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Sulfation of *O*-demethyl apixaban: Enzyme identification and species comparison

Lifei Wang, Nirmala Raghavan, Kan He, Joseph M. Luetzgen, W Griffith Humphreys,
Robert M Knabb, Donald J Pinto, and Donglu Zhang,

Pharmaceutical Candidate Optimization (LW, DZ, NR, KH, WGH)

Discovery Biology (JML, RMK)

Chemistry (DJP)

Bristol-Myers Squibb Research & Development, Princeton, NJ 08543

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Running title: human sulfotransferases for metabolizing apixaban

Address correspondence to:

Lifei Wang

Pharmaceutical Candidate Optimization,

Bristol-Myers Squibb, PO BOX 4000,

Princeton, New Jersey 08543.

Phone: 609-252-5657.

Email: Lifei.Wang@bms.com

Abbreviations used: Cl_{int} , intrinsic clearance; DCNP, 2,6-dichloro-4-nitrophenol; HLM, human liver microsomes; IS, internal standard; LC/MS, liquid chromatography/mass spectrometry; MS/MS, tandem mass spectrometry; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; SRM, selective reaction monitoring; SULT, sulfotransferase.

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ABSTRACT

Apixaban, a potent and highly selective factor Xa inhibitor, is currently under development for treatment of arterial and venous thrombotic diseases. The *O*-demethyl apixaban sulfate is a major circulating metabolite in humans but circulates at lower concentrations relative to parent in animals. The aim of this study was to identify the sulfotransferases (SULTs) responsible for the sulfation reaction. Apixaban undergoes *O*-demethylation catalyzed by P450 enzymes to *O*-demethyl apixaban, and then is conjugated by sulfotransferases to form *O*-demethyl apixaban sulfate. Of the five human cDNA-expressed SULTs tested, SULT1A1 and 1A2 exhibited significant levels of catalytic activity for formation of *O*-demethyl apixaban sulfate and SULT1A3, 1E1, and 2A1 showed much lower catalytic activities. In human liver S9, quercetin, a highly selective inhibitor of SULT1A1 and SULT1E1, inhibited *O*-demethyl apixaban sulfate formation by 99%; 2, 6-Dichloro-4-nitrophenol (DCNP), another inhibitor of SULT1A1, also inhibited this reaction by >90%; Estrone, a competitive inhibitor for SULT1E1, had no effect on this reaction. The comparable K_m values for formation of *O*-demethyl apixaban sulfate were 41.4 μM (human liver S9), 36.8 μM (SULT1A1), and 70.8 μM (SULT1A2). Because of the high level of expression of SULT1A1 in liver and its higher level of catalytic activity for formation *O*-demethyl apixaban sulfate, SULT1A1 might play a major role in humans for formation of *O*-demethyl apixaban sulfate. *O*-Demethyl apixaban was also investigated in liver S9 of mice, rats, rabbits, dog, monkeys, and humans. The results indicated that liver S9 samples from dog, monkeys, and humans had higher activities for formation of *O*-demethyl apixaban sulfate than those of mice, rats, and rabbits.

INTRODUCTION

Apixaban (BMS-562247, Figure 1) is an 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 (FXa) inhibitor, which inhibits both free and prothrombinase-bound FXa 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 man (Lassen et al., 2007; Büller et al., 2008). After oral administration of apixaban to human subjects, the parent compound was the major circulating component and *O*-demethyl apixaban sulfate (Figure 1) was a significant metabolite in human plasma; however, it was not as abundant relative to parent in plasma samples of mice, rats, female rabbits, and dogs (Zhang et al., 2009). Apixaban probably underwent *O*-demethylation catalyzed by P450 enzymes to *O*-demethylated apixaban that was then conjugated by sulfotransferases to form a sulfate metabolite.

Sulfation is a major conjugation reaction of drug metabolism, and the sulfotransferases (SULTs) are responsible for sulfation biotransformation of many xenobiotics and endogenous substrates such as steroids and neurotransmitters in humans (Blanchard et al., 2004; Nagata and Yamazoe, 2000; Rikke and Roy, 1996). The process of sulfation involves the transfer of a sulfonyl group of PAPS to a hydroxyl or amino group of a molecule. This reaction generally results in a decrease in biological activity and an increase in hydrophilicity of xenobiotics (drugs) or endogenous compounds, so as to facilitate their excretion. SULTs play an important role in normal human homeostasis and

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drug metabolism (Gamage et al., 2006; Glatt et al., 2000). For example, SULT1E1 is responsible for the high affinity sulfation of β -estradiol (E2), estrone, and 17 α -ethinylestradiol (Adjei et al., 2003; Falany et al., 1995; Schrag et al., 2004). SULT1A1 catalyzes the sulfation of many xenobiotics and is expressed in many tissues including liver and small intestine (Chen et al., 2002; LeWitt, 2004).

Sulfotransferases are a superfamily of enzymes that catalyze the sulfate conjugation of various antibiotics and xenobiotics. Five gene families of SULTs, including SULT1, SULT2, SULT3, SULT4, and SULT5, have been identified in mammals. There is less than 45% amino acid sequence identity between families (Blanchard et al., 2004). In humans, thirteen SULTs have been identified, all belonging to the SULT1, SULT2, or SULT4 families (Gamage et al., 2006; Glatt et al., 2000; Glatt et al., 2001). Human SULT1A1 shows high homology with SULT1A2 (96%). Expression of SULT1A1, 1A3, 1B1, 1B2, 1E1, 1E4, 2A1, and 2A3 in adult human livers was documented and characterized (Honma et al., 2002; Richard et al., 2001; Tabrett et al., 2003). SULT1A1 is the dominant SULT1A protein expressed in human liver (Gamage et al., 2006), and there are three important allelic variants, SULT1A1*1 (wild type), SULT1A1*2 and SULT1A1*3. SULT1A1*2 is defined by an Arg213His amino acid change (G to A change at nucleotide 638), and SULT1A1*3 variant is defined by a Met223Val amino acid change (A to G change at nucleotide 667) (Carlini et al., 2001). SULT1A1 allele frequencies have been reported in different populations (Carlini et al., 2001): 65.6% (white), 47.7% (African American), and 91.4% (Chinese) for SULT1A1*1; 33.2% (white), 29.4% (African American), and 8.0% (Chinese) for SULT1A1*2; 1.2% (white), 22.9% (African American), and 0.6% (Chinese) for SULT1A1*3. These alloenzymes are

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associated with altered enzymatic activities (Nagar et al., 2006; Raftogianis et al., 1997). The general trend of V_{\max} values estimated in previous study was $1 > 3 > 2$, with trend of V_{\max}/K_m values varied with substrates (Raftogianis et al., 1997). A total of 6 alloenzymes of SULT1A2 have been reported (Raftogianis et al., 1999). Human SULT1A3 showed 93% similarity to SULT1A1 (Blanchard et al., 2004; Gamage et al., 2006). No genetic polymorphisms have been reported for SULT1A3 and SULT1E1 at the DNA level. SULT2A1 is also a major enzyme located in human liver and involved in drug metabolism (Meloche et al., 2002).

In this study, the sulfation activities of human cDNA-expressed SULTs as well as liver S9 from mice, rats, rabbits, dogs, monkeys, and humans on *O*-demethyl apixaban were investigated to determine the principal SULT enzymes involved in this sulfation reaction and to compare enzyme activities between species.

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MATERIALS AND METHODS

Materials. Apixaban (purity >99%), *O*-demethyl apixaban (purity >95%), and *O*-demethyl apixaban sulfate (purity >85%) were synthesized at Bristol-Myers Squibb (BMS, Princeton, NJ). The structures of apixaban and *O*-demethyl apixaban sulfate are shown in Figure 1. 3'-Phosphoadenosine 5'-phosphosulfate (PAPS), magnesium chloride, all SULT inhibitors (quercetin dihydrate, estrone, and 2, 6-dichloro-4-nitrophenol (DCNP)) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Pooled human liver S9 fraction (20 subjects) was purchased from BD Biosciences (Woburn, MA). Pooled liver S9 fractions of mice, rats, dogs, and monkeys were purchased from Xenotech LLC (Lenexa, KS). Male and female rabbit liver S9 was purchased from InVitro Technologies, Inc. (Baltimore, MD). The highly active cytosolic extracts of Sf-9 insect cells infected with a baculovirus strain containing human cDNA encoding specific human sulfotransferases (SULT1A1, 1A2, 1A3, 1E1, 2A1) were purchased from Invitrogen Corporation (Carlsbad, CA). Human factor Xa and alpha-thrombin were purchased from Haematologic Technologies (Essex Junction, VT). Human pancreatic trypsin was purchased from (Calbiochem, Darmstadt, Germany). For factor Xa, thrombin, and trypsin, the respective peptide substrates were S2765 (N-alpha-Benzoyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-para-nitroaniline-dihydrochloride), S2366 (L-Pyroglutamyl-L-prolyl-L-arginine-para-nitroaniline hydrochloride), and S2222 (N-Benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-para-nitroaniline hydrochloride and its glutamyl methyl ester (1:1 mixture)), which were purchased from DiaPharma Group, Inc (West Chester, OH). Acetonitrile was purchased from Burdick & Jackson Inc (Muskegon, MI). All organic solvents and water were of HPLC grade. *O*-Demethyl apixaban stock solutions (5 mM)

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were prepared in acetonitrile/water (2.5:1, v/v) for the concentration-dependent metabolite formation studies. The stock solutions of apixaban (0.1 mM) and *O*-demethyl apixaban sulfate (1 mg/mL) were prepared in acetonitrile/water (1:1, v/v). The 250 μ M stock solutions of quercetin, estrone, and DCNP were separately prepared in ethanol.

Metabolite profiling in rat and human plasma. Pooled plasma samples (at 24 h) of rats and humans following oral administration of [14 C]apixaban were obtained from previous studies (Zhang et al., 2009; Raghavan et al., 2009). Plasma samples were extracted in duplicate by addition of 4 mL of acetonitrile/methanol (1:1, v/v) to 1 mL of plasma while the sample was mixed on a vortex mixer. After centrifugation at 2000 x g for 1 h, each supernatant fraction was removed and saved. The precipitate was resuspended in 2 mL of acetonitrile and 1 mL of methanol. Following centrifugation of the mixture for 30 min at 2000 x g, the supernatant fraction was removed and combined with the first supernatant. The precipitate was resuspended in 2 mL acetonitrile. Following centrifugation of the mixture at 2000 x g for 30 min, the supernatant fraction was removed and combined with the first and second supernatants. The combined supernatant fraction was evaporated to dryness under nitrogen and reconstituted in 0.15 mL of acetonitrile and 0.05 mL of methanol. Following centrifugation at 2000 x g for 5 min, a portion of 100 μ L supernatant was injected into the HPLC for metabolite profiling and identification.

Assessment of pharmacological activities of *O*-demethyl apixaban and *O*-demethyl apixaban sulfate. The enzyme activities of factor Xa, alpha-thrombin, and trypsin were determined as described previously (Pinto et al., 2007; Wong et al., 2008). Briefly, the final concentration of human factor Xa, human alpha-thrombin, or human pancreatic trypsin in the assays was 0.1 nM, 0.125 nM, and 0.625 nM, respectively. The inhibition of

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factor Xa thrombin, or trypsin was evaluated with substrate S2765 (100 μ M), S2366 (200 μ M), or S2222 (100 μ M). Stock solutions of *O*-demethyl apixaban or *O*-demethyl apixaban sulfate were prepared in 20 mM phosphate buffer pH 7.0 and kept frozen till the day the assays were performed. Assays were conducted at room temperature in 96-well microtiter plate spectrophotometers (Molecular Devices, Sunnyvale, CA) with simultaneous measurement of enzyme activities in control and *O*-demethyl apixaban or *O*-demethyl apixaban sulfate-containing solutions. Assays were initiated by adding enzyme to buffered solutions containing synthetic substrates in the presence or absence of *O*-demethyl apixaban or *O*-demethyl apixaban sulfate. Hydrolysis of the substrate resulted in the release of pNA (*para*-nitroaniline), which was monitored spectrophotometrically by measuring the increase in absorbance at 405 nm. The rate of absorbance change is proportional to the enzyme activity. A decrease in the rate of absorbance change in the presence of *O*-demethyl apixaban or *O*-demethyl apixaban sulfate is indicative of enzyme inhibition. Assays were conducted under conditions of excess substrate and *O*-demethyl apixaban or *O*-demethyl apixaban sulfate over enzyme. If negligible inhibition was observed at the highest inhibitor concentration tested, the inhibitory constant was conservatively estimated by assuming 20% inhibition at the highest dose. *O*-Demethyl apixaban sulfate was evaluated for inhibition of human factor Xa at concentrations up to 750 μ M, and for inhibition of human thrombin and human trypsin at concentrations up to 30 μ M. *O*-Demethyl apixaban was evaluated for inhibition of the factor Xa at range of 3-10,000 nM and for inhibition of the trypsin at a range of 1-30,000 nM.

Sulfation of *O*-demethyl apixaban by human cDNA-expressed SULTs. The sulfation activity of *O*-demethyl apixaban was determined using human cDNA-expressed SULTs

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(SULT1A1, 1A2, 1A3, 1E1, and 2A1). The incubation mixtures (0.5 mL, in triplicate) contained 50 mM potassium phosphate buffer (pH 7.0), 5 mM MgCl₂, SULT (15 µg protein), 30 µM *O*-demethyl apixaban, and 2.5 mM PAPS. Acetonitrile in the incubation mixtures was 0.5%. Before addition of PAPS, the mixtures were pre-incubated at 37°C for 5 min. After 30 min incubation at 37°C with shaking (100 rpm), ice-cold acetonitrile (0.5 mL) was added to each incubation to stop the reaction and the internal standard (IS, apixaban) was added to each sample to a final concentration of 100 nM. After centrifugation at 2000 x *g* for 15 min, an aliquot of supernatant (10 or 20 µL) was used for LC/MS analysis.

Sulfation assays in human liver S9 in presence of SULT inhibitors. The sulfation activity of *O*-demethyl apixaban was determined in human liver S9 in the presence of SULT inhibitors. The incubation mixtures (0.5 mL, in triplicate) contained 50 mM phosphate buffer (pH 7.0), 5 mM MgCl₂, human liver S9 (100 µg protein), 30 µM *O*-demethyl apixaban, single SULT inhibitor, and 2.5 mM PAPS. The chemical inhibitors used were quercetin dihydrate (1 µM) for SULT1A1 and 1E1, estrone (1 µM) for SULT1E1 and 1A3, and DCNP (0.5 µM) for SULT1A1. Acetonitrile and ethanol in the incubation mixtures were 0.5% (v/v) and 0.4% (v/v), respectively. Before addition of PAPS, the mixtures were pre-incubated at 37°C for 5 min. After 30 min incubation at 37°C with shaking (100 rpm), ice-cold acetonitrile (1 mL) was added to each incubation to stop the reaction and the internal standard (IS, apixaban) was added to each sample to a final concentration of 100 nM. After centrifugation at 2000 *g* for 15 min, an aliquot of supernatant (10 or 20 µL) was used for LC/MS analysis. Control incubations (without inhibitor or PAPS) were performed under similar conditions.

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Sulfation assays in liver S9 of different species. Sulfation activities of *O*-demethyl apixaban were determined using liver S9 fractions from mice, rats, and rabbits, dogs, monkeys, or humans, and *O*-demethyl apixaban as substrate. Incubation mixtures (0.5 mL, in triplicate) contained 50 mM potassium phosphate buffer (pH 7.5), 5 mM MgCl₂, liver S9 (100 µg protein), 30 µM *O*-demethyl apixaban, and 2.5 mM PAPS. Before addition of PAPS, the mixtures were pre-incubated at 37°C for 5 min. After 30 min incubation at 37°C with shaking (100 rpm), ice-cold acetonitrile (0.5 mL) was added to each incubation to stop the reaction and the internal standard (IS, apixaban) was added to each sample to a final concentration of 100 nM. After centrifugation at 2000 x *g* for 15 min, an aliquot of supernatant (10 or 20 µL) was used for LC/MS analysis.

Substrate concentration-dependent sulfate formation. For enzyme kinetic studies, the incubation mixtures (0.5 mL) contained 50 mM phosphate buffer (pH 7.5), 5 mM MgCl₂, human liver S9 (150 µg protein) or expressed SULTs (20 pmole), *O*-demethyl apixaban, and 2.5 mM PAPS. The final concentration of acetonitrile in these incubation mixtures was 0.5% (v/v). Before PAPS addition, the mixtures were pre-incubated at 37°C for 5 min. After PAPS addition, the samples were then incubated at 37°C for 30 min with shaking (100 rpm), then ice-cold acetonitrile (0.5 mL) was added to each sample to stop reaction and the internal standard (IS, apixaban) was added to each sample to a final concentration of 100 nM. An aliquot of 20 µL of supernatant was used for LC/MS analysis. In order to determine linear conditions for protein concentration and incubation time, *O*-demethyl apixaban (10 µM) was incubated up to 50 min with 100, 200, 300, and 600 µg of human liver S9 protein/mL; *O*-demethyl apixaban (10 µM) was incubated up to 50 min with human cDNA-expressed SULTs at 8 to 20 µg protein/mL: 8.8 µg protein/mL

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for SULT1A1, 12 μg protein/mL for SULT1A2, or 600 μg protein/mL for human liver S9 and 50 min of incubation time was in a linear range, and these conditions were used for enzyme kinetic studies. Thirteen substrate concentrations, 0.01, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 30, 50, and 100 μM were evaluated in triplicate for expressed SULT1A1 and SULT1A2; eleven substrate concentrations, 0.5, 1, 2, 5, 10, 20, 30, 50, 100, 200, and 300 μM were evaluated in triplicate for human liver S9.

Identification and quantification of sulfate metabolite. Metabolites in human plasma samples of rats and human were analyzed as described previously (Zhang et al., 2009). Metabolites in samples of incubations were analyzed by LC/MS/MS method. For incubation samples, after termination of the incubation, an internal standard was added to the reaction mixture. LC/MS analysis was performed on a Finnigan LTQ mass spectrometer (ThermoFisher Scientific, San Jose, CA) with an ESI probe in a positive ion mode. The HPLC system was a HEWLETT[®] PACKARD Agilent 1100 series system equipped with two pumps, an autoinjector, a UV detector, and an ACE[®] 3 C18 column (4.6 mm x 150 mm). The mobile phase consisted of two solvents: A) 0.4% formic acid in water, pH 3.2, and B) 100% acetonitrile. The gradient employed was as follows: Solvent B started at 5%, then linearly increased to 20% at 5 min, to 30% at 50 min, to 35% at 55 min, to 90% at 65 min, held at 90% for 2 min, and then decreased to 5% at 69 min. The mobile phase flow rate was 0.7 mL/min. The HPLC effluent was directed to the mass spectrometer through a valve set to divert the flow to waste from 0 to 5 min. The capillary temperature used for analysis was set at 250°C. The gas flow rate of nitrogen, spray current, and voltages were adjusted to give maximum sensitivity using apixaban as a standard. For metabolite quantification in incubation samples, the detection was

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performed at m/z 526 \rightarrow 446 (*O*-demethyl apixaban sulfate), m/z 446 \rightarrow 429 (*O*-demethyl apixaban), and m/z 460 \rightarrow 443 (apixaban). The retention time of *O*-demethyl apixaban sulfate, *O*-demethyl apixaban, and apixaban were 10.50, 11.53, and 13.04 min, respectively. The quantification of *O*-demethyl apixaban sulfate was achieved with the same LC/MS system, HPLC column, and analysis gradient conditions. A standard curve of 8 points of *O*-demethyl apixaban sulfate, ranging from 0.001 to 100 $\mu\text{g/mL}$, was generated by linear regression ($r = 0.98$). An aliquot (10-20 μL) of supernatant was injected for LC/MS analysis. The concentration of *O*-demethyl apixaban sulfate in each incubation sample was quantified from the peak area ratio (m/z 526 to 446) based on the calibration curve of *O*-demethyl apixaban sulfate.

Data Analysis. The formation rates of *O*-demethyl apixaban sulfate from incubations with a broad substrate concentration range were evaluated by fitting the data to the Michaelis-Menten equation, $V = V_{\text{max}} * S / (K_m + S)$. Linear regression and nonlinear regression analyses were performed by SigmaPlot (SigmaPlot, version 8, SPSS science, Chicago, IL). The formation rate (pmol/min/ μg protein of SULT or human liver S9) of *O*-demethyl apixaban sulfate in each incubation sample was calculated and plotted. Enzyme kinetic parameters were estimated using SigmaPlot nonlinear regression analysis to obtain K_m values and V_{max} values.

For substrate hydrolysis by each protease was determined at room temperature by fitting the data to the Michaelis-Menten equation. For K_m determination initial hydrolysis rates from 0 to 5 minutes were used. For control, *O*-demethyl apixaban, and *O*-demethyl apixaban sulfate, the steady-state hydrolysis rates of 25 to 30 minutes were used. The IC_{50} was calculated by fitting the percent activity versus control: $\text{IC}_{50} = 100 * [1 - (V_i/V_o)]$, where:

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V_i is the observed velocity in the presence of the inhibitor; V_o is the observed velocity in the absence of the inhibitor. The following relationship was used to calculate K_i values: $K_i = IC_{50} / (1 + S/K_m)$ for a competitive inhibitor, where: IC_{50} is the concentration of inhibitor that produces 50% inhibition; K_i is the dissociation constant of the enzyme and inhibitor complex; S is the concentration of substrate; K_m is the Michaelis-Menten constant for the substrate.

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RESULTS

Metabolite profiling and identification. The HPLC radiochromatographic profiles of plasma samples (at 24 h) from rats and humans are shown in Figures 2. In rat plasma profile, apixaban was the dominant component (98%) and *O*-demethyl apixaban sulfate was minor (1.8%); in human plasma, major circulating components were apixaban (61%) and *O*-demethyl apixaban sulfate (34%). *O*-demethyl apixaban was a minor metabolite detected by LC/MS analysis only in plasma samples at early time points (6 h) (Raghavan et al., 2009, Zhang et al., 2009).

The LC/MS analyses of plasma and incubation samples showed that a metabolic peak at 10.50 min had a molecular ion $[M+H]^+$ at m/z 526 and a product ion at m/z 446 (526-80), indicating the loss of 80 Da. Based on this information, a sulfate conjugate of *O*-demethyl apixaban was proposed. Subsequent synthesis of an authentic standard (Raghavan et al., 2009) and comparison to LC/MS confirmed the proposed structure. This metabolite peak was detected in the incubations of liver S9 (mice, rats, rabbits, dogs, monkeys and humans), SULT1A1, 1A2, 1A3, 1E1, and 2A1. A second peak at 11.53 min had a molecular ion $[M+H]^+$ at m/z 446, and LC/MS/MS analysis showed a product ion at m/z 429. This second peak was *O*-demethyl apixaban. Apixaban (at 13.04 min, internal standard) showed a molecular ion $[M+H]^+$ at m/z 460 and a major fragment ion at m/z 429.

Pharmacological activities of *O*-demethyl apixaban and *O*-demethyl apixaban sulfate.

The enzyme activities for factor Xa, thrombin, and trypsin were determined using para-nitroaniline coupled peptides as substrates (S2765, S2366, and S2222). Under these conditions, K_m values were 23.6, 220, and 31 μ M for factor Xa, thrombin, and trypsin, respectively. The significant inhibition for factor Xa was observed only at high

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concentrations of *O*-demethyl apixaban and *O*-demethyl apixaban sulfate (>20 μ M). The value of IC_{50} and K_i were approximately 300 and 58 μ M, respectively, for *O*-demethyl apixaban sulfate. *O*-Demethyl apixaban sulfate at concentrations up to 30 μ M did not inhibit thrombin or trypsin amidolytic activity towards their respective peptide substrates. For both enzymes, The K_i values of *O*-demethyl apixaban sulfate can be conservatively estimated to be greater than 10 μ M. *O*-Demethyl apixaban had a K_i value of 20 nM for inhibition of factor Xa, 250-fold less potent than apixaban (0.08 nM) (Pinto et al., 2007) and a K_i value of >15,000 nM for inhibition of trypsin.

Sulfation activity by various recombinant SULTs. Among five recombinant human SULTs screened for the sulfation activity, all generated *O*-demethyl apixaban sulfate. In human cDNA-expressed SULTs, the sulfation activities followed a decreasing order of $SULT1A1 > SULT1A2 \gg SULT1E1 > SULT1A3 \approx SULT2A1$ (Table 1 and Figure 3).

Inhibition studies. The sulfation activity of *O*-demethyl apixaban was determined with human liver S9 in the presence of SULT inhibitors (Table 2 and Figure 4). Quercetin (1 μ M) inhibited the formation of *O*-demethyl apixaban sulfate by 99%; DCNP (0.5 μ M), a high selective inhibitor for SULT1A1 ($IC_{50} < 0.1 \mu$ M), also inhibited the formation of *O*-demethyl apixaban sulfate by >90%; estrone, which has a high selectivity for SULT1E1, showed no effect at 1 μ M concentration on the formation of *O*-demethyl apixaban sulfate. The inhibition results indicated that SULT1A1 is likely the major sulfotransferase catalyzing the sulfation of *O*-demethyl apixaban in humans.

Species comparison of *O*-demethyl apixaban sulfation. The formation of *O*-demethyl apixaban sulfate was observed in all liver S9 incubations and the formation rates are shown in Table 3 and Figure 5. Relatively low levels of sulfation activities (from ~1 to 11

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pmole/min/mg protein) were found in liver S9 of mice, rats, male rabbits, female rabbits. Dog, monkey, and human liver S9 all showed significantly higher levels of sulfation activity for *O*-demethyl apixaban than mice, rat, and rabbit liver S9.

Substrate concentration-dependent metabolite formation. The formation rates of *O*-demethyl apixaban sulfate were measured over a range of substrate concentrations (0.5 to 300 μM) in human liver S9 and expressed SULT1A1 and SULT1A2. Formation of *O*-demethyl apixaban sulfate exhibited hyperbolic kinetics and the data was fitted to the Michaelis-Menten equation. The K_m values for sulfation of *O*-demethyl apixaban were similar (37-41 μM) in human liver S9 and SULT1A1 (Table 4, Figure 6); however, the V_{\max} value in SULT1A1 was 53-fold higher than that in human liver S9 and 5-fold higher than that in SULT1A2. The higher catalytic efficiency (V_{\max}/K_m) of SULT1A1 suggests that SULT1A1 is likely to play a major role for formation of *O*-demethyl apixaban sulfate in humans.

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DISCUSSION

The radioactivity profiles of plasma at 24 h post dose showed that both apixaban and *O*-demethyl apixaban sulfate were prominent circulation components in humans and while apixaban was dominant component in rats. *O*-Demethyl apixaban was a minor metabolite detected by LC/MS analysis only in plasma samples of early time points (6 h) (Raghavan et al., 2009, Zhang et al., 2009). In addition, *O*-Demethyl apixaban and *O*-demethyl apixaban sulfate did not significantly inhibit purified human factor Xa. The affinity constants (K_i) were 20 nM and 58 μ M for *O*-demethyl apixaban and *O*-demethyl apixaban sulfate, respectively, over 250-fold less potent than apixaban (Pinto et al., 2007). In contrast, the K_i of apixaban for human factor Xa is 0.08 nM. Based on K_i values and human plasma profiles (apixaban accounting for over 61% radioactivity), the antithrombotic effects of *O*-demethyl apixaban and *O*-demethyl apixaban sulfate are negligible in humans in clinical treatment. These results indicate that *O*-demethyl apixaban and *O*-demethyl apixaban sulfate did not possess any activity that could contribute to the pharmacological activity of apixaban. *O*-Demethyl apixaban sulfate did not produce significant inhibition of human thrombin or trypsin at concentrations up to 30 μ M. The lack of affinity for factor Xa, thrombin, and trypsin suggest that *O*-demethyl apixaban sulfate is also unlikely to inhibit other serine proteases at low μ M concentrations that achieved after administration of apixaban in humans (Raghavan, et. al., 2009).

The identification of the SULTs involved in the sulfation of *O*-demethyl apixaban was carried out with initial screening of metabolic turnover by cDNA-expressed enzymes followed by evaluation of the effects of chemical inhibitors on sulfation reaction in human liver S9. Upon initial screening with cDNA-expressed enzymes, multiple SULTs

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(SULT1A1, 1A2, 1A3, 1E, and 2A1) were capable of forming *O*-demethyl apixaban sulfate; however SULT1A1 and 1A2 had a higher catalytic efficiency than other enzymes tested. Quercetin and DCNP were selective inhibitors of SULT1A1 with IC_{50} values of approximately 100 nM (Walle et al., 1995; Schrag et al., 2004). In the present study, results showed that quercetin (1 μ M) and DCNP (0.5 μ M) selectively inhibited the formation of *O*-demethyl apixaban sulfate by >90% in human liver S9 incubations at 20 and 100 μ M of *O*-demethyl apixaban (Figure 4). In contrast, estrone which is a high affinity inhibitor for SULT1E1 with a K_m value of approximate 6 nM (Schrag et al., 2004), had no significant effect on the formation of *O*-demethyl apixaban sulfate in human liver S9 incubations at 20 and 100 μ M of *O*-demethyl apixaban (Figure 3). These data suggested that SULT1A1 plays an important role in the formation of *O*-demethyl apixaban sulfate.

SULT1A1 had a K_m value of 36.8 μ M for the formation of *O*-demethyl apixaban sulfate, similar to that in human liver S9 (41.4 μ M). The estimated K_m value for SULT1A2 was 70.8 μ M. The Cl_{int} values (V_{max}/K_m) of SULT1A1 and SULT1A2 for the formation of *O*-demethyl apixaban sulfate were 13 and 1.2 mL/min/mg protein, respectively, suggesting that the formation of *O*-demethyl apixaban sulfate in human liver was efficient. Since the Cl_{int} value of SULT1A1 was 10-fold higher than that of SULT1A2 and there is a high expressed level of SULT1A1 in the human liver, these findings suggest that SULT1A1 may play a significant role in apixaban metabolism and elimination.

The in vitro sulfation activities of *O*-demethyl apixaban were highly variable among species, with humans, monkeys, and dogs showing higher levels of the activity than rabbits, rats, and mice. These in vitro results are somewhat consistent with different

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amounts of the sulfate metabolite found *in vivo*, although the metabolite profile of dogs and monkeys would have been predicted to be more similar to human than that actually found. This metabolite represented approximate 3% of dose following a single 20 mg oral dose of [¹⁴C]apixaban in humans, but was only detected as a very minor metabolite in mice, rats, or dogs following oral administration of [¹⁴C]apixaban in these animals (Zhang et al., 2009). It is possible that there was significant formation of *O*-demethyl apixaban sulfate in the liver and subsequently excreted in the bile and then hydrolyzed back to *O*-demethyl apixaban in intestines of animal species.

In summary, liver S9 fractions from human and animal species generated *O*-demethyl apixaban sulfate in incubations with *O*-demethyl apixaban. The studies with cDNA-expressed enzymes, SULT chemical inhibitors, and kinetic analysis showed that *O*-demethyl apixaban sulfate was mainly formed by SULT1A1.

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Legends for figures:

- Figure 1. Chemical structures of apixaban, *O*-demethyl apixaban, and *O*-demethyl apixaban sulfate.
- Figure 2. Metabolite profiles of rat and human plasma samples at 24 h after an oral dose of [¹⁴C]apixaban. Details of sample analyses are described in the section of *Materials and Methods*.
- Figure 3. Formation activities of *O*-demethyl apixaban sulfate in human cDNA-expressed SULTs. Human cDNA-expressed SULTs were incubated with 30 μM *O*-demethyl apixaban in the presence of PAPS at 37°C for 30 min. Details of the incubation and sample analyses are described in the section of *Materials and Methods*.
- Figure 4. Formation activities of *O*-demethyl apixaban sulfate in human liver S9 incubations in the presence of SULT inhibitors. Human liver S9 were incubated with 20 or 100 μM *O*-demethyl apixaban in the presence of PAPS and SULT inhibitors at 37°C for 30 min. Details of the incubation and sample analyses are described in the section of *Materials and Methods*.
- Figure 5. Formation activities of *O*-demethyl apixaban sulfate in the liver S9 of mice, rats, rabbits, dogs, monkeys, and humans. The liver S9 were incubated with 30 μM *O*-demethyl apixaban in the presence of PAPS at 37°C for 30 min. Details of the incubation and sample analyses are described in the section of *Materials and Methods*.

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Figure 6. Substrate concentration-dependent formation of *O*-demethyl apixaban sulfate in incubations of human liver S9, SULT1A1, and SULT1A2. Details of the incubation and sample analyses are described in the section of *Materials and Methods*.

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Table 1. Formation of *O*-demethyl apixaban sulfate in incubations of human cDNA-expressed SULTs.

Samples	Formation of <i>O</i> -demethyl apixaban sulfate (nmol/min/mg protein) (Mean \pm SD)
SULT1A1	160.0 \pm 50.0 (n = 3)
SULT1A2	21.0 \pm 1.7 (n = 3)
SULT1A3	0.15 \pm 0.02 (n = 3)
SULT1E1	1.3 \pm 0.4 (n = 3)
SULT2A1	0.16 \pm 0.03(n = 3)

Human cDNA-expressed SULTs were incubated with 30 μ M *O*-demethyl apixaban in the presence of PAPS at 37°C for 30 min. Details of the incubation and sample analyses are described in the section of *Materials and Methods*.

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Table 2. Formation of *O*-demethyl apixaban sulfate in incubations of human liver S9 with SULT inhibitors.

Treatment group	<i>O</i> -Demethyl apixaban (μM)	% of remaining enzyme activity
Control	20	100
	100	100
Quercetin (1 μM)	20	0.12
	100	0.54
Estrone (1 μM)	20	90.80
	100	135.36
DCNP (0.5 μM)	20	5.08
	100	8.37

Human liver S9 were incubated with 20 or 100 μM *O*-demethyl apixaban in the presence of PAPS and SULT inhibitors at 37°C for 30 min. Details of the incubation and sample analyses are described in the section of *Materials and Methods*.

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Table 3. Formation of *O*-demethyl apixaban sulfate in incubations of liver S9 of mice, rats, rabbits, dogs, monkeys, and humans.

Samples	Formation of <i>O</i> -demethyl apixaban sulfate (pmol/min/mg protein) (Mean \pm SD)
Mouse	0.9 \pm 0.7 (n = 6)
Rat	11.4 \pm 13.3 (n = 6)
Rabbit (M)	3.8 \pm 0.4 (n = 3)
Rabbit (F)	2.6 \pm 0.7 (n = 3)
Dog	244.8 \pm 95.3 (n = 6)
Monkey	780.4 \pm 536.5 (n = 6)
Human	586.2 \pm 260.36 (n = 6)

The liver S9 were incubated with 30 μ M *O*-demethyl apixaban in the presence of PAPS at 37°C for 30 min. Details of the incubation and sample analyses are described in the section of *Materials and Methods*.

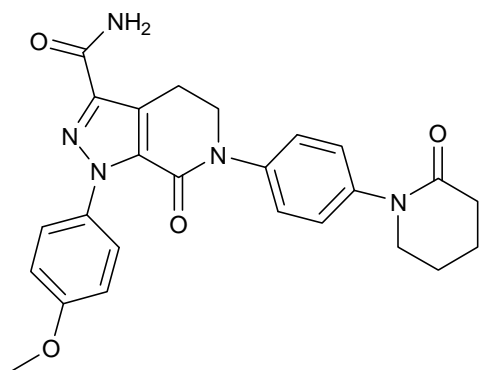
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Table 4. Enzyme kinetic parameters of sulfation in the incubations with *O*-demethyl apixaban in human liver S9, human cDNA-expressed SULT1A1 and SULT1A2

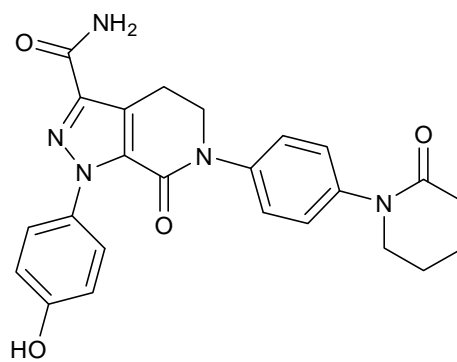
	K_m (μM) (Mean ± SD, n =3)	V_{max} (nmole/min/mg protein) (Mean ± SD, n = 3)	CL_{int} (V_{max}/K_m) (mL/min/mg protein) (Mean ± SD, n = 3)
Human liver S9	41.4 ± 24.7	7.0 ± 0.8	0.2 ± 0.08
SULT1A1	36.8 ± 28.2	370.0 ± 150.0	13.0 ± 6.0
SULT1A2	70.8 ± 53.3	70.0 ± 18.0	1.2 ± 0.7

Substrate concentration-dependent formation of *O*-demethyl apixaban sulfate were evaluated in triplicate with human liver S9, human cDNA-expressed SULT1A1 and SULT1A2. Substrate concentrations ranged from 0.01 to 300 μM and samples were incubated at 37°C for 30 min. Details of the incubation and sample analyses are described in the section of *Materials and Methods*.

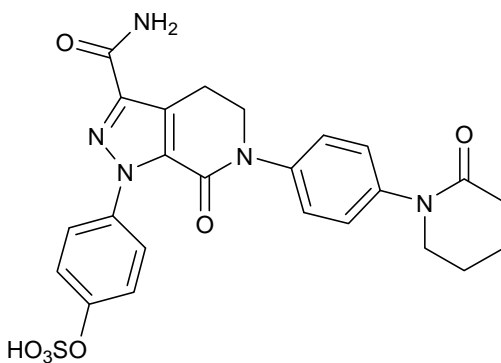
Figure 1.



Apixaban



***O*-Demethyl apixaban**



***O*-Demethyl apixaban sulfate**

Figure 2.

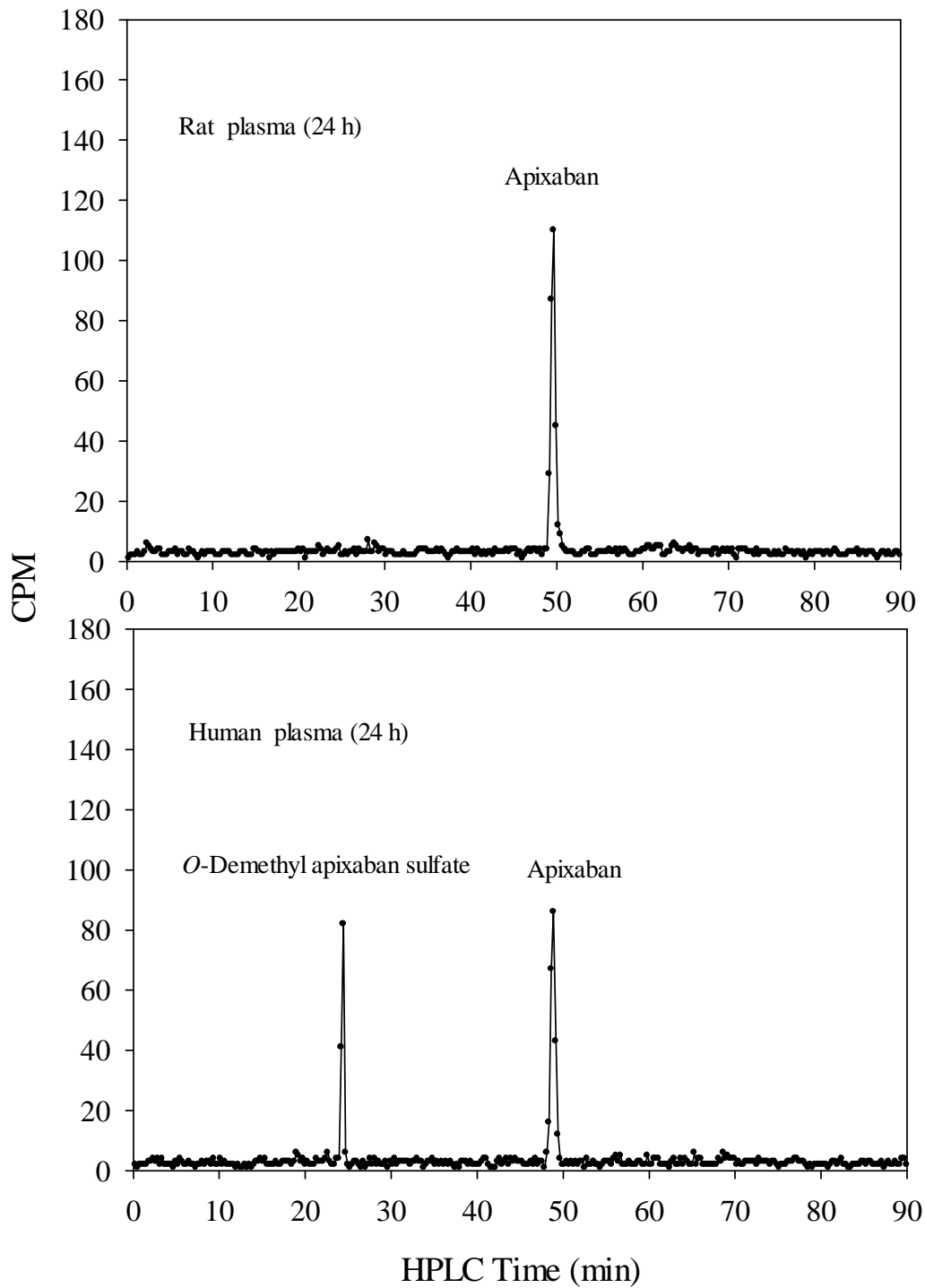
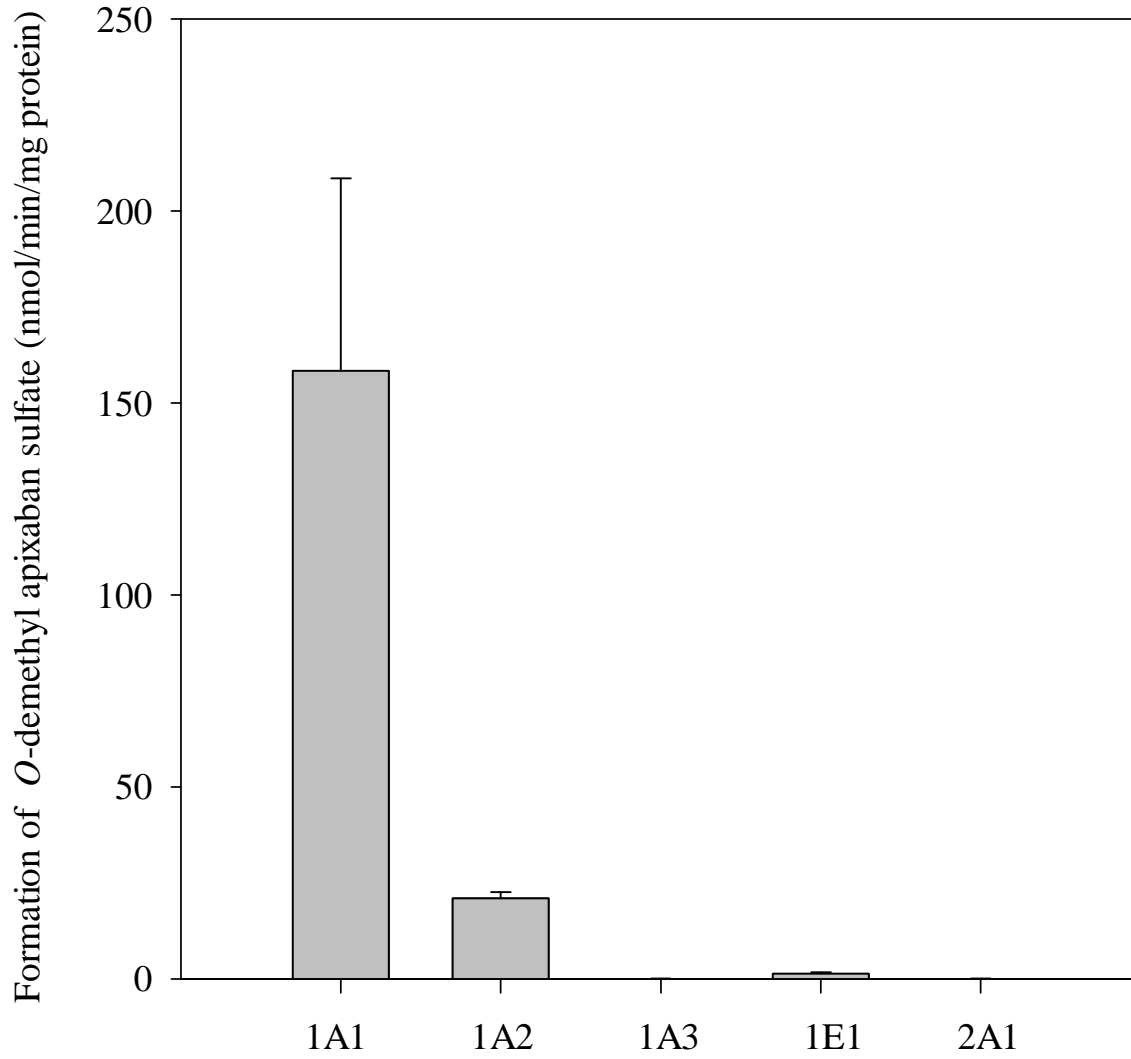


Figure 3.



n = 3 for each; CV (%) were from 8% to 33%;

Figure 4.

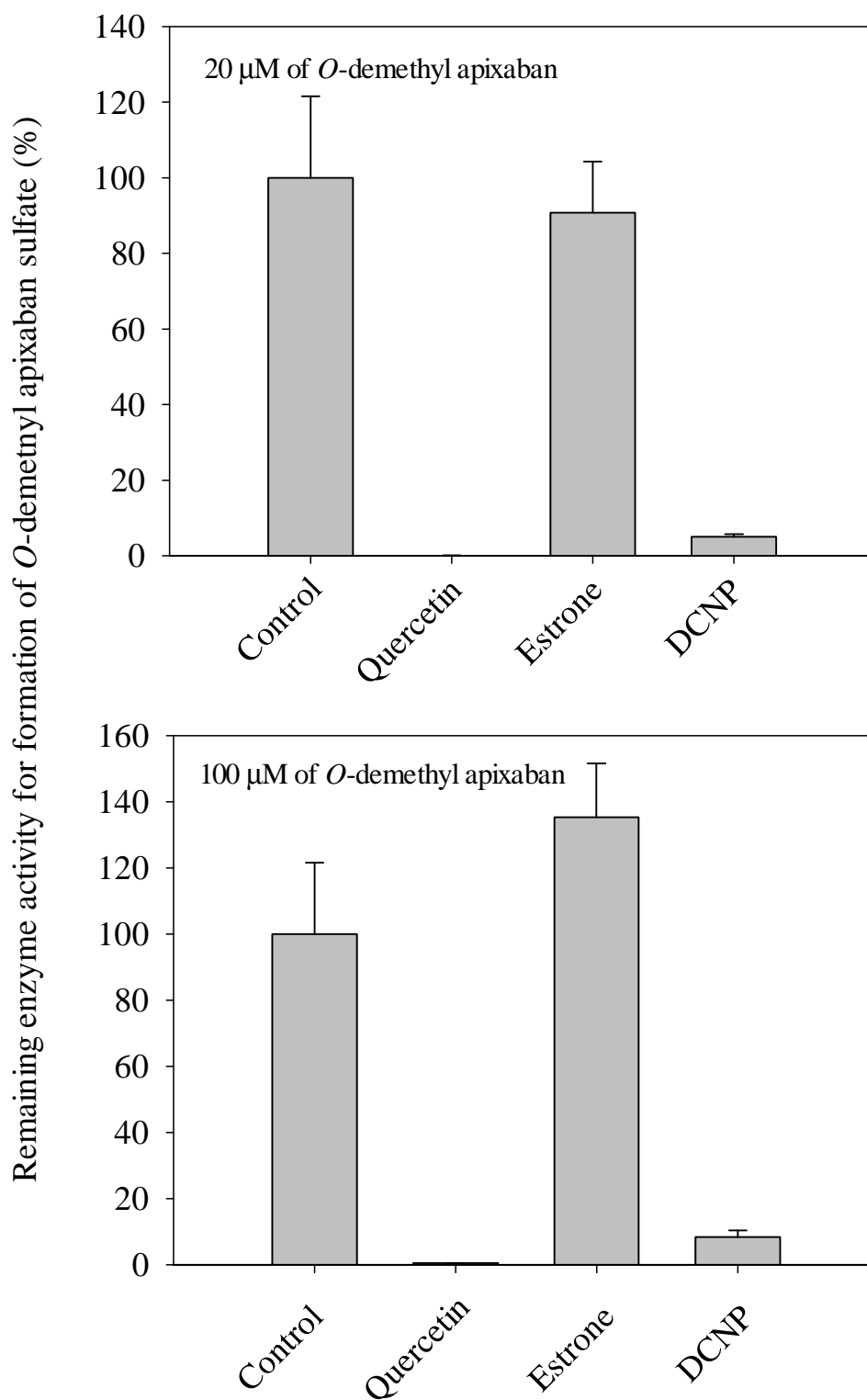
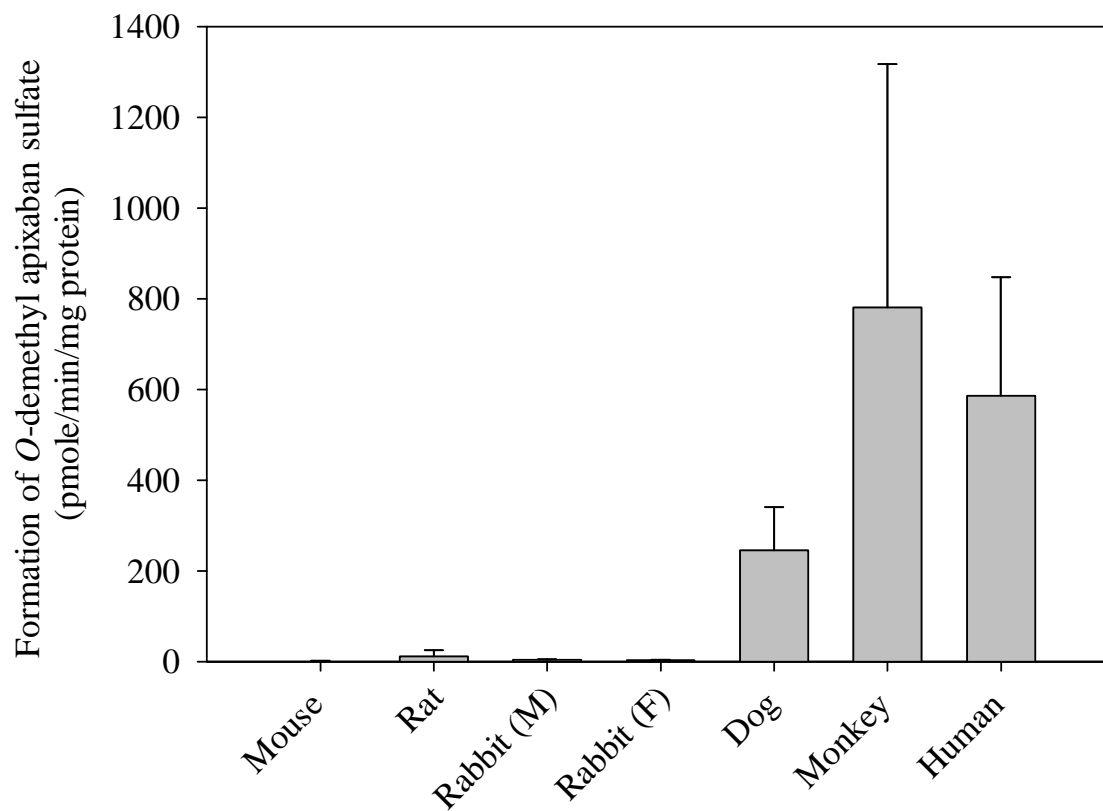


Figure 5.



n = 6 for mouse, rat, dog, monkey, and human liver S9; CV (%) were from 44% to 118%;
n = 3 for male and female rabbit liver S9; CV (%) were from 9.8% to 26%.

Figure 6.

