Urinary dose recovery seemed to be lower in rabbits than in other species.

Male mice excreted approximately 85% of the administered dose within 12 h after the oral dose of [14C]apixaban (approximately 71% in feces and 14% in urine). In male rats and dogs, approximately 78 and 86%, respectively, of the administered dose was excreted within 48 h after the oral dose of [14C]apixaban, with approximately 6.7 and 12.6% excreted in urine. In humans, approximately 23% of the dose was excreted in urine within 48 h of dosing, and approximately 45% of dose was excreted in feces within 96 h postdose. Urinary dose recovery seemed to be lower in rabbits than in other species.

Metabolic Profiles of [14C]Apixaban in Plasma, Urine, Bile, and Feces. Apixaban metabolite occurrence and identities are listed in Table 2. Profiles and tabulations of the distribution of radioactivity in plasma, urine, and feces for samples obtained without bile collection are listed in Tables 3 and 4. The identities of apixaban and the metabolites in different matrices were verified by LC-MS together with radioactivity detection.

Plasma. The relative distribution of radioactive metabolites in plasma after oral administration of [14C]apixaban is summarized in Table 3 and Supplemental Fig. S1A. The percent extraction of radioactivity in all of the plasma samples was >85%. Apixaban was the major radioactive component in plasma from mice, rats, and dogs, but a minor component in rabbits. Metabolite M1 was not detected in mouse plasma, was present at only low relative abundance (<1% of radioactivity) in plasma from rats and dogs, and was a prominent component in rabbit plasma. Its metabolic precursor, M2 (O-demethyl apixaban), was a prominent metabolite in plasma from mice, rats, rabbits, and dogs. Other animal plasma metabolites were M7, M13, and M14 as minor components in mice, M4 and M7 as minor components in rats, and M14 as a major component in rabbits. For comparison, apixaban and metabolite M1 (O-demethyl apixaban sulfate) were the major radioactive components in human plasma. The relative abundance of M1 increased from 2 to 28% of the radioactivity in the 1 h versus 12 h human plasma samples.

Urine. The relative distribution of radioactive metabolites in feces after oral administration of [14C]apixaban is summarized in Table 4 and Supplemental Fig. S1B. Apixaban constituted most of the radioactivity in urine from mice, rats, and dogs, but M2 was the major component in rabbit urine. No metabolites including M2 and M13 in mice, M2 and M4 in rats, M1, M14, and M16 in rabbits, and M2, M7, M4, and M8 in dogs accounted for more than 1% of the dose in urine from these species. In human urine, apixaban constituted most of the radioactivity (22.5% of the dose), along with small amounts of M1 and M7 (each less than 2% of the dose).

Bile. The relative distribution after the oral dose of [14C]apixaban in pooled bile of rat and human is shown in the Supplemental Fig. S1A. The percent extraction of radioactivity from fecal samples was >80%. The most abundant radioactive compounds found in feces of mice, rats, rabbits, and dogs were unchanged apixaban followed by metabolites M2 and M7. In human feces, unchanged apixaban and metabolite M2 (O-demethyl apixaban) were the major radioactive components, representing 34 and 12% of the dose, respectively. Metabolites M1, M4, M7, and M13 were also found in human feces at amounts representing 1 to 4% of the dose.

Excretion of Radioactive Dose. After oral administration, the relative distribution of radioactive metabolites in feces was excreted in rat bile but not in rat feces because it was hydrolyzed to M2 in the intestine or feces. In human bile, the major radioactive components were unchanged apixaban and metabolites M1, M7, and M10. Metabolites M2 and M13 were also present in lesser quantities. The difference between the profiles of radioactivity in human feces and bile probably resulted from the partial hydrolysis of the sulfate conjugates M10 to M13 and M1 to M2 by intestinal contents in feces.

Elimination of [14C]Apixaban in Rats After Intravenous Administration. The disposition of [14C]apixaban was determined in intact and BDC rats (15 mg/kg) after intravenous administration. Urinary, fecal, and I recoveries (0–24 h) of radioactivity were 20.7, 12.7, and 1.7% of the dose, respectively, from intact rats. Urinary, biliary, fecal, and GI recoveries of radioactivity were 46.6, 23.0, 24.9, and 0.4%, respectively, from the modified rats. The parent was the major radioactive component in urine, bile, and feces in both intact and BDC rats. The parent compound represented approximately 22%
of the dose in feces of BDC rats, after intravenous administration, suggesting direct excretion of apixaban from GI tracts in rats. M2, M4, and M7 were the metabolites observed in feces of intact rats. The biliary metabolite profile of BDC rats after intravenous administration was similar to that of BDC rats after oral administration of [14C]apixaban, and those metabolites included M1, M2, M7, and M9 as the major ones and M3 and M4 as the minor ones (Fig. 1).
Table 3

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Proposed Structure</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>M8 (hydroxy O-demethyl apixaban)</td>
<td><img src="image" alt="M8 Structure" /></td>
<td>Rat: urine, bile&lt;br&gt;Mouse: N.D.&lt;br&gt;Rabbit: N.D.&lt;br&gt;Dog: urine, feces&lt;br&gt;Monkey: N.D.&lt;br&gt;Human: N.D.</td>
</tr>
<tr>
<td>M9 (hydroxy apixaban)</td>
<td><img src="image" alt="M9 Structure" /></td>
<td>Rat: urine, feces, bile&lt;br&gt;Mouse: urine&lt;br&gt;Rabbit: N.D.&lt;br&gt;Dog: N.D.&lt;br&gt;Monkey: N.D.&lt;br&gt;Human: N.D.</td>
</tr>
<tr>
<td>M10 (hydroxy O-demethyl apixaban sulfate)</td>
<td><img src="image" alt="M10 Structure" /></td>
<td>Rat: N.D.&lt;br&gt;Mouse: plasma&lt;br&gt;Rabbit: N.D.&lt;br&gt;Dog: N.D.&lt;br&gt;Monkey: N.D.&lt;br&gt;Human: plasma, feces</td>
</tr>
<tr>
<td>M13 (hydroxy O-demethyl apixaban)</td>
<td><img src="image" alt="M13 Structure" /></td>
<td>Rat: N.D.&lt;br&gt;Mouse: plasma, urine&lt;br&gt;Rabbit: N.D.&lt;br&gt;Dog: N.D.&lt;br&gt;Monkey: N.D.&lt;br&gt;Human: feces</td>
</tr>
<tr>
<td>M14 (O-demethyl apixaban glucuronide)</td>
<td><img src="image" alt="M14 Structure" /></td>
<td>Rat: N.D.&lt;br&gt;Mouse: plasma&lt;br&gt;Rabbit: plasma, urine&lt;br&gt;Dog: N.D.&lt;br&gt;Monkey: N.D.&lt;br&gt;Human: N.D.</td>
</tr>
</tbody>
</table>

N.D., not detected; RH, rat hepatocytes; MH, mouse hepatocytes; DH, dog hepatocytes; MKH, monkey hepatocytes; HH, human hepatocytes.

M16 as an uncharacterized oxygenated metabolite was found in rabbit urine.

The structures of metabolites were identified based on LC-MS analyses and mass spectral comparison with those of parent and known metabolites.

Lactam ring-opened metabolites M3 and M5 were detected in hepatocyte incubations but not in vivo in mouse or human.

Table 3

Distribution of radioactive metabolites in pooled plasma after an oral dose of [14C]apixaban to intact mice, rats, rabbits, dogs, and humans

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Mouse 1 h</th>
<th>Mouse 4 h</th>
<th>Rat 1 h</th>
<th>Rat 4 h</th>
<th>Rabbit 1 h</th>
<th>Rabbit 4 h</th>
<th>Dog 1 h</th>
<th>Dog 4 h</th>
<th>Dog 12 h</th>
<th>Human 1 h</th>
<th>Human 6 h</th>
<th>Human 12 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apixaban</td>
<td>97.3</td>
<td>75.2</td>
<td>95.5</td>
<td>94.6</td>
<td>91.4</td>
<td>9.1</td>
<td>8.9</td>
<td>95.9</td>
<td>93.7</td>
<td>92.1</td>
<td>98.4</td>
<td>83.2</td>
</tr>
<tr>
<td>M1</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.4</td>
<td>0.9</td>
<td>23.4</td>
<td>4.2</td>
<td>N.D.</td>
<td>0.2</td>
<td>0.4</td>
<td>1.6</td>
<td>15.8</td>
<td>28.4</td>
</tr>
<tr>
<td>M2</td>
<td>0.6</td>
<td>3.1</td>
<td>0.6</td>
<td>0.9</td>
<td>1.1</td>
<td>29.2</td>
<td>17.4</td>
<td>1.1</td>
<td>2.5</td>
<td>2.5</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>M4</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.7</td>
<td>0.8</td>
<td>0.7</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
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</tr>
<tr>
<td>M7</td>
<td>0.4</td>
<td>2.8</td>
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<td>0.4</td>
<td>0.4</td>
<td>N.D.</td>
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<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
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<td>N.D.</td>
<td>N.D.</td>
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<td>M13</td>
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<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
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<td>N.D.</td>
<td>N.D.</td>
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<tr>
<td>M14</td>
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<td>N.D.</td>
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<td>53.3</td>
<td>N.D.</td>
<td>N.D.</td>
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<td>N.D.</td>
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<td>Others*</td>
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<td>1.2</td>
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<td>1.0</td>
<td>N.D.</td>
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<td>N.D.</td>
</tr>
<tr>
<td>Total#</td>
<td>100</td>
<td>100</td>
<td>97.1</td>
<td>97.1</td>
<td>94.5</td>
<td>100</td>
<td>100</td>
<td>97.0</td>
<td>96.5</td>
<td>96.0</td>
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<td>100</td>
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</tbody>
</table>

N.D., not detected.

*Unidentified components.

#Total was <100% because of unidentified minor peaks, each representing <0.1%. 

Human data for subjects without bile collection have been presented previously (Raghavan et al., 2009) and are included here for comparison. The samples at all other time points for rabbit, 12 h for mouse, and 24 and 48 h for all species were not analyzed because of low levels of radioactivity.
In Vitro Metabolism. Control incubations demonstrated the viability of the hepatocyte preparations as the activities for oxidative and conjugative metabolism of model substrates matched historical levels. Values for percentages of substrate metabolized at the end of the incubation period for hepatocytes from mice, rats, dogs, monkeys, and humans, respectively, were 16, 11, 83, 92, and 90%, respectively, using 7-ethoxycoumarin as an oxidation substrate and 29, 64, 76, 99, and 83%, respectively, using 7-hydroxycoumarin as a conjugation substrate.

Apixaban metabolite structures are shown in Table 2, and the distribution of the metabolites in the hepatocyte incubations is shown in Table 5. Overall, [14C]apixaban showed a low rate of in vitro metabolism. The metabolite profiles of all five species were qualitatively similar, with unchanged apixaban remaining as the major radioactive component. Seven radioactive metabolites, designated M1 to M7, were characterized or identified in vitro. M6 was proposed as a carboxylic acid derivative resulting from hydrolysis of the amide bond and was only observed in the mouse and rat hepatocytes but not in any other hepatocytes or any in vivo matrices. Six human heptocyte metabolites, M1 to M5 and M7, were all found to be present in animal hepatocyte incubations. The major in vitro metabolic routes for apixaban included O-demethylation (M2) followed by sulfate conjugation (M1) and monooxidation to form hydroxy apixaban (M4 and M7).

Identification of Apixaban Metabolites. Metabolites were characterized by LC-MS and LC-MS/MS analyses from plasma, urine, bile, and feces of all species. The proposed prominent metabolic pathways of apixaban are shown in Fig. 2. The proposed structures of

### TABLE 4

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Mouse</th>
<th>Rat</th>
<th>Rabbit</th>
<th>Dog</th>
<th>Human</th>
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<tr>
<td>Apixaban</td>
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<td>73.2</td>
<td>12.1</td>
<td>65.9</td>
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<tr>
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<td>Trace</td>
<td>N.D.</td>
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<td>2.9</td>
<td>1.4</td>
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<tr>
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<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
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<td>0.5</td>
<td>0.3</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>M5</td>
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<td>3.5</td>
<td>0.2</td>
<td>2.2</td>
<td>N.D.</td>
</tr>
<tr>
<td>M6</td>
<td>N.D.</td>
<td>N.D.</td>
<td>Trace</td>
<td>N.D.</td>
<td>0.1</td>
</tr>
<tr>
<td>M7</td>
<td>0.1</td>
<td>0.5</td>
<td>0.3</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
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<td>0.1</td>
<td>0.5</td>
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<td>0.4</td>
<td>N.D.</td>
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<tr>
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<td>0.1</td>
<td>0.5</td>
<td>0.3</td>
<td>0.4</td>
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</tr>
<tr>
<td>M10</td>
<td>0.1</td>
<td>0.5</td>
<td>0.3</td>
<td>0.4</td>
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<td>0.4</td>
<td>N.D.</td>
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<tr>
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<td>0.4</td>
<td>N.D.</td>
</tr>
<tr>
<td>M13</td>
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<td>0.3</td>
<td>0.4</td>
<td>N.D.</td>
</tr>
<tr>
<td>M14</td>
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<td>N.D.</td>
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<tr>
<td>M15</td>
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<tr>
<td>M16</td>
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<td>0.5</td>
<td>0.3</td>
<td>0.4</td>
<td>N.D.</td>
</tr>
<tr>
<td>Others</td>
<td>Trace</td>
<td>N.D.</td>
<td>Trace</td>
<td>N.D.</td>
<td>Trace</td>
</tr>
<tr>
<td>Total</td>
<td>15.2</td>
<td>83.9</td>
<td>13.0</td>
<td>71.8</td>
<td>1.8</td>
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</tbody>
</table>

**N.D., not detected; Trace, radioactive peak observed but <0.1%.
Unidentified components.**

**Total was <100% because of unidentified minor peaks, each representing <0.1%.
Including M14 (0.22%) and M16 (0.03%).**
Apixaban metabolites from the in vitro and in vivo samples in which they were found are shown in Table 2.

Apixaban. Apixaban showed a molecular ion \([\text{M} + \text{H}]^+\) at \(m/z\) 460 and fragment ions at \(m/z\) 443, 417, 402, 199, and 184 in LC/MS and MS/MS analyses.

Metabolites M2 and M1. M1 and M2 showed molecular ions \([\text{M} + \text{H}]^+\) at \(m/z\) 526 and 446 and major fragment ions at \(m/z\) 446 and 429, respectively. As described previously (Raghavan et al., 2009), M1 was identified as a sulfate conjugate of M2. Metabolite M2 was identified as O-demethyl apixaban.

Metabolite M3. M3 showed a molecular ion \([\text{M} + \text{H}]^+\) at \(m/z\) 492 and major fragment ions at \(m/z\) 474 and 378. M3 was consistent with oxidation followed by lactam ring opening (Table 2). The major fragment ion at \(m/z\) 378 was consistent with the structure of 1-

N.D., not detected.

*a* Unidentified peaks.

**FIG. 2.** Proposed prominent metabolic pathways of apixaban in animals and humans.
apixaban, suggesting that these were products of monooxidation. Metabolites M4, M7, and M9 showed a molecular ion $[M + H]^+$ at m/z 476, 16 amu higher than apixaban, suggesting that these were products of monooxidation. M4, M7, and M9 had fragment ions at m/z 459, 441, 415, 371, 199, and 177 in the MS2 spectrum and a characteristic fragment ion at m/z 199 in the MS3 spectrum for the proposed fragment ion shown in the Supplemental Fig. S3, A to C. Based on the retention time and LC-MS/MS fragmentation pattern, M4, M7, and M9 were identified as isomers of hydroxylated apixaban. A hydrogen exchange experiment was conducted with M7. LC-MS analysis of M7 after hydrogen/deuterium exchange yielded $[M + D]^+$ at m/z 480, an increase of 4 amu to its molecular ion, indicating that M7 contained one additional exchangeable proton in addition to two exchangeable protons in apixaban. This result further supported M7 containing a hydroxyl group.

Metabolites M4, M7, and M9. Metabolites M4, M7, and M9 showed a molecular ion $[M + H]^+$ at m/z 476, 16 amu higher than apixaban, suggesting that these were products of monooxidation. M4, M7, and M9 had fragment ions at m/z 459, 441, 415, 371, 199, and 177 in the MS2 spectrum and a characteristic fragment ion at m/z 199 in the MS3 spectrum for the proposed fragment ion shown in the Supplemental Fig. S3, A to C. Based on the retention time and LC-MS/MS fragmentation pattern, M4, M7, and M9 were identified as isomers of hydroxylated apixaban. A hydrogen exchange experiment was conducted with M7. LC-MS analysis of M7 after hydrogen/deuterium exchange yielded $[M + D]^+$ at m/z 480, an increase of 4 amu to its molecular ion, indicating that M7 contained one additional exchangeable proton in addition to two exchangeable protons in apixaban. This result further supported M7 containing a hydroxyl group.

Metabolite M7 (approximately 80 μg) was generated and isolated from a large incubation of $^{14}$C-apixaban in a cDNA-expressed CYP3A4 incubation. The site of hydroxylation was determined by proton-NMR analysis. The proposed structure based on the NMR data was consistent with hydroxylation at carbon 30. By Comparing the aliphatic region of the one-dimensional $^1$H NMR spectrum of the metabolite with that of the parent compound, the resonance for the chemical shift of the signal for the methylene group at position 30 moved downfield from 1.90 to 4.24 ppm, and its integration changed from two protons to one proton (Fig. 3). The coupling pattern in the double quantum-filtered correlation spectroscopy experiment confirmed this hydroxylation at carbon 30. The cross peak between protons 28, 29, and 31 are labeled as regions A, B, and C.
Metabolite M5. M5 showed a molecular ion \([M + H]^+\) at \(m/z\) 478 and major fragment ions at \(m/z\) 458, 432, and 2342. M5 was consistent with lactam ring opening (Table 2). The major fragment ion at \(m/z\) 378 was consistent with the structure of 1-(4-hydroxyphenyl)-7-oxo-6-(4-(2-oxyopiperidin-1-yl)phenyl) amine. Upon HPLC analysis on a reverse-phase C18 column, this metabolite had a much shorter retention time with a mobile phase at pH 6.8 than at pH 3.2, supporting a carboxylic acid moiety in this metabolite. Based on the HPLC retention and MS fragmentation patterns, M5 was proposed as a metabolite resulting from lactam ring opening.

Metabolite M6. M6 showed a molecular ion \([M + H]^+\) at \(m/z\) 461, 1 amu higher than the parent, suggesting loss of a nitrogen as judged by the nitrogen rule. The major fragment ion was at \(m/z\) 417, consistent with loss of 44 amu, a carboxylic acid moiety. These data are consistent with amide hydrolysis of apixaban (Table 2). In addition, upon HPLC analysis on the reverse-phase C18 column, this metabolite had a much shorter retention time with a mobile phase at pH 6.8 than at pH 3.2, supporting a carboxylic acid moiety in this metabolite. Based on these HPLC retention and fragmentation patterns, M6 was proposed as a carboxylic acid resulting from amide hydrolysis.

Metabolite M13 and M8. M13 and M8 showed molecular ions \([M + H]^+\) at \(m/z\) 462, 16 amu higher than M2 by LC-MS. The addition of 16 amu from the protonated molecular ion of \(m/z\) 446 suggests that M13 is hydroxylated O-demethyl apixaban. The fragmentation ions of M8 were at \(m/z\) 445, 419, 401, 363, 333, and 185 (\(m/z\) at 185 was a characteristic fragment ion for O-demethyl apixaban) (Raghavan et al., 2009). Accurate mass measurement of M13 gave a molecular ion of 462.1777 and a derived formula of \(C_{24}H_{23}N_{2}O_{6}\). The difference between the measured mass and the theoretical mass was \(-1.7\) Da. These data supported M8 and M13 being isomers of hydroxylated O-demethyl apixaban.

Metabolite M10. Metabolite M10 showed a molecular ion \([M + NH_4]^+\) at \(m/z\) 559 that was 80 amu higher than M13 by accurate mass LC-MS. An accurate mass measurement of M10 gave a molecular ion of 559.1628 and a derived formula of \(C_{24}H_{27}N_3O_6\). The difference between the measured mass and the theoretical mass was \(-1.7\) Da. The metabolite was proposed as a sulfate conjugate of hydroxylated O-demethyl apixaban.

Metabolite M14. M14 showed a molecular ion \([M + H]^+\) at \(m/z\) 622, and LC-MS/MS analysis showed a product ion at \(m/z\) 446. The loss of 176 from the protonated molecular ion of \(m/z\) 622 suggests that M14 is a glucuronide of M2. Based on the retention time and LC-MS/MS fragmentation, M14 was proposed as a glucuronide of O-demethyl apixaban.

Discussion

This study reports the comparative disposition and in vitro and in vivo metabolic profiles of \(^{14}C\)apixaban in mice, rats, rabbits, dogs, and humans. Animal species used for toxicity testing of apixaban were used for these metabolism studies. Metabolism of apixaban in hepatocytes incubations appeared to be slow, and the relatively low rate of turnover in hepatocytes was consistent with the low clearance of apixaban in corresponding species (Shantsila and Lip, 2008; Raghavan et al., 2009). Metabolism of apixaban in human hepatocytes produced primarily an O-demethylation metabolite (M2), a sulfmate metabolite (M1), and a hydroxylated metabolite (M7). Similar metabolites were produced in incubations of apixaban with hepatocytes from animal species (mouse, rat, dog, or monkey). In vivo, prominent oxidative metabolic pathways for apixaban were O-demethylation to form M2 and hydroxylation to form M7 and M4 in all species. The glucuronide of O-demethyl apixaban (M14) was a circulating metabolite in plasma of mouse but was not detected in mouse urine or feces probably because of a low concentration of this metabolite in mouse extera. Different from rat, dog, or human, the rabbit showed that the glucuronide of O-demethyl apixaban (M14) was a prominent component in plasma. A significant amount of this glucuronide was found in the rabbit urine samples although potential intestinal hydrolysis might underestimate the amount of this glucuronide in feces. The glucuronidation pathway for metabolism of apixaban was only found in the mouse and rabbit. In addition, the minor hydroxylation metabolite M9 was only detected in the mouse and rat, and the hydroxy O-demethyl metabolite M8 was only detected in the rat and dog. The metabolism of apixaban in vivo was more complicated than that seen in vitro with hepatocytes in terms of relative abundance and sequential metabolism. For humans, additional oxidative (formation of M13) and sulfate conjugation pathways (formation of M10) were found in in vivo samples. In addition, the presence of a major circulating human metabolite (M1) would not have been predicted from the in vitro results. The major in vivo metabolic pathways for apixaban were qualitatively similar across species but quantitatively different between species. There were sequential metabolites such as M8, M10, M13, and M14 that were not detected in the in vitro incubations, yet there were also the very minor in vitro metabolites M5 and M6, involving the lactam ring opening or amide hydrolysis, that were not identified in vivo probably because very low quantities of these metabolites were formed in vivo. The in vitro metabolite M3 was only found in the rat and dog.

O-Demethyl apixaban sulfate (M1) was a second major radioactive component in human plasma after oral administration. This metabolite was found at much lower abundance relative to the parent in the plasma of mouse, rat, or dog. It is unclear what drives the difference in disposition and makes this metabolite a prominent circulating metabolite in humans, but not in animals as all species were capable of the demethylation and sulfate conjugation reactions based on the in vitro data (Wang et al., 2009). In addition, M1 was a prominent metabolite in rat bile. Metabolites M1 and M2 were tested and would be inactive factor Xa inhibitors (Wang et al., 2009). Although M1 was disproportionately circulating in humans compared with animal species with the given doses, no additional safety testing was needed because it was a stable and inactive conjugation metabolite (Food and Drug Administration Guidance for Industry: Safety Testing of Drug Metabolites, February 2008, http://www.fda.gov/Drugs/Guidances/).

Based on the radioactivity profiles of urine, feces, and bile, direct intestinal and urinary excretion as well as oxidative metabolism appeared to be important routes of elimination for apixaban in rats. After intravenous administration of \(^{14}C\)apixaban to bile duct-cannulated rats, the large portion (approximately 22%) of the dose recovered as the parent drug in the BDC rat feces suggests direct excretion of the drug through the gastrointestinal tract as an important elimination pathway of apixaban in rats. The importance of the gastrointestinal excretion of apixaban in other species is not known at this time. Evidence has accumulated in the literature to suggest that direct intestinal secretion may play a major part in elimination of a number of structurally diverse drugs including digoxin, paclitaxel (Taxol), and doxorubicin (Mayer et al., 1996; Rabbab et al., 1996; Sparreboom et al., 1997; van Asperen et al., 2000; Merino et al., 2005; Garner et al., 2008). Although direct intestinal excretion was not commonly documented in humans because of the difficulty in making this assessment in humans, apixaban was demonstrated to show a significant level of direct excretion from gastrointestinal tracts through a bile cannulation study in rats.

The in vivo analysis showed that the parent compound was the major drug-related component in plasma of mouse, rat, dog, and human after oral administration of apixaban. In contrast, apixaban was...
only detected at early time points in rabbits after oral administration and was quickly cleared from the circulation. Apixaban had a very poor oral bioavailability (of approximately 3%) in rabbits. The systemic clearance of apixaban was also high in the rabbit (2.55 l/h/kg, representing approximately 61% of hepatic blood flow). This pharmacokinetic profile in rabbit was very different from that in the rat, dog, and human in which apixaban showed low clearance (representing <10, <3, and <4% of hepatic blood flow, respectively), low volume of distribution, and good oral bioavailability (34, 88, and 60%, respectively) (Shantisila and Lip, 2008). Such a distinct pharmacokinetic profile was attributed to extensive first-pass metabolism of apixaban in the rabbit compared with low metabolic clearance in other species.

In summary, these studies demonstrated that the metabolic pathways in the toxicological species were generally similar to those seen in humans although rabbit showed a much higher metabolic clearance than other species. Apixaban was the most prominent drug-related component in circulation of mice, rats, dogs, and humans. Apixaban clearance was demonstrated to be mediated by oxidative metabolism as well as direct renal and gastrointestinal excretion.

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References


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