Apixaban Metabolism and Pharmacokinetics after Oral Administration to Humans

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ABSTRACT:
The metabolism and disposition of [14C]apixaban, an orally bioavailable, highly selective, and direct acting/reversible factor Xa inhibitor, was investigated in 10 healthy male subjects without (group 1, n = 6) and with bile collection (group 2, n = 4) after a single 20-mg oral dose. Urine, blood, and feces samples were collected from all subjects. Bile samples were also collected for 3 to 8 h after dosing from group 2 subjects. There were no serious adverse events or discontinuations due to adverse effects. In plasma, apixaban was the major circulating component and O-demethyl apixaban sulfate, a stable and water-soluble metabolite, was the significant metabolite. The exposure of apixaban (C_{max} and area under the plasma concentration versus time curve) in subjects with bile collection was generally similar to that in subjects without bile collection. The administered dose was recovered in feces (group 1, 56.0%; group 2, 46.7%) and urine (group 1, 24.5%; group 2, 28.8%), with the parent drug representing approximately half of the recovered dose. Biliary excretion represented a minor elimination pathway (2.44% of the administered dose) from group 2 subjects within the limited collection period. Metabolic pathways identified for apixaban included O-demethylation, hydroxylation, and sulfation of hydroxylated O-demethyl apixaban. Thus, apixaban is an orally bioavailable inhibitor of factor Xa with elimination pathways that include metabolism and renal 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). However, 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 (PK) 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; Kubitza and Haas, 2006). Clinical proof of principle for the efficacy and potential of factor Xa inhibitors is available from fondaparinux, an indirect factor Xa inhibitor (Walenga et al., 1998; Samama and Gerotziafas, 2003; Simoons et al., 2004; Wong et al., 2006; Yusuf et al., 2006a,b; Schumacher et al., 2007) [prescribing information for Arixtra (fondaparinux sodium) injection, http://us.gsk.com/products/assets/us_arixtra.pdf].

Apixaban is an orally bioavailable, highly selective, direct acting/reversible factor Xa inhibitor in late-stage clinical development for the prevention and treatment of thromboembolic diseases. Apixaban has a high affinity for human factor Xa with a relatively low affinity for...
thrombin and trypsin; the high affinity and selectivity of apixaban may translate into improved pharmacologic characteristics versus those of the preceding oral factor Xa compounds, leading to an improved treatment option (Pinto et al., 2007; Wong et al., 2008).

Positive 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 (Lassen et al., 2007; Büller et al., 2008). Apixaban demonstrated linear pharmacokinetics in the range of 2.5- to 25-mg oral doses in humans (Frost et al., 2007a). Here, we evaluated the metabolism and PK of apixaban after oral administration in healthy volunteers.

Materials and Methods

Study Design. This was an open-label single-dose study in healthy male subjects 18 to 45 years of age. All subjects were in good health as determined by medical history, physical examination, vital sign and electrocardiogram assessment, and clinical laboratory tests. Subjects with a previous medical history of coagulopathy or adverse reaction to anticoagulant or antiplatelet agents were excluded. Subjects who met all inclusion and exclusion criteria and provided informed written consent were admitted to the clinical facility. Subjects were assigned to one of two groups, group 1 (n = 6) or group 2 (n = 4). In addition to other study procedures, bile samples were collected from subjects in group 2 after a single dose of 20 mg of [14C]apixaban containing 108.8 µCi of radioactivity as an oral solution in polyethylene glycol 400-ethanol (85:15, v/v). All subjects remained in the clinical facility for 12 days. Urine and fecal samples were collected continuously over 24-h intervals up to 240 h after or until radioactivity in the sample was ≤1% of the administered dose. Bile samples were collected by continuous suction through a nasogastric tube at 3 to 4, 6 to 6, and 6 to 8 h postdose from subjects in group 2. Blood samples were collected at 1, 6, 12, 24, 48, and 96 h postdose in all subjects for biotransformation analysis. Blood samples for measuring apixaban and radioactivity were collected at 0, 0.5, 1, 1.5, 2, 4, 6, 8, 12, 24, 36, 48, 96, 120, 144, 168, and 192 h postdose. Subjects were discharged from the clinic in the afternoon of day 13 provided that the day 7 measurement of radioactivity in feces was ≤1% of administered radioactivity. To ensure defecation before discharge a 30-ml dose of milk of magnesia was administered on day 6. All subjects were closely monitored for adverse events throughout the study.

The study was performed in accordance with local regulations and the principles of the Declaration of Helsinki, its amendments, and Good Clinical Practice. The protocol and informed consent were approved by the New England Institutional Review Board (Wellesley, MA). The clinical phase of the study was conducted at Clinical Applications Laboratories Inc. (San Diego, CA).

Radiolabeled Drug and Chemicals. Apixaban, BMS-562247 (purity 99.1%), and [14C]apixaban were synthesized at Bristol-Myers Squibb (Princeton, NJ). The specific radioactivity of [14C]apixaban was 5.44 µCi/mg (98.6% radiochemical purity).

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), and Ecolite liquid scintillation cocktail was purchased from MP Biomedicals Inc. (Irvine, CA). All other organic solvents and reagents were of high-performance liquid chromatography (HPLC) grade.

Synthesis of Metabolite Standards. Preparation of 1-(4-hydroxyphenyl)-7-oxo-6-(4-(2-oxopiperidin-1-yl)phenyl)-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]pyridine-3-carboxamide (O-demethyl apixaban). Apixaban (4.4 g, 9.5 mmol) was mixed with pyridine hydrochloride (6.6 g, 57 mmol) and heated at 200°C for 5 h. The reaction was cooled, and solid materials were suspended in a mixture of CH2Cl2-water (1:1, v/v, 100 ml). Sodium bicarbonate (10 g) was added to this suspension, and the insoluble solid precipitate was filtered to give 7.3 g of white powder. Approximately 4 g of this solid powder was subjected to purification via silica gel column chromatography (methanol-CH2Cl2, 1:9, v/v) to give 0.76 g of the phenol product. The balance of the crude phenol powder was suspended in aqueous NH4OH and washed with dilute acid (1 N HCl, 100 ml), dried (MgSO4), and purified by chromatography as described above to give additional phenol product (0.117 g). The overall yield for O-demethyl apixaban was 30.9%.

1H NMR (CDCl3) δ (ppm): 7.34 (2H, d, J = 8.8 Hz), 7.33 (2H, d, J = 8.8 Hz), 7.25 (2H, d, J = 8.6 Hz), 6.80 (1H, s), 6.76 (2H, d, J = 8.8 Hz), 6.35 (1H, s), 5.44 (1H, s), 4.10 (2H, t, J = 6.6 Hz), 5.87 (2H, t, J = 5.87 Hz), 3.38 (2H, t, J = 6.6 Hz), 2.57 (2H, t, J = 5.38 Hz), 1.94 (4H, m); HRMS calculated [M + H]+ 446.1828; found [M + H]+ 446.1817.

Preparation of 4-(3-carbamoyl-7-oxo-6-(4-(2-oxopiperidin-1-yl)phenyl)-4,5,6,7-tetrahydropyrazolol[3,4-c]pyridin-1-yl)phenyl hydrogen sulfate (O-demethyl apixaban sulfate). To O-demethyl apixaban (0.48 g, 1.07 mmol) in pyridine (2 ml) was added sulfur trioxide in triethylamine (0.45 g, 3.2 mmol), and the reaction was stirred at room temperature for 72 h. An additional (0.1 g, 0.71 mmol) of sulfur trioxide-triethylamine mixture was added, and the reaction was stirred for an additional 24 h. The reaction was diluted with diethyl ether, and the precipitated solid was filtered off. The filtrate was dissolved in water (50 ml), and the pH was adjusted with 1 N NaOH until pH = 8. The aqueous solution was then extracted with CH2Cl2/toluene (25 ml washes) to remove unreacted phenol. The pH was again adjusted with 1 N HCl (pH = 5), and this solution was used directly onto a reverse-phase HPLC system using water-CH3CN with 0.05% trifluoroacetic acid for purification. The isolated product fractions were concentrated in vacuum and freeze-dried. Analysis of the recovered solid showed some phenol O-demethyl apixaban present. The sample was again repurified via reverse-phase HPLC using water with 0.01 M ammonium acetate buffer and CH3CN. The pure fractions were concentrated with a stream of N2 and freeze-dried to give 231 mg (39%) of the ammonium salt of O-demethyl apixaban sulfate (purity 90.0%).

3H NMR (dimethyl sulfoxide-δ6, δ ppm): 7.74 (1H, s), 7.49 (2H, d, J = 8.8 Hz), 7.42 (1H, s), 7.38 (2H, d, J = 8.8 Hz), 7.29 (2H, d, J = 8.8 Hz), 7.24 (2H, d, J = 8.8 Hz), 7.09 (5H, br s), 4.08 (2H, t, J = 6.6 Hz), 3.62 (2H, t, J = 5.6 Hz), 3.23 (2H, t, J = 6.6 Hz), 2.41 (2H, t, J = 6.1 Hz), 1.87 (4H, m); HRMS calculated C19H27N4O8S [M + H]+ 526.1396; found [M + H]+ 526.1379.

Collection and Preparation of Blood, Bile, Urine, and Feces Samples. Blood samples. Blood samples were collected via an indwelling catheter by or direct venipuncture using Vacutainers containing K3EDTA as coagulant and immediately placed on ice. Duplicate gravimetric aliquots (~100 mg) of whole blood samples were combusted in a sample oxidizer before scintillation counting for total radioactivity (TRA). The remaining blood sample was centrifuged to harvest plasma. All plasma samples were frozen and stored at −20°C. Duplicate gravimetric aliquots (~100 mg) of each plasma sample were analyzed for total radioactivity.

Pooled plasma samples (at 1, 6, 12, 24, 48, and 96 h) from each group were prepared separately by mixing an equal volume (0.5 ml) of plasma sample from each subject. Each pooled plasma sample was extracted in duplicate at each time point by addition of 4 ml of acetonitrile-methanol (1:1, v/v) to 1 ml of plasma, whereas the sample was mixed on a vortex mixer; each supernatant fraction was removed and saved. The precipitate was then resuspended in 2 ml of acetonitrile and 1 ml of methanol. After centrifugation the supernatant fraction was removed and combined with the first supernatant. The final precipitate was resuspended in 2 ml of acetonitrile. After centrifugation the supernatant fraction was removed and combined with the first and second supernatants. Extraction recovery of radioactivity (percentage of sample) from pooled human plasma after an oral dose of [14C]apixaban was calculated as radioactivity in the extract divided by radioactivity in the sample. The average extraction recovery from the replicate (n = 2) plasma samples was 85 to 95% for all time points. The combined supernatant fraction was evaporated to dryness under nitrogen and reconstituted in 0.15 ml of acetonitrile and 0.05 ml of methanol. After centrifugation, a 0.06- to 0.1 ml portion of supernatant was injected into the HPLC system.

Bile samples. Bile samples were collected from group 2 subjects using a direct venipuncture catheter or by direct venipuncture using Vacutainers containing K3EDTA as coagulant and immediately placed on ice. Duplicate gravimetric aliquots (~100 mg) of each plasma sample were analyzed for total radioactivity.

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contraction and enhance bile flow. Bile was collected for 3 to 8 h postdose. The bile samples were immediately frozen and stored at −70°C. Before analysis, the bile was thawed at room temperature and diluted with water (1:1). Portions of 0.1 ml were injected into the HLPC system for ion trap analysis and 0.03 ml was used for quadrupole time of flight (Q-TOF) mass spectrometry (MS) analysis.

**Urine and fecal samples.** All urine and feces samples were collected, frozen, and stored at −20°C. Pooled urine samples were prepared by combining 10% of each collection by volume from all subjects from each group. Before analysis, the urine was thawed at room temperature. Centrifugation was then performed and 0.1 ml of urine was injected into the HLPC system. Water-reagent ethanol (50:50, v/v) was added to each fecal sample to form an approximate 20% (w/w) feces-solvent mixture, which was homogenized using a probe-type homogenizer. Fecal homogenates were stored at −20°C. Pooled feces sample were prepared by combining 5% by weight from all collections from each subject of each group. Each pooled fecal homogenate sample (0.5 ml) was extracted in replicates by addition of 1.5 ml of methanol-acetonitrile (1:2, v/v) while the sample was mixed on a vortex mixer. The extraction was repeated, and the supernatants were combined. Extraction recovery from the replicate (n = 4) fecal samples was 86 to 93%.

**Measurement of Radioactivity.** The levels of total radioactivity in blood, plasma, urine, bile, and feces were determined by liquid scintillation counting after an aliquot of each sample was combusted. Sample combustion was performed with a sample oxidizer (PerkinElmer Life and Analytical Sciences). The resulting $^{14}$CO$_2$ was trapped with Carbo-Sorb E (PerkinElmer Life and Analytical Sciences) and mixed with Permafluor E+ (PerkinElmer Life and Analytical Sciences) scintillation fluid, and the radioactivity was quantified over 5 min using liquid scintillation counting. Liquid scintillation counter data were automatically corrected for counting efficiency using an external standardization technique and an instrument-stored quench curve was generated from a series of sealed quench standards.

The combustion efficiency was determined before combustion of experimental samples using a commercial $^{14}$C standard. In particular, $^{14}$C-fortified scintillation fluid or fecal homogenate samples fortified with the same amount of radioactivity at three levels of radioactivity (low, medium, and high standards) were combusted and then counted. The average disintegrations per minute recovered after combustion were then compared in the scintillation fluid or the fecal homogenate to determine the combustion efficiency value. Combustion efficiency was within 100 ± 5% (100 ± 10% for feces).

**Measurement of Apixaban Concentrations.** The concentrations of apixaban in plasma were determined by a validated LC-MS/MS method using 0.2 ml of plasma and $^{13}$CD$_3$-labeled apixaban as the internal standard. The lower limit of quantitation of apixaban in plasma under these conditions was 1 ng/ml.

**TABLE 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Pharmacokinetic Parameters</th>
<th>C$_{max}$</th>
<th>AUC$_{0-24}$</th>
<th>AUC$_{t}$</th>
<th>$T_{max}$</th>
<th>t½</th>
<th>CL/F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total radioactivity</td>
<td>All subjects (n = 10)</td>
<td>509 (16)</td>
<td>5305 (24)</td>
<td>5872 (23)</td>
<td>1.00 (0.50–2.00)</td>
<td>8.2 ± 1.69</td>
<td>3405</td>
</tr>
<tr>
<td>Group 1 (n = 6)</td>
<td>513 (20)</td>
<td>5087 (26)</td>
<td>5664 (24)</td>
<td>1.00 (0.50–2.00)</td>
<td>8.2 ± 1.59</td>
<td>3531</td>
<td></td>
</tr>
<tr>
<td>Group 2 (n = 4)</td>
<td>504 (11)</td>
<td>5649 (22)</td>
<td>6198 (23)</td>
<td>1.25 (0.50–2.00)</td>
<td>8.2 ± 2.09</td>
<td>3226</td>
<td></td>
</tr>
<tr>
<td>Apixaban</td>
<td>All subjects (n = 10)</td>
<td>469 (17)</td>
<td>4050 (25)</td>
<td>4101 (25)</td>
<td>1.00 (0.50–2.00)</td>
<td>12.7 ± 8.55</td>
<td>4876</td>
</tr>
<tr>
<td>Group 1 (n = 6)</td>
<td>480 (20)</td>
<td>4055 (30)</td>
<td>4100 (30)</td>
<td>1.00 (0.50–2.00)</td>
<td>12.2 ± 8.48</td>
<td>4878</td>
<td></td>
</tr>
<tr>
<td>Group 2 (n = 4)</td>
<td>453 (12)</td>
<td>4041 (20)</td>
<td>4103 (20)</td>
<td>1.25 (0.50–2.00)</td>
<td>13.5 ± 9.91</td>
<td>4874</td>
<td></td>
</tr>
</tbody>
</table>

Cl, clearance; F, bioavailability.
After solid extraction on a 3M Empore C8-SD 96-well solid extraction plate, HPLC on a Shimadzu LC-10AT system (Shimadzu Scientific Instruments, Kyoto, Japan) was performed isocratically on a Phenomenex Luna C18(2) analytical column (2.1 × 50 mm, 5 µ). The mobile phase contains 0.1% formic acid in acetonitrile and water (60:40, v/v) at 0.3 ml/min. The mass analysis was performed on a Sciex API3000 mass spectrometer (PE-Sciex, Concord, ON, Canada). The detection was by positive ion electrospray tandem mass spectrometry with the mass transition of m/z 460 to 443.

Stability Testing of M1. Synthetic metabolite M1 was fortified in human plasma at 10 and 500 ng/ml concentrations in triplicate. The metabolite was extracted from 100 µl of plasma by protein precipitation using 300 µl of acetonitrile and methanol (3:1, v/v). The supernatant was then diluted and analyzed by LC-MS/MS with a mass transition of 526.2 to 446.1 in a manner similar to that used for analysis of apixaban. Concentration standards were prepared similarly at 5, 10, 50, 100, 500, 800, and 1000 ng/ml synthetic M1. The stable isotope-labeled apixaban was used as the internal standard.

Pharmacokinetic Analysis. The mean plasma concentration versus time data for radioactivity and unchanged apixaban were analyzed with a noncompartmental method (Perrier and Gibaldi, 1982). The peak plasma concentration (Cmax) and the time to reach peak concentration (Tmax) were recorded directly from experimental observations. Total radioactivity was converted to apixaban nanogram-equivalents based on radioactive specific activity of [14C]apixaban (5.44 µCi/mg). The area under the plasma concentration versus time curve from 0 to T (AUC0–T), where T equals the time of the last measured plasma concentration, was calculated by a combination of conventional trapezoidal and log-trapezoidal methods using Kinetica (version 4.4). AUC to infinity (AUC0–∞) as determined by the sum of AUC0–T and the extrapolated area. The extrapolated area was determined by dividing the last measured concentration by the slope of the terminal log-linear phase. The terminal slope (α) of the plasma concentration-time profile was determined by the method of least squares (log-linear regression of at least three data points). The terminal half-life was estimated as ln2/α.

Metabolite exposure was estimated from the relative distribution of metabolite in the plasma, the specific activity (5.44 µCi/mg) of the administered apixaban, and the total concentration of radioactivity in plasma sample ob-

FIG. 2. Radiochromatograms of pooled plasma samples from subjects of group 1 (A) without bile collection and group 2 (B) with bile collection after oral administration of [14C]apixaban at the selected time points: 1, 6, 12, 24, and 48 h.

After solid extraction on a 3M Empore C8-SD 96-well solid extraction plate, HPLC on a Shimadzu LC-10AT system (Shimadzu Scientific Instruments, Kyoto, Japan) was performed isocratically on a Phenomenex Luna C18(2) analytical column (2.1 × 50 mm, 5 µ). The mobile phase contains 0.1% formic acid in acetonitrile and water (60:40, v/v) at 0.3 ml/min. The mass analysis was performed on a Sciex API3000 mass spectrometer (PE-Sciex, Concord, ON, Canada). The detection was by positive ion electrospray tandem mass spectrometry with the mass transition of m/z 460 to 443.

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Metabolite exposure was estimated from the relative distribution of metabolite in the plasma, the specific activity (5.44 µCi/mg) of the administered apixaban, and the total concentration of radioactivity in plasma sample ob-

FIG. 3. Radiochromatograms of pooled urine (0–240 h), bile (3–8 h), and fecal samples (0–240 h) from subjects of group 1 (A) without bile collection and group 2 (B) with bile collection after oral administration of [14C]apixaban.
tained for biotransformation analysis at limited time points (1, 6, 12, 24, and 48 h).

**Metabolite Profiles.** Sample analysis by HPLC was performed on a Shimadzu LC-10AT system equipped with a photodiode array UV detector. An Ace 3 C18 column (3 μm, 150 × 4.6 mm; MAC-MOD Analytical, Inc., Chadds Ford, PA) was used, with a mobile phase flow rate of 0.7 ml/min. An Ace 3 C18 column (3 μm, 150 × 2.1 mm; Mod-Mac Analytical, Inc.) was used for Q-TOF MS analysis, with a mobile phase flow rate of 0.17 ml/min. The retention times of reference standards were confirmed by their UV spectra. The HPLC solvent system was a gradient of two solvents of 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 and 50 to 100% B in 5 min; and hold 100% B for 5 min.

For quantification of radioactivity, the HPLC effluent was collected in 0.26-min intervals using a Gilson model 204 fraction collector (Gilson Medical Electronics, Middleton, WI). The plates were dried in an automatic environmental speed vacuum and counted for radioactivity for 10 min (counts per minute) per sample using a Packard TopCount NXT microplate scintillation and luminescence counter (PerkinElmer Life and Analytical Sciences). Radiochromatograms were reconstructed from the radioactivity in each collection versus collection times. 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.

Metabolism profiles were prepared by plotting the counts per minute values against time after injection. Radioactivity peaks in the metabolism profiles were reported as a percentage of the total radioactivity collected during the entire HPLC run.

**Metabolite Identification.** Bile, urine, and the extracts of pooled plasma and fecal samples were analyzed by LC-MS/MS using a Finnigan LTQ ion trap mass spectrometer (Thermo Fisher Scientific). The HPLC eluate was directed to the mass spectrometer through a valve set to divert the flow from 0 to 5 min.

**Recovery of Radioactive Dose.** Maximal excretion of [14C]apixaban in urine and feces was from 0 to 24 h and 24 to 48 h, respectively, in both subject groups. The recovery of the radioactive dose in bile of group 2 was 2.44% (0.25, 0.82, and 1.37% of dose for the 3–4, 4–6, and 6–8 h collections, respectively). The excretion of radioactivity in urine and feces over the 0 to 292 h collection period from group 1 was 24.5 and 56.0% of the dose, respectively, and from group 2 over the 0 to 240 h collection period was 28.8 and 46.7% of the dose, respectively. The 96-h sample was not analyzed because of low radioactivity.

**Pharmacokinetic Parameters.** A summary of estimated pharmacokinetic parameters is given in Table 1, and the plasma concentration versus time curve for apixaban and total radioactivity are shown in Fig. 1. Apixaban plasma concentration-time profiles were comparable between groups 1 and 2, indicating that the bile collection procedures had minimal if any impact on apixaban PK. Apixaban was rapidly absorbed, and Cmax was achieved at 1 h on average after oral dose administration. After Cmax was reached, the apixaban plasma concentration-time profile exhibited an initial rapid decline and then a more gradual terminal phase, with an average T1/2 of 12.7 h. The mean Cmax and AUC(0→∞) values for total plasma radioactivity were 509 ng-Eq/ml and 5872 ng-Eq · h/ml, respectively, versus 469 ng-Eq/ml and 4101 ng · h/ml, respectively, for apixaban. Apixaban plasma AUC(0→∞) represented approximately 70% of total radioactivity AUC(0→∞), indicating that unchanged apixaban accounted for the majority of circulating radioactivity. Correspondingly, the apparent clearance of TRA represented approximately 68% of that of apixaban (Table 1). The half-life of TRA seemed to be shorter than that of apixaban, 8.2 versus 12.7 h, which was probably due to differences in LLOQ. For most of subjects, plasma exposure was less than the TRA LLOQ within 36 h after dose administration versus 96 h for apixaban LLOQ by LC-MS/MS analysis. The mean blood to plasma radioactivity concentration ratios for both groups were relatively constant at approximately 0.71 to 0.83 at 0.5, 1, 1.5, 2, 4, 8, and 12 h postdose, indicating more radioactivity in plasma than in blood per unit volume. The concentration of O-demethyl apixaban sulfate showed a Tmax at 6 h followed by a near monophasic decline that was similar to the parent.

**Identification of Metabolites.** Based on the radiochromatographic profiles of metabolites in urine, bile, feces, and plasma (Figs. 2 and 3), the prominent peaks were investigated and assigned on the basis of mass spectrometry data and comparisons with chemically synthesized reference standards. A description of the information used for the assignment of each metabolite is detailed below.

**Apixaban.** Apixaban showed a molecular ion [M + H]+ at m/z 460 and fragment ions at m/z 443, 417, 282, 241, 199, and 184 in LC/MS and MS/MS analyses.

**Metabolite M1.** M1 showed a molecular ion [M + H]+ at m/z 526 that was 80 amu higher than that of M2, suggesting a sulfate-conjugated derivative of O-demethylated apixaban. Fragment ions included m/z 446 and 429 (=446 – 17). LC-MS/MS analysis indicated that fragmentation of M1 was due to loss of 80 amu, and accurate mass measurement of M1 gave a molecular ion [M + NH4]+ of 543.1666 and a derived formula of C20H27NO2S. The difference between the measured mass and the theoretical mass was ~0.4 mDa, further supporting the conclusion that M1 was O-demethylated apixaban.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>RT</th>
<th>Group 1 (n = 6)</th>
<th>Group 2 (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td>1 h</td>
<td>6 h</td>
</tr>
<tr>
<td>M1</td>
<td>24.4</td>
<td>1.64</td>
<td>15.8</td>
</tr>
<tr>
<td>M2</td>
<td>28.6</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>M7</td>
<td>27.6</td>
<td>0.75</td>
<td>N.D.</td>
</tr>
<tr>
<td>M10</td>
<td>19.0</td>
<td>0.34</td>
<td>N.D.</td>
</tr>
<tr>
<td>Apixaban</td>
<td>48.9</td>
<td>98.4</td>
<td>83.2</td>
</tr>
<tr>
<td>Others</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
<td>100</td>
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RT, retention time; N.D., not detected; N.A., not applicable.
sulfate. This metabolite was subsequently synthesized, and the retention time and the fragmentation pattern of the standard matched those of M1 in humans.

**Metabolite M2.** M2 showed a molecular ion [M+H]+ at m/z 446 and a major fragment ion at m/z 429. Other fragment ions of M2 included m/z 282, 240, 227, and 185. M2 was 14 amu lower than apixaban, consistent with demethylation of the methoxy moiety. The fragment at m/z 185 was consistent with 1-(4-methoxyphenyl)-4,5-ethylenepyrazole-3-carboxamide structure, supporting the fragmentation of apixaban, consistent with demethylation of the methoxy moiety. O-Demethyl apixaban was subsequently synthesized, and the retention time and the fragmentation pattern of the standard matched those of M2 in humans.

**Metabolites M4 and M7.** Metabolites M4 and M7 both showed a molecular ion [M+H]+ at m/z 476, which was 16 amu higher than apixaban. M4 and M7 had a major fragment ion at m/z 459 in the MS/MS spectrum and at m/z 199 (=185 + 14) in the MS3 spectrum. Other fragment ions included m/z 298, 280, 241, 199, and 184. The fragment at m/z 199 indicated the 1-(4-methoxyphenyl)-pyrazole-3-carboxamide core structure of apixaban was not the site for hydroxylation. On the basis of the retention times and LC-MS/MS fragmentation, M4 and M7 were proposed as two isomers of hydroxylated apixaban.

**Metabolite M10.** Metabolite M10 showed a molecular ion [M + NH₄]⁺ at m/z 559. This metabolite showed a fragment ion at m/z 462 corresponding to a loss of 80 + 17 amu, suggesting a metabolite of the sulfate conjugate. The fragment ion at m/z 462 was 16 amu higher than M2. Other fragment ions of M10 included m/z 542, 525, 462, and 445. Accurate mass measurement of M10 gave a molecular ion of 559.1628 and a derived formula of C₁₂H₁₄N₂O₅S. The difference between the measured mass and the theoretical mass was −1.7 mDa. These results were consistent with a sulfate of hydroxylated O-demethyl apixaban.

**Metabolite M13.** Metabolite M13 showed a molecular ion [M + H]+ at m/z 462 that was 16 amu higher than M2 by LC/MS, consistent with a hydroxylated O-demethyl apixaban. The major fragment ion of M13 was at m/z 445 (M17). Accurate mass measurement of M13 gave a molecular ion of 462.1777 and a derived formula of C₁₁H₁₄N₂O₅. The difference between the measured mass and the theoretical mass was −1.3 mDa, further supporting the conclusion that M13 was a hydroxylated O-demethyl apixaban.

**Metabolic Profiles of [¹⁴C]Apixaban in Plasma, Bile, Urine, and Feces.** The highest level of radioactivity in plasma occurred at 1 h after apixaban administration and decreased over time (Fig. 1). The 96-h plasma sample was not analyzed because of the low level of radioactivity. The HPLC radiochromatographic profiles of plasma from groups 1 and 2 showed that M1 contributed an increasing fraction of the TRA at late time points (Fig. 2, A and B). The relative distributions of radioactive metabolites in pooled plasma after oral administration of [¹⁴C]apixaban are summarized in Table 2. Apixaban and metabolite M1 were the major radioactive components in group 1 and group 2 plasma samples, and M1 represented approximately 25% of the estimated parent AUC₀–₄₈ h (Table 3). O-Demethyl apixaban (M2), hydroxy apixaban (M7), and sulfate of hydroxylated O-demethyl apixaban (M10) were minor metabolites with relative contribution of <1% at each time point (Table 4).

**Apixaban was the major component in pooled urine and feces samples from group 1 and group 2 subjects. Approximately 50% of the recovered oral dose was parent compound (Table 4). The promi-
reement metabolites were M1, M2, M7, and M13 in feces of groups 1 and 2 subjects. Metabolites M1 and M7 were present in urine samples of both group 1 and group 2 subjects. The HPLC radiochromatographic profiles of pooled urine, bile (group 2 only), and feces from group 1 and group 2 subjects are shown in Fig. 3, A and B, respectively.

Apixaban and metabolite M1 were the major components in bile collected at 3 to 8 h (Table 4). The HPLC radiochromatographic profile of pooled bile is shown in Fig. 3B. Apixaban and the identified metabolites together accounted for 98% of the radioactivity in the human bile samples.

Safety. Apixaban was well tolerated in this study. Four adverse events (AEs) were reported by a total of four subjects. AEs were considered mild (eye allergy, headache, and hyperhidrosis) or moderate (dyspepsia) in intensity. Only one adverse event was considered possibly related to the study medication (moderate dyspepsia in group 2), and all others were considered not related or not likely to be related. All AEs resolved without treatment.

Discussion

This study reports the results of a clinical study designed to determine the metabolite profiles and routes of elimination for [14C]apixaban. Data showed that after oral administration of apixaban, the majority of the recovered dose was in feces (56% of the recovered dose), with urinary excretion also representing a significant elimination pathway (24.5–28.8% of the recovered dose). On the basis of the dose recovery in urine and the fraction of dose excreted as metabolites of apixaban in feces, at least 43 to 46% of the radioactive dose was absorbed after oral administration of [14C]apixaban in humans. Pharmacokinetic analyses demonstrate rapid absorption of apixaban after oral administration in healthy male subjects.

The ratio of apixaban AUC(0–12 h) to TRA AUC(0–12 h) in plasma was 66 to 72%, indicating that the parent was the major drug-related component in circulation. This finding was confirmed by radioactivity profiling of plasma samples, which showed that in plasma, apixaban constituted approximately 98% of total radioactivity at 1 h and >50% at all other time points. Furthermore, the similarity between the apixaban plasma concentration-time profile and the plasma radioactivity-time profile in conjunction with the rapid decline of TRA in plasma (TRA t1/2 of 8.2 h) suggests that there were no significant long-lasting metabolites present in circulation. The pharmacokinetic profile of apixaban observed in the current study was consistent with previous findings (Frost et al., 2007b). Less than 3% of the dose was excreted in bile collected between 3 and 8 h postdose, which occurred when the plasma concentration of apixaban was greater than 200 ng/ml, suggesting that biliary excretion was a minor elimination pathway for apixaban. Of the total dose recovered in urine (24.5–28.8%), 83 to 88% was apixaban and 80% was recovered within 24 h after dosing, which paralleled the disappearance of apixaban in plasma for that period. Together, these data indicated that renal excretion of apixaban was a significant route of apixaban elimination.
Analysis of the metabolite profile of apixaban showed that the parent compound is the major drug-related component in plasma, urine, and feces in humans, but there are also several metabolites, of which the most prominent one was O-demethyl apixaban sulfate. Apixaban metabolites accounted for approximately less than 32% of the total recovered dose. Identification of these metabolites suggested that the metabolic pathways for apixaban include O-demethylation, hydroxylation, and sulfation of hydroxylated O-demethyl apixaban (Fig. 4). O-Demethyl apixaban sulfate was the prominent circulating metabolite, representing approximately 25% of the estimated parent AUC(0–24 h). O-Demethyl apixaban sulfate was tested for its ability to inhibit factor Xa, and results indicated that M1 was inactive against the pharmaceutical target of apixaban (data not shown).

In vitro studies with liver microsomes and hepatocytes of human and animal species indicated that the primary human metabolites identified in this study were produced in in vitro systems and that there were no unique metabolites formed in humans (data not shown). The presence of O-demethyl apixaban sulfate in the human feces indicates the relative stability of this conjugate in gastrointestinal tracts during its excretion.

Multiple factors may contribute to the <100% recovery of the radioactive dose from human subjects after oral administration of [14C]apixaban in this study, including loss of samples either during the collection or processing periods or the inherent difficulty in adequately sampling nonhomogeneous processed fecal samples for determination of total radioactivity. Studies in rat and dog models have indicated good mass balance and no tissue accumulation of radioactivity (data not shown). The problems sometimes associated with attaining complete mass balance in human absorption, distribution, metabolism, and elimination studies have been documented in a recent review of the subject (Roffey et al., 2007).

The current study demonstrates that apixaban and its metabolites are excreted by multiple elimination pathways, including renal excretion and metabolism. The multiple elimination pathways suggest that patients with hepatic or renal impairment may be treated with apixaban and that the likelihood of drug-drug significant interactions may be low. Further specifically designed clinical studies are required to fully clarify whether apixaban may be a benefit in those special populations.

In conclusion, oral single-dose administration of 20 mg of apixaban was safe and well tolerated. Apixaban is eliminated via metabolism and renal excretion in humans. The minimal extent of elimination of radioactivity in the bile through the 3 to 8 h collection period suggests that the biliary route plays a minor role in the overall disposition of apixaban.

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References


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