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

Donglu Zhang, Kan He,1 Nirmala Raghavan, Lifei Wang, James Mitroka, Brad D. Maxwell, Robert M. Knabb, Charles Frost, Alan Schuster, Feng Hao, Zheming Gu, W. Griffith Humphreys, and Scott J. Grossman


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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. 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 (O-demethyl apixaban) and M14 (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; 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.
proof of principle for the efficacy and potential of factor Xa inhibitors is available from fondaparinux, an indirect factor Xa inhibitor (Wa-
lenge et al., 1988; Samama and Gerotziafas, 2003; Simoons et al.,
2004; Wong et al., 2006; Yusu7 et al., 2006a,b; Schumacher et al.,
2007) for prescribing information for fondaparinux sodium (Xrix-
tra) injection, see http://us.gsk.com/products/assets/us_arixtra.pdf).

Apixaban is a promising oral anticoagulant in late-stage clinical
development for the prevention and treatment of venous thrombo-
embolism, stroke prevention in atrial fibrillation, and secondary preven-
tion 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 ef-
cy 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 pulmo-
nary 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 of apixaban
was 0.31 and 0.2 to 0.29 l/kg in rats and dogs, respectively. The
systemic clearance of apixaban was low, <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
(Irvine, 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 K3EDTA. 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 (30 μCi/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), 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. 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, 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 bicarbonate 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 col-
clected 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 5325 (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 admin-
istration. 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 in 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
these 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 biotransfor-
mation 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 [sincalide (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 plate cultures (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-ethoxycoumarin 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 acetone/ether, 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 ml of acetone/ether-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 acetone/ether 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 combusted. Fecal samples were thawed and a water-ethanol (50:50, v/v) solution was added to form an approximate 20% (w/w) feces/solvent homogenate. The volume of bile and urine and weight of feces collected over each interval and the concentrations of radioactivity in the corresponding samples were used to calculate the cumulative percentage of the administered dose recovered in the bile, urine, and feces for the estimation of biliary, urinary, and fecal elimination of radioactivity.

**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 acetone/ether-methanol (1:1, v/v) to 1 ml of plasma, and the sample was mixed 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 acetone 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 acetonitrile and 1 ml of methanol. After centrifugation of the precipitate mixture for 30 min at 2000g, 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 acetone/ether 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 at room temperature and mixed 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 quadrupole time 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-acetonitrile (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% acetonitrile (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 quantification of radioactivity, HPLC effluent was collected into Deepwell LumaPlate-96 96-well plates in 0.26-min 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 TopCount 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 at each time point 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.

**Metabolite Identification and Quantification.** Bile, urine, and the extracts of pooled plasma and fecal samples were analyzed by LC-MS using a Finnigan LTQ ion trap mass spectrometer (Thermo Fisher Scientific). LC-MS analysis was performed with an electrospray ionization probe in the positive ion mode. The HPLC eluate was directed to the mass spectrometer through a valve set to divert the flow from 0 to 5 min. The eluate flow was directed to the mass spectrometer from 5 min until the end of the HPLC run. The capillary temperature for analysis was set to 230°C. The nitrogen gas flow rate, spray current, and voltages were adjusted to give the maximum sensitivity for apixaban. Bile and plasma samples were also analyzed using quadrupole time of flight mass spectrometry (Waters). The collision energies used for MS and MS/MS were 10 and 25 V, respectively. The mass reference was leucine enkephalin.

**Incubation, Isolation, and NMR Analysis of M7.** [14C]Apixaban at a concentration of 250 μM (1 μCi/μg) was incubated with CDNA-expressed CYP3A4 Supersones 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 μl. Aliquots of 2.5 μl 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, acetone/nitrite (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-μl 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 acetone/nitrite 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 acetone/nitrite. 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 TXI 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
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

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

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.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Mouse</th>
<th>Rat</th>
<th>Rabbit</th>
<th>Dog</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
<td>4 h</td>
<td>1 h</td>
<td>4 h</td>
<td>12 h</td>
</tr>
<tr>
<td>Apixaban</td>
<td>97.3</td>
<td>75.2</td>
<td>95.5</td>
<td>94.6</td>
<td>91.4</td>
</tr>
<tr>
<td>M1</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.4</td>
<td>0.9</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>
</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>
</tr>
<tr>
<td>M7</td>
<td>0.4</td>
<td>2.8</td>
<td>0.3</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>M10</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.4</td>
<td>N.D.</td>
</tr>
<tr>
<td>M13</td>
<td>0.7</td>
<td>5.8</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>M14</td>
<td>0.4</td>
<td>2.4</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Others*</td>
<td>0.6</td>
<td>10.8</td>
<td>N.D.</td>
<td>N.D.</td>
<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>
</tr>
</tbody>
</table>

N.D., not detected.

*Unidentified components.

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

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**Table 2—Continued.**

<table>
<thead>
<tr>
<th>Drug or Metabolite</th>
<th>Proposed Structure</th>
<th>Source</th>
</tr>
</thead>
</table>
| M8 (hydroxy O-demethyl apixaban) | ![Proposed Structure](image) | Rat: urine, bile  
Mouse: N.D.  
Rabbit: N.D.  
Dog: urine, feces  
Monkey: N.D.  
Human: N.D. |
| M9 (hydroxy apixaban) | ![Proposed Structure](image) | Rat: urine, feces, bile  
Mouse: urine  
Rabbit: N.D.  
Dog: N.D.  
Monkey: N.D.  
Human: N.D. |
| M10 (hydroxy O-demethyl apixaban sulfate) | ![Proposed Structure](image) | Rat: N.D.  
Mouse: plasma  
Rabbit: N.D.  
Dog: N.D.  
Monkey: N.D.  
Human: plasma, feces |
| M13 (hydroxy O-demethyl apixaban) | ![Proposed Structure](image) | Rat: N.D.  
Mouse: plasma, urine  
Rabbit: N.D.  
Dog: N.D.  
Monkey: N.D.  
Human: feces |
| M14 (O-demethyl apixaban glucuronide) | ![Proposed Structure](image) | Rat: N.D.  
Mouse: plasma  
Rabbit: plasma, urine  
Dog: N.D.  
Monkey: N.D.  
Human: N.D. |

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.

2 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.
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 hepatocyte 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.
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 \([M + H]^{+}\) 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 \([M + H]^{+}\) 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 \([M + H]^{+}\) 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-

**TABLE 5**

Distribution of radioactive metabolites in incubations of [14C]apixaban with hepatocytes from mouse, rat, dog, monkey, and human

Control incubations contained 0.3 to 0.7% of M7 and 2.7 to 4.4% of other unidentified peaks.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Mouse</th>
<th>Rat</th>
<th>Dog</th>
<th>Monkey</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 (\mu)M</td>
<td>10 (\mu)M</td>
<td>1 (\mu)M</td>
<td>10 (\mu)M</td>
<td>1 (\mu)M</td>
</tr>
<tr>
<td>Apixaban</td>
<td>91.2</td>
<td>93.3</td>
<td>88.1</td>
<td>91.1</td>
<td>86.6</td>
</tr>
<tr>
<td>M1</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.1</td>
<td>N.D.</td>
</tr>
<tr>
<td>M2</td>
<td>0.7</td>
<td>0.5</td>
<td>0.5</td>
<td>0.6</td>
<td>1.4</td>
</tr>
<tr>
<td>M3</td>
<td>0.2</td>
<td>0.2</td>
<td>N.D.</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>M4</td>
<td>0.1</td>
<td>0.2</td>
<td>N.D.</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>M5</td>
<td>1.1</td>
<td>0.8</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.5</td>
</tr>
<tr>
<td>M6</td>
<td>0.1</td>
<td>0.1</td>
<td>0.3</td>
<td>0.6</td>
<td>N.D.</td>
</tr>
<tr>
<td>M7</td>
<td>0.4</td>
<td>0.4</td>
<td>0.6</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>Others*</td>
<td>6.2</td>
<td>4.5</td>
<td>10.5</td>
<td>6.5</td>
<td>10.1</td>
</tr>
</tbody>
</table>

N.D., not detected.

*Unidentified peaks.

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Fig. 2. Proposed prominent metabolic pathways of apixaban in animals and humans.
(4-hydroxyphenyl)-7-oxo-6-(4-(2-oxopiperidin-1-yl)phenyl) amine. Upon HPLC analysis on a reverse-phase C18 column, the 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 time and MS fragmentation patterns, M3 was proposed as a metabolite resulting from oxidation followed by ring opening.

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 [¹⁴C]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 ¹H 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-oxopiperidin-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 \(C_{24}H_{22}N_2O_4\). The difference between the measured mass and the theoretical mass was \(-1.3\) mDa. 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. Accurate mass measurement of M10 gave a molecular ion of 559.1628 and a derived formula of \(C_{24}H_{22}N_2O_4\). The difference between the measured mass and the theoretical mass was \(-1.7\) mDa. The metabolite was proposed as a sulfate conjugate of hydrated 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 \([14C]\)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 sulfate 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 excreta. 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 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 \([14C]\)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 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) (Shantsila 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.

Acknowledgments. We thank Robert Espina for conducting NMR analysis.

References
Wang L, Raghavan N, He K, Luettgen JM, Humphreys WG, Knabb RM, Pinto D, and Zhang D (2009) Sulfation of R[(2S, 7S)-1R, 5R, 6S, 4S]-4% of hepatic blood flow, respectively), low volume of distribution, and good oral bioavailability (34, 88, and 60%, respectively) (Shantsila 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.

Acknowledgments. We thank Robert Espina for conducting NMR analysis.

References