Characterization of ADME properties of \([^{14}\text{C}]\)asunaprevir (BMS-650032) in humans

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Abstract
1. Asunaprevir (ASV, BMS-650032), a highly selective and potent NS3 protease inhibitor, is currently under development for the treatment of chronic hepatic C virus infection. This study describes in vivo biotransformation in humans and the identification of metabolic enzymes of ASV.
2. Following a single oral dose of \([^{14}\text{C}]\)ASV to humans, the majority of radioactivity (>73% of the dose) was excreted in feces with <1% of the dose recovered in urine. Drug-related radioactivity readily appeared in circulation and the plasma radioactivity was mainly attributed to ASV. A few minor metabolites were observed in human plasma and are not expected to contribute to the pharmacological activity because of low levels. The area under the curve (AUC) values of each circulating metabolite in humans were well below their levels in animals used in the long-term toxicological studies. In bile and feces, intact ASV was a prominent radioactive peak suggesting that both metabolism and direct excretion played important roles in ASV clearance.
3. The primary metabolic pathways of ASV were hydroxylation, sulfonamide hydrolysis and the loss of isoquinoline. In vitro studies with human cDNA expressed CYP enzymes and with human liver microsomes (HLM) in the presence of selective chemical inhibitors demonstrated that ASV was primarily catalyzed by CYP3A4 and CYP3A5.

Keywords
ADME, asunaprevir, direct acting antiviral, HCV, reaction phenotyping

Introduction
Chronic hepatitis C virus (HCV) infection continues to be a serious disease, affecting an estimated 2% of the world’s population (Armstrong et al., 2006). The disease is asymptomatic in most patients but often progresses to hepatic manifestations including cirrhosis, liver failure and hepatocellular carcinoma (Perz et al., 2006; Zoulim et al., 2003). The treatment for HCV infection is advancing rapidly in recent years. Interferon-based therapy (in combination with ribavirin) was the standard treatment for many years but suffered from low sustained viral response (SVR) particularly in patients with genotype 1 virus (Manns et al., 2007; Munir et al., 2010; Saxi, 2011), and severe side effects that often lead to the treatment discontinuation (Fried, 2002). Based on recent advances in the understanding of HCV life cycle and genomic organism, the focus of HCV therapy has turned to direct acting antiviral (DAA) agents that inhibit specific viral enzymes involved in viral translation and replication (Ferenci, 2012; Holler et al., 2009; Poordad & Khunger, 2011). Among them, NS3 serine protease is responsible for processing four cleavages along the viral polyprotein to produce functional proteins and plays a key role in the maturation of HCV (Goudreaux & Llinàs-Brunet, 2005; Manns et al., 2007). In efforts to develop DAAs for HCV treatment, NS3 protease has become a compelling target for small molecular inhibitors (Kwong et al., 2008; McPhee et al., 2003; Sheldon et al., 2007). A number of NS3 protease inhibitors have been discovered, with many of them either in clinical trials or been approved for marketing (Khalid & Bacon, 2011; Kwong et al., 2011; Venkatraman & Njoroge, 2009).

Asunaprevir (ASV, BMS-650032) is a highly selective and potent NS3 protease inhibitor and a promising DAA in the late-stage clinical development for the treatment of chronic HCV infection (Li et al., 2011; McPhee et al., 2010, 2012). Results from preclinical studies demonstrated that ASV has been effective in the treatment of HCV infection with manageable safety profile (Chayama et al., 2012; Lok et al., 2012; Pasquinelli et al., 2012). In the clinic, ASV has been tested in combination with other DAAs including
daclatasvir, an NS5A polymerase inhibitor, and beclabuvir, a nonnucleoside NS5B polymerase inhibitor. Combination of daclatasvir and ASV was highly effective in patients infected with genotype 1b virus (McPhee et al., 2012; Pasquinelli et al., 2012) and is approved in Japan.

An absorption, distribution, metabolism and excretion (ADME) study of a drug candidate has become an essential part of drug discovery and development. These studies are often conducted in both humans and preclinical species used in long-term safety evaluation to confirm that human circulating metabolites are also present in toxicology species. In addition, the primary metabolic pathways of a compound can be determined based on metabolite structures identified in excreta which are critical to assess drug interaction potential of the compound. This article describes pharmacokinetics, metabolism and disposition of ASV following a single oral dose of [14C]ASV to healthy volunteers. In addition, in vitro studies were conducted to identify human enzymes involved in the metabolism of ASV.

Materials and methods

Materials

[14C]ASV (Figure 1, BMS-650032, 14.52 μCi/mg, radiochemical purity 99.5%), namely, tert-butyl ((S)-1-((2S,4R)-4-((7-chloro-4-methoxyisouquinolin-1-yl)oxy)-2-(((1R,2S)-1-((cyclopropylsulfonyl)carbamoyl)-2-vinylcyclopropyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)carbamate, was supplied by the radiochemistry group of discovery chemistry, Bristol-Myers Squibb Research & Development, Wallingford, CT. Non-radiolabeled ASV, stable-labeled d9-ASV and reference standards of M6 (O-demethylation metabolite) and M9 (loss of isoquinoline) were supplied by Bristol-Myers Squibb Research & Development, Wallingford, CT. More structure information of metabolite standards is available in the Supplemental Information. Pooled human liver microsomes (HLM, 20 donors of mixed gender, 20 mg protein/mL) and human cDNA-expressed CYP enzymes (1 nmol/mL) were purchased from BD Bioscience (Woburn, MA). All chemicals, namely, furafylline, tranylcypromine, orphenadrine, montelukast, sulfaphenazole, benzylrhizanol, quinidine, diethyldithiocarbamate, ketoconazole, 1-aminoanbenzotriazole (1-ABT) and β-nicotinamide adenine dinucleotide phosphate-reduced form (NADPH) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Potassium phosphate and formic acid were obtained from EM Science (Gibbstown, NJ).

EOSzotriazole (1-ABT) and phenytoin, quinidine, diethyldithiocarbamate, ketoconazole, 1-aminobenzotriazole (1-ABT) and β-nicotinamide adenine dinucleotide phosphate-reduced form (NADPH) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Potassium phosphate and formic acid were obtained from EM Science (Gibbstown, NJ).

Dosing and sample collection in human ADME study

All human subjects were provided written consent before participation in the study and the protocol for human study was approved by the Institutional Review Board and Radiation Safety Committee at the investigational site.

The human ADME study was a non-randomized, open label, single dose study. Nine healthy male subjects, aged 18–49 years, were assigned to two groups: group 1 (six subjects without bile collection) and group 2 (three subjects with bile collection at 3–8 h post-dose). In the morning on Day 1, after fasting for a minimum of 10 h, each subject (groups 1 and 2) received a single dose of 200 mg [14C]ASV in an oral solution containing approximately 100 μCi of radioactivity. The dosing vehicle consisted of polyethylene glycol 400 (50% w/w)/ethanol (35% w/w)/polysorbate 80 (15% w/w). The radiation exposure due to the radioactive dose to healthy volunteers was supported by tissue distribution study of [14C]ASV in LE pigmented rats, where organ exposures of radioactivity were determined with quantitative whole-body autoradiography. Dosimetry predictions were determined according to recommendations of the International Committee on Radiation Protection and Medical Internal Radiation Dose Committee of the Society of Nuclear Medicine. Administration of a 100 μCi oral dose of [14C]ASV would expose human subjects to a total committed effective dose-equivalent of 1.29 mrem, which is approximately 0.04% of the 3000 mrem single-dose limit specified for whole-body exposure by the Code of Federal Regulations.

All subjects remained in the clinical facility for 10 days and were closely monitored for adverse events throughout the study. Subjects were discharged from the clinic in the morning of Day 10 provided that at least 90% of the total dose of radioactivity had been collected or the measurement of combined radioactivity in a 24-h interval collection of urine and feces was ≤1% of the administered radioactivity over the prior two consecutive days. A single oral dose (30 ml) of magnesium hydroxide was administered on the evening of Day 10 to ensure defecation before release from the clinical facility.

Serial blood samples were collected for analysis of ASV and total radioactivity (TRA) over a 6-day period at pre-dose and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36, 48, 72, 96, 120 and 144 h post-dose. Extra blood samples for biotransformation analysis were collected at pre-dose and at 0.5, 1, 1.5, 2, 4, 8, 12, 24, 48 and 96 h post-dose. All blood samples were collected into vacutainer tubes containing EDTA as the anticoagulant. Plasma was prepared from the blood samples by centrifugation at 1000 g for 15 min at 4°C. The plasma samples were then stored at −20°C until analysis. Complete urinary and fecal output was collected from each subject at 24-h intervals over the 10-day period and analyzed for TRA and biotransformation.

![ASV](image-url)

Figure 1. The structure of ASV. The site labeled with C-14 is indicated with an asterisk.
For subjects in group 2, bile was collected for TRA and biotransformation analysis under suction using a weighted oral-gastro-duodenal tube. Approximately 1 h after study drug administration, the terminal end of the tube was positioned at the vertical limb of the duodenal loop (confirmed via imaging). Bile was collected continuously from 3–8 h post-dose, using continuous suction. Subjects were allowed to receive IV fluid replacement but not food, during the course of the bile collection.

Representative pooled urine, bile and fecal samples were prepared by combining a constant percentage of volume (urine or bile), or weight (fecal homogenate) across subjects. Plasma samples were segregated by collection time, and equal volumes of plasma were combined from each subject. Mass balance studies of [14C]ASV in animals, and samples collection and pooling were described in the Supplemental Information.

Analysis of radioactivity

The radioactivity in urine, bile and fecal homogenate was quantified with a LS6000 or LS6500 liquid scintillation counter (LSC) (Beckman Instruments, Inc., Fullerton, CA). Samples of urine (200–300 mL) and bile (100 mL) were mixed with 15 mL of Emulsifier-Safe scintillation fluid (PerkinElmer, Inc., Boston, MA) and were analyzed directly with LSC. Each sample was counted for 10 min and disintegrations per min (DPM) were determined. Fecal homogenates were combusted with a Model 307 sample oxidizer (PerkinElmer, Inc., Boston, MA). The resulting \(^{14}\text{CO}_2\) was trapped into 9 mL of Carbo-Sorb solvent (PerkinElmer, Inc., Boston, MA) and mixed with 9 mL of Emulsifier-Safe scintillation fluid (PerkinElmer, Inc., Boston, MA) before analysis with LSC. Plasma samples (200 \(\mu\)L) were mixed with 10-mL Ecolite\textsuperscript{TM} liquid scintillation fluid (MP Biomedicals, Irvine, CA) and radioactivity was measured with a Tri-Carb 3100TR liquid scintillation counter (Perkin Elmer, Fullerton, CA), using a low level quench curve supplied by the manufacture, and a counting time of 2 h. The radioactivity in plasma, urine, bile and feces was reported as \(\mu\)-g-equivalent/mL of ASV.

Quantitative analysis of ASV in plasma

The plasma concentration of ASV was determined with an API 4000 triple quadrupole mass spectrometer coupled with a Shimadzu HPLC system, following the procedure previously described (Jiang et al., 2015). Briefly, the internal standard (IS) d\(_9\)-ASV (50 \(\mu\)L at 20 ng/mL) was added to 200 \(\mu\)L of plasma or QC samples followed by a brief vortexing. Ammonium acetate buffer (50 \(\mu\)L at 0.2 M, pH 7) and MTBE (900 \(\mu\)L) were then added. The mixture was gently mixed, followed by centrifugation for 5 min at 3000 rpm. The organic layer was removed and evaporated to dryness under nitrogen at 40 °C. The residue was reconstituted in 150 \(\mu\)L of methanol:water:formic acid (75:25:0.1, v:v:v) and a portion of supernatant (10 \(\mu\)L) was analyzed with LC–MS/MS. Chromatographic conditions and MS detection for ASV and the internal standard were described previously (Jiang et al., 2015).

Preparation of biological samples for metabolite profiling

Pooled plasma (1–5 mL) was extracted with three volumes of methanol/acetonitrile (50:50 v/v). The mixtures were vortex mixed, sonicated for 5 min and centrifuged at 4000 g for 15 min. The supernatant was collected into a new tube, and the pellet was extracted two additional times with 2 mL of methanol/acetonitrile:water (1.5:1.5:1 v/v/v). The combined supernatant was evaporated to dryness under a stream of nitrogen and the residue was suspended in 0.20 mL of methanol:water (1:1 v/v). The suspension was centrifuged at 14 000 g for 10 min. A portion of supernatant (50–80 \(\mu\)L) was injected into LC–MS/MS for biotransformation profiling and mass-spectral analysis.

Pooled fecal homogenates (approximately 1 g) were extracted with 2 mL of methanol/acetonitrile (50:50 v/v). The mixtures were vortex mixed, sonicated for 5 min and centrifuged at 4000 g for 15 min. The supernatant was collected into a new tube, and the remaining pellet was extracted twice with 2 mL of methanol/acetonitrile:water (1:1:1 v/v/v). The combined supernatant was evaporated to dryness under a stream of nitrogen. The residue was re-suspended in 0.50 mL of methanol:water (1:1 v/v) and centrifuged at 14 000 g for 10 min. A portion of supernatant (50–80 \(\mu\)L) was injected into the LC–MS for biotransformation profiling and mass-spectral analysis.

Pooled bile samples were diluted 5-fold with water and the diluted samples were centrifuged at 14 000 g for 10 min. A portion of supernatant (50–80 \(\mu\)L) was injected into the LC–MS/MS for biotransformation profiling and mass-spectral analysis.

Radiochromatographic profiling and mass spectrometry analysis of metabolites

Biotransformation profiling and metabolite identification were performed on an Agilent 1100 HPLC system interfaced with a Thermo-Finnigan LTQ ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA). Chromatographic separation was achieved with a Phenomenex C18 HPLC column (4.6 × 250 mm, 5 \(\mu\)m, Phenomenex, Torrance, CA). A stepped gradient of two solvent systems A and B was used for HPLC profiling. Solvent A consisted of 0.1% formic acid in water and solvent B was acetonitrile. The HPLC flow rate was 1.0 mL/min. The mobile phase composition started at 10% B, increased to 80% B over the course of 60 min, followed by a column wash with 80% B for 5 min and returned to the initial condition of 10% B over the course of 2 min. The HPLC elute was split via a flow splitter (Dionex Co., Sunnyvale, CA) where 75% of elute was collected into Deepwell LumaPlate plates at 0.25 min intervals per well. The remaining 25% of the elute was directed into a Thermo-Finnigan LTQ ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA) for metabolite identification. The plates were dried in a Speed-Vac (Savant Instruments Inc., Holbrook, NY) and the radioactivity was counted for 5 min per well with a TopCount microplate analyzer (PerkinElmer Biosciences, Downers Grove, IL) to quantify radioactivity. Radiochromatographic profiles were prepared by plotting the net counts per minute (CPM) values obtained from the
TopCount versus time after injection using Microsoft Excel (Version 2007, Microsoft Corporation, Redmond, WA).

Mass spectrometry analysis was performed on the Thermo-Finnigan LTQ ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA). The ESI source was set in positive ion mode and analytical conditions were set as follows: voltage, 4 kV; current, 10 μA; and capillary temperature, 275 °C. MS data were collected for a mass range of 250–1000 amu, and MS/MS data were acquired with the following parameters: isolation width, 1.5 amu; collision energy, 35 eV; and activation time, 30 ms. The nitrogen flow rate, spray current, and voltages were adjusted to give maximum sensitivity. MS and MS/MS analyses were performed to identify the drug-related compounds.

Assessment of metabolite exposures in humans at steady state

Plasma samples were collected from healthy volunteers at 1, 1.5, 2, 3, 4, 6, 8 and 12 h post-dose following daily doses of 200 mg ASV in tablets for 10 days. Plasma samples were pooled and extracted with organic solvents according to the procedures described for ADME samples. The concentrations of drug-related materials were determined using a similar LC–MS/MS method to that of ASV. Briefly, the concentration of ASV was determined with an MRM method using d9-ASV as internal standard, and the metabolite/parent MS peak area ratio was determined for each metabolite. Since metabolites may have different MRM responses as compared to parent compound, a correction factor of each metabolite was obtained by comparing the areas of the MS peak from this study and the corresponding MS and radioactive peaks from the ADME study. The individual MRM transitions were m/z 748.2 → m/z 648.2 for ASV, m/z 764.2 → m/z 664.2 for M3 and M7, m/z 780.2 → m/z 680.2 for M4, m/z 557.2 → m/z 457.2 for M9, m/z 573.2 → m/z 473.2 for M12 and M25, m/z 645.2 → m/z 545.2 for M8, m/z 565.2 → m/z 465.2 for M15, m/z 661.2 → m/z 561.2 for M20.

Isolation and characterization of M12

Human fecal homogenates (100 mL) from subjects in human ADME study were extracted with 200 mL ethyl acetate. The organic phase was separated and dried under nitrogen and the residue was re-dissolved in 5 mL water/methanol mixture (4:1, v/v). Metabolite M12 was isolated with a preparative Shimadzu HPLC system (Shimadzu, Kyoto, Japan) using a preparative Synergy Polar RP HPLC column (Phenomenex, Torrance, CA, 21.2 × 150 mm, 4.0 μm). The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B), and the flow rate was 10 mL/min. The mobile phase composition started at 10% B, increased to 90% B over the course of 30 min, followed by a column wash with 90% B for 5 min. Fractions containing M12 were collected and dried under nitrogen. The structure of M12 was confirmed with MS/MS and 1H NMR analysis (Supplemental Information).

The stability of M12 was tested in human plasma and HLM. [14C]M12 (0.2 mL, 0.21 μCi/mL) was mixed separately with human plasma (1 mL) or HLM (1 mL), and the mixtures were incubated at 37 °C. Aliquots (200 mL) were removed following incubation for 0, 2, 5 and 24 h. The aliquots from HLM incubation mixture were immediately treated with 200 μL cold ACN, vortex mixed and centrifuged at 14 000 rpm for 10 min. Plasma aliquots were treated with the same procedure as that for human ADME samples. Supernatants were removed for radioactivity counting and LC/MS profiling.

HLM-mediated metabolism of [14C]ASV and M6

HLM-mediated metabolism was carried out at 37 °C in a shaking water bath with a final volume of 0.5 mL. The incubation mixtures consisted of [14C]ASV (10 μM, 58 nCi/mL) or M6 (5 μM), HLM (1.0 mg protein/mL), NADPH (1.0 mM), MgCl2 (0.5 mM) and potassium phosphate buffer (100 mM, pH 7.4). Negative control experiments were conducted under similar conditions but either lacking proteins or NADPH. Additional incubations of M6 with HLM were conducted under similar conditions with the addition of nucleophilic trapping reagent (GSH, 5 mM). The mixtures were allowed to react for 1 h and were quenched with the addition of 0.5 mL cold acetonitrile. The samples were vortex mixed and centrifuged for 10 min at 14 000 rpm. Supernatant from the incubation of [14C]ASV was profiled with LC–MS/MS. Incubation mixtures of M6 were analyzed with LC–UV–MS/MS and the levels of drug-related materials were estimated based on their UV absorption at 254 nm.

Incubations of ASV with human cDNA-expressed CYP enzymes

ASV was incubated in triplicate with human cDNA-expressed CYP enzymes (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5) at 37 °C in a shaking water bath. The reaction mixtures consisted of individual CYP enzyme (100 pmol/mL), ASV (2 or 20 μM), NADPH (1.0 mM), MgCl2 (0.5 mM) and phosphate buffer (0.1 M, pH 7.4) with a final volume of 0.5 mL. The mixtures were allowed to react for 20 min before being quenched with cold acetonitrile (0.5 mL). The samples were mixed with vortexing and centrifuged for 10 min at 14 000 rpm.

Concentrations of M3, M6, M7, M9 and M12 were determined with an API 4000 Qtrap mass spectrometer coupled with a Shimadzu Class VP HPLC system. The chromatographic conditions and MS parameters were same as those for the quantitation of ASV. Standards of M3, M7 and M12 were not available. Thus, an incubation mixture of [14C]ASV with HLM, with known concentration of each metabolite from radio-profiling, was used as the analyst standard. The standard samples were prepared by adding IS (d9-ASV, 50 ng/mL) followed by serial dilutions. The mixtures were vortex mixed and centrifuged, and 20 μL aliquots were injected into LC–MS/MS along with the study samples. Standard curves were fitted with a quadratic regression equation weighted by the reciprocal of the concentration (1/x). The selected reaction monitoring (SRM) transitions were m/z 764.2 → m/z 664.2 for M3 and M7, m/z 734.2 → m/z 634.2 for M6, m/z 557.2 → m/z 457.2 for M9, m/z 573.2 → m/z 473.2 for M12 and m/z 757.2 → m/z 657.2 for IS.

HLM incubation in the presence of P450 chemical inhibitors

All experiments were conducted in triplicate with a final volume of 0.5 mL. The incubation mixtures consisted of HLM
(250 µg protein/mL), ASV (2 or 20 µM), NADPH (1 mM), MgCl₂ (0.5 mM), phosphate buffer (0.1 M, pH 7.4) and individual inhibitors. Chemical inhibitors were furafylline (10 µM) for CYP1A2, tranylcypromine (2 µM) for CYP2A6, orphenadrine (50 µM) for CYP2B6, montelukast (3 µM) for CYP2C8, sulfaphenazole (10 µM) for CYP2C9, quinidine (1 µM) for CYP2D6, diethylthiocarbamate (50 µM) for CYP2E1, ketoconazole (1 µM) for CYP3A4/5 and 1-ABT (1 mM) for all CYPs. Incubations without inhibitors were also conducted as the positive controls. For incubations with direct chemical inhibitors (tranylcypromine, montelukast, sulfaphenazole, quinidine and ketoconazole), all ingredients were mixed together except NADPH. The mixtures were warmed to 37 °C and NADPH was then added to the mixtures to initiate the reactions. The mixtures were allowed to react for 30 min before being quenched with cold acetonitrile (0.5 mL) containing IS (d₉-ASV, 50 ng/mL). For incubations with time-dependent inhibitors (furafylline, orphenadrine, diethylthiocarbamate and 1-ABT), the inhibitors were pre-incubated with HLM in the presence of NADPH at 37 °C for 15 min before the substrate was added. The mixtures were incubated for additional 20 min at 37 °C before being quenched with cold acetonitrile (0.5 mL) containing IS (d₉-ASV, 50 ng/mL). The quenched reaction mixtures were centrifuged for 10 min at 14,000 rpm. A portion of the supernatant (50–80 µL) was injected into LC/MS to determine the concentration of M3, M6, M7, M9 and M12.

**ASV concentration-dependent metabolite formation**

The kinetics of ASV oxidation in cDNA expressed that CYP3A4 and 3A5 were determined over a range of ASV concentrations. The protein concentrations of CYP3A4 and 3A5 were 10 and 20 pmol/mL, respectively. Eight ASV concentrations (1, 2, 5, 10, 20, 30, 40 and 60 µM) were evaluated in triplicate. The incubation mixtures consisted of ASV, NADPH (1 mM), MgCl₂ (0.5 mM) and protein (CYP3A4, 10 pmol/mL; or CYP3A5, 20 pmol/mL) in phosphate buffer (100 mM, pH 7.4). The mixtures were allowed to react for 30 min before being quenched with cold acetonitrile (0.5 mL). The samples were mixed by vortexing and centrifuged for 10 min at 14,000 rpm. A portion of the supernatant (50–80 µL) was injected into LC–MS/MS to determine the metabolite concentrations.

**Data analysis**

The pharmacokinetic parameters of ASV and TRA were determined using Kinetica 4.4.1 (Thermo Electron Corporation, Philadelphia, PA) with a noncompartmental method. The following single-dose PK parameters were derived: maximum plasma concentration (Cmax), time to reach maximum observed plasma concentration (Tmax), area under the concentration–time curve from time 0 extrapolated to infinity (AUC0–inf), AUC from time 0 to the last quantifiable concentration (AUC0–T), terminal elimination half-life (t1/2), renal clearance (CLR) and apparent total body clearance (CL/F). Km and Vmax values of CYP3A4 and 3A5 were estimated by fitting the ASV oxidation activities to the Michaelis–Menten equation using a non-linear regression analysis in SigmaPlot.

**Results**

**Plasma concentration–time profiles**

The mean plasma concentration–time profiles of ASV and TRA following a single oral dose of [14C]ASV are shown in Figure 2, and pharmacokinetic parameters are summarized in Table 1. TRA and ASV readily appeared in systemic circulation with Tmax occurring before 2 h in subjects from both the groups following oral dose. After the absorption phase, ASV declined at a faster rate than TRA with t1/2 ~22 h in group 1 subjects, compared to t1/2 40 h for TRA. The AUC₀–T of ASV in plasma represented 26.3% and 27.9% of TRA in group 1 and 2 subjects, respectively, with the remaining radioactivity presumably contributed from metabolites.

**Excretion of radioactive dose**

The mean percent recovery of radioactivity in urine, feces and bile is summarized in Table 2. Approximately 84% and 73% of the dose were recovered in feces from subjects in groups 1 and 2, respectively. The recovery of radioactivity in urine was minimum, with <0.3% dose over the entire collection period (0–240 h). In subjects with bile collection (group 2), approximately 8% of the dose was recovered in bile over 3–8 h post-dose collection period.

**Metabolite profiles**

Representative radiochromatograms of plasma, feces and bile are shown in Figure 3, and the distribution of metabolites is summarized in Table 3. Urine samples were not profiled for metabolite analysis because of low concentration of radioactivity. Biotransformation studies of ASV in animals were also conducted. Experimental details and representative radiochromatograms of plasma, feces, urine and bile from animal studies are included in the Supplemental Information.

**Plasma**

The recovery of radioactivity in pooled plasma after extraction with organic solvents was greater than 90%. Unchanged ASV was the most abundant drug-related materials in human plasma, representing ~73% of plasma TRA at 2 h post-dose. A number of monohydroxy metabolites (M3 and M7) and dihydroxy metabolite (M4), along with M8 (sulfonamide...
hydrolysis), M9 (loss of isoquinoline), M12 (hydroxylation of M9) and M25 (hydroxylation of M9) were observed in plasma from humans with the concentration of each metabolite 5% of the parent. The area under the curve (AUC) values of the prominent human circulating metabolites, estimated based on their fraction of TRA in plasma, were well below 10% of plasma TRA, and were lower in humans than in animals (Table 4). To evaluate the metabolite exposure at steady state, plasma samples from healthy volunteers following multiple doses of ASV were collected and analyzed to estimate the concentration of the metabolites (total of nine metabolites). Plasma concentrations of these metabolites were increased by 3–12-fold after multiple doses of ASV.

Feces

Similar metabolite profiles were observed in feces from subjects with or without bile collection. Unchanged ASV comprised of approximately 9% of the radioactivity excreted in feces from group 1 subjects, or 7.5% of the dose. The most abundant metabolites in feces were M8 and M12, accounted for 14.6% and 8.3% of the dose, respectively. Other prominent drug-related components were M3, M7, M22 (dihydroxylation) and M24 (dihydroxylation), along with M9 and its secondary metabolites M25 and M26. In addition, metabolites resulted from the hydroxylation of M8, including M11, M19 and M20, were observed in the feces.

Bile

Unchanged ASV was the most prominent drug-related component, representing 26.6% of the radioactivity excreted in bile. Numerous metabolites were detected in bile. Among them, five metabolites (M3, M4, M7, M22 and M24) were generated from mono- or bis-oxidation that together representing 20.7% of the radioactivity in human bile. Metabolite M9 and its secondary metabolites (M12, M25 and M26) together represented 17.1% of biliary radioactivity. Other prominent metabolites included M8 (4.8% of biliary radioactivity) and its secondary metabolites.

Identification of metabolites

LC–MS/MS analysis was the primary tool to generate the radio-profiles and to elucidate the structures of the metabolites. Structures were proposed based on the MS of the molecular ions, MS/MS fragmentation patterns and by comparison of HPLC retention times to those of the reference standards. A list of the metabolites observed in humans, along with the MS spectrum of each metabolite, is shown in Table 5. The proposed structures and the metabolic pathways are illustrated in Figure 4. The rationale for structural assignment is described in the Supplemental Information.

Metabolite M12 was an epoxide based on the characterization with 1H NMR (Supplemental Information), as illustrated in Figure 4. The metabolite was formed through the loss of the isoquinoline moiety of ASV and epoxidation on the vinyl group. To test its stability, [14C]M12 was isolated from human feces and incubated with HLM and human plasma at 37°C up to 24 h. The radioactivity in HLM and plasma was completely recovered after extraction with organic solvents. In addition, the radio-chromatograms of the organic extracts showed a single peak of M12.

Identification of human enzymes involved in the metabolism of ASV

As an initial step towards the identification of human metabolic enzyme, in vitro metabolism of ASV in HLM was conducted. Results suggested that ASV was relatively stable in HLM with 75.9% of the parent drug remaining after 1 h incubation. The primary metabolites observed in the

| Group | Analyte | C<sub>max</sub> (ng/mL) geometric mean (CV) | AUC<sub>0-T</sub> (ng h/mL) geometric mean (CV) | AUC<sub>inf</sub> (ng h/mL) geometric mean (CV) | T<sub>max</sub> (h) median (min–max) | t<sub>1/2</sub> (h) mean (SD) | CLT/F (L/h) geometric mean (CV) |
|-------|---------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|-----------------|--------------------------|
| Group 1 (n = 6) | ASV 367.3 (72) 1084.0 (63) | 1087.3 (63) 1.75 (1.0–4.0) | 22.45 (6.366) | 183.9 (48) |
| Group 1 (n = 3) | TRA 491.5 (65) 4119.9 (50) | 4843.6 (48) 1.75 (1.5–4.0) | 39.71 (14.000) | 41.3 (54) |

NR: not reportable: two out of three subjects in group 2 had AUC extrapolated over 20% of AUC(INF).

For TRA data, the concentration unit is ng-equiv ASV/mL, AUC unit is ng-equiv ASV h/mL.

Table 2. Recovery of radioactive dose in subjects from groups 1 and 2 (with bile-collection at 3–8 h post-dose) after oral administration of [14C]ASV.

| Species | No. of subjects | Collection interval (h) | Urine | Bile<sup>a</sup> | Feces | Total |
|---------|----------------|------------------------|-------|-----------------|-------|-------|
| Group 1 | 6              | 0–240                  | 0.24 ± 0.18 | N.A.<sup>b</sup> | 83.86 ± 9.43 | 84.10 ± 9.61 |
| Group 2 | 3              | 0–240                  | 0.19 ± 0.04 | 8.14 ± 4.14 | 73.13 ± 1.66 | 81.46 ± 5.84 |

<sup>a</sup>Collected from 3 to 8 h post-dose.

<sup>b</sup>N.A., not applicable.

Table 1. Pharmacokinetic parameters of TRA and ASV after a single oral dose of 200 mg [14C]ASV to healthy subjects.

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Human ADME of asunaprevir

Human ADME of asunaprevir
incubation mixture were M3, M7, M6 (O-demethylation), M9 and M8 (Figure 3d). Other metabolites resulting from further oxidation of the primary metabolites included M4, M11 and M12. In the absence of NADPH, only M8 and M9 were detected in the incubation mixture. The formation of M8 was not influenced by NADPH, but M9 was formed at much lower level (<20%) in HLM without the cofactor. Metabolite M6 was not observed in humans following dose administration of [14C]ASV even though this was a prominent metabolite in HLM incubation, presumably because of further metabolism. To understand the downstream metabolism of M6, it was incubated with HLM in the presence of NADPH. Metabolite M6 was converted rapidly to M9 and M12 with >90% of the substrate metabolized after incubation. No other metabolites were detected in the mixture. On the other hand, only a small portion of M6 (<5%) was oxidized in HLM in the absence of the cofactor. As a side product, the isoquinoline was not detected in incubation mixture. Instead, a downstream metabolite of isoquinone (proposed structure illustrated in Figure 5) with a protonated molecular ion at m/z 228.6, and major MS fragments at m/z 213.6 and m/z 186.6, was observed in the incubation mixture. When GSH was added to the incubation mixture of M6 with HLM in the presence or absence of NADPH, no covalent adduct with GSH was observed.

To evaluate the activity of each P450 enzyme, human cDNA-expressed P450s were incubated with ASV. The formation of the oxidative metabolites including M3, M6, M7, M9 and M12 was monitored with LC–MS/MS. Among the enzymes tested, CYP3A4 and CYP3A5 displayed highest activity for the formation of these metabolites. Other enzymes including CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6 and 2E1 showed little or no activity for the metabolism of ASV (Figure 6). To further confirm the primary CYP enzymes involved in ASV metabolism, selective inhibitors of the CYP enzymes were evaluated for their inhibitor effects on the formation of metabolites (Figure 7). In the presence of ketoconazole, a selective inhibitor of CYP3A4/5, the formation of hydroxy metabolites (M3 and M7) and metabolites through the loss of isoquinoline (M9 and M12) were inhibited by 95% and 89%, respectively. Likewise, a time-dependent inhibitor of CYP3A4/5 (troleandomycin) inhibited the formation of metabolites by >90%. Inhibitors for the other CYP enzymes did not inhibit the metabolism of ASV to a meaningful extent.

To further evaluate the relative contribution of CYP3A4 and 3A5 in the metabolism of ASV, the enzyme kinetic parameters of oxidation by each enzyme were determined. The $K_m$ values for the formation of hydroxy metabolites were 24 and 18 μM, and $V_{max}$ values were 18 and 9 (pmol/min/pmol protein) for CYP3A4 and CYP3A5, respectively (Figure 8).

**Discussion**

This study aimed to characterize biotransformation of [14C]ASV following a single oral dose to healthy volunteers and to identify enzymes involved in the metabolism. For a comparison, biotransformation of [14C]ASV in preclinical species following a single dose of [14C]ASV at no adverse effect levels (NOAEL) was also described and included in the Supplemental Information. ASV pharmacokinetic behavior observed in the current study was largely consistent with the findings in previous studies (Eley et al., 2013). ASV was rapidly absorbed in humans with $T_{max}$ within 2 h after oral administration. The clearance of TRA was much slower than ASV suggesting the presence of persistent metabolites, e.g. M3, M12, M15, M20, etc. whose exposures were increased significantly after multiple doses of ASV. In addition, the AUC of ASV accounted for only a small portion of plasma TRA (<20%), with the rest attributed to metabolites. This was confirmed by radio-profiles of plasma where numerous metabolites were observed along with ASV as the most abundant drug-related species. These metabolites were present at low levels with the percent of relative distribution generally below 5% of the parent or total drug-related materials, and were observed in at least one preclinical species (Supplemental Information). The metabolite exposures in humans at steady state (200 mg ASV twice daily for
10 days) were also evaluated with LC–MS/MS (Supplemental Information). The estimated exposure values (AUC) of each prominent circulating metabolite in humans at steady state were well below their levels in animal plasma, suggesting that systemic exposures of these metabolites were covered in the toxicological species. The biological activities of these metabolites have not been tested. However because of low plasma concentration, these minor metabolites are not expected to contribute significantly to the pharmacology of ASV.

Recovery of radioactivity was nearly complete after 240 h post-dose in humans. The majority of radioactivity was excreted in feces, with minimal recovery in urine. Therefore, it is likely that renal impairment would not significantly alter renal clearance of ASV. A group of subjects with bile collection (3–8 h post-dose) were included in the study because in a previous study, a second peak was observed in PK profiles ~10 h post-dose at different dose panels (Eley et al., 2013). This was presumably due to enterohepatic recirculation of intact parent and/or conjugative metabolites. Even though bile collection only provided a snapshot in the entire collection period, the metabolite profile in bile provided valuable information to address the potential of enterohepatic recirculation. A good amount of radioactivity was excreted in bile with ~27% of biliary radioactivity attributed to the intact parent drug. The presence of unchanged ASV suggests the likelihood of enterohepatic recirculation even though there was no obvious second PK peak in the current study. Overall, excretory profiles of radioactivity suggested that biliary excretion followed by fecal elimination was the major elimination route of drug-derived radioactivity. Additionally, in a study conducted previously

### Table 3. Relative distribution of metabolites in plasma, feces and bile in humans after oral administration of [14C]ASV.

| Metabolite | Plasma (% radioactivity) | Feces | Bile |
|------------|--------------------------|-------|------|
| P          | 73.2                     | 52.7  | 8.9  |
| M3         | 5.5                      | 1.4   | 9.2  |
| M4         | 3.0                      | 9.2   | 3.1  |
| M7         | 1.6                      | 5.7   | 14.6 |
| M8         | 3.0                      | 2.8   | 1.8  |
| M9         | 3.6                      | 0.5   | 3.6  |
| M11        | ND                       | ND    | 6.5  |
| M12        | 1.4                      | 0.5   | 10.0 |
| M15        | 1.1                      | 9.0   | 4.5  |
| M19        | ND                       | ND    | 1.1  |
| M20        | ND                       | ND    | 4.3  |
| M21        | ND                       | ND    | 0.4  |
| M22        | ND                       | ND    | 3.6  |
| M24        | ND                       | ND    | 5.2  |
| M25        | 0.3                      | 8.7   | 6.7  |
| M26        | ND                       | ND    | 0.5  |
| Total      | 96.0                     | 93.6  | 85.4 |

### Table 4. Metabolite exposures (AUC) in plasma from animals and humans following oral doses of [14C]ASV.

| Metabolite | Mouse plasma | Rat plasma | Dog plasma | Human plasma |
|------------|--------------|------------|------------|--------------|
| TRA        | 1.15 × 10⁵   | 1.04 × 10⁵ | 4.55 × 10⁴ | 2.97 × 10³  |
| ASV        | 6.57 × 10⁴   | 7.96 × 10⁴ | 3.34 × 10⁴ | 1.08 × 10³  |
| M3         | 5.14 × 10²   | 9.62 × 10² | 4.03 × 10³ | 6.18 × 10⁵  |
| M4         |              | 9.63 × 10² |            |              |
| M7         | 7.63 × 10²   | 7.21 × 10² | 2.13 × 10² | 6.61 × 10⁵  |
| M8         | 1.45 × 10²   | 9.97 × 10² | 7.95 × 10² | 3.41 × 10⁵  |
| M9         | 7.95 × 10²   |            | 1.67 × 10² | 9.80 × 10⁵  |
| M12        | 3.37 × 10²   |            |            | 3.20 × 10⁵  |
| M15        | 4.24 × 10²   |            | 2.24 × 10² | 4.26 × 10⁵  |
| M20        | 1.06 × 10³   | 4.62 × 10² | 6.54 × 10² | 2.56 × 10⁵  |
| M25        | 4.28 × 10²   | 1.83 × 10² |            | 9.16 × 10⁵  |

# References

Eley et al., 2013.
where BDC dogs were administered IV doses of [14C]ASV (3 mg/kg), ~10% of radioactivity was recovered as unchanged ASV in the feces, suggesting that direct intestinal excretion played a role in the clearance of ASV (Mosure et al., 2015). The potential role of transporters in the biliary and intestinal secretion has been investigated and will be published in a separate manuscript.

Radioprofiles of bile and feces from humans were qualitatively similar to those from animals (Supplemental Information). Metabolites comprised the majority of the radioactivity in bile and feces suggesting that parent drug underwent extensive metabolism prior to excretion. Most of the metabolites in bile and feces were generated through oxidative metabolism and hydrolysis, with approximately 50% of dose was cleared through oxidative metabolism (hydroxylation and loss of isoquinoline). The contribution of conjugative metabolism to the overall clearance of ASV was minimal. The level of unchanged ASV in feces was relatively low compared to that in bile presumably because biliary excreted ASV was re-absorbed in GI and ultimately metabolized. ASV was well absorbed in humans with minimum absorption of 76.6% after oral administration, estimated by excluding the recovery of unchanged ASV in feces from total recovery of radioactivity. The relative abundance of M8 in bile (4.8%) was significantly lower than that in feces (21.2%) indicating bacteria-mediated hydrolysis of ASV in the GI tract, which is also supported by the factor that M8 was not formed in hepatocyte incubations (Supplemental Information).

Metabolite M12 was prominent in humans, and MS and NMR analyses revealed that it was an epoxide. As epoxides are reactive in general, the stability of M12 was tested in incubations with HLM and human plasma. The results suggested that M12 was stable in both

| Metabolite | [M+H]⁺ | Major fragment | Metabolic pathway(s) | Species a |
|------------|--------|----------------|----------------------|-----------|
| ASV        | 648    | 692, 648, 535  | –                    | Mouse, rat, dog, monkey |
| M1         | 1071   | 971, 842, 664, 551 | Hydroxylation, GSH adduct | Dog |
| M3         | 764    | 708, 664, 535, 478 | Hydroxylation | Mouse, rat, dog, monkey |
| M4         | 780    | 724, 680, 597, 567 | Hydroxylation | Mouse, rat, monkey |
| M5         | 764    | 708, 664, 551, 478 | Hydroxylation | Mouse, rat, dog, monkey |
| M7         | 764    | 708, 664, 551, 478 | Hydroxylation | Mouse, rat, dog, monkey |
| M8         | 645    | 589, 545, 436, 380 | Sulfonamide hydrolysis | Mouse, rat, dog, monkey |
| M9         | 557    | 501, 457, 380, 344 | Loss of isoquinoline | Mouse, rat, dog, monkey |
| M11        | 661    | 589, 545, 452, 432, 380 | Sulfonamide hydrolysis, hydroxylation | Mouse, rat, dog, monkey |
| M12        | 573    | 555, 517, 473, 360 | Hydroxylation, Amide hydrolysis, carboxylation | Mouse, rat, dog, monkey |
| M15        | 565    | 509, 564, 356, 332 | Loss of isoquinoline, hydroxylation | Mouse, rat, dog, monkey |
| M19        | 661    | 643, 605, 561, 448 | Sulfonamide hydrolysis, hydroxylation | Mouse, rat, dog, monkey |
| M20        | 661    | 643, 605, 561, 432 | Sulfonamide hydrolysis, hydroxylation | Mouse, rat, dog, monkey |
| M21        | 780    | 724, 721, 680, 597, 567 | Hydroxylation | Mouse, rat, dog, monkey |
| M22        | 780    | 724, 721, 680, 597, 567 | Hydroxylation | Mouse, rat, dog, monkey |
| M24        | 780    | 724, 721, 680, 597, 567 | Hydroxylation | Mouse, rat, dog, monkey |
| M25        | 573    | 555, 517, 473, 360 | Loss of isoquinoline, hydroxylation | Mouse, rat, monkey |
| M26        | 573    | 555, 517, 473, 360 | Loss of isoquinoline, hydroxylation | Mouse, rat, monkey |

Table 5. Mass spectral analysis of ASV and its metabolites.

aMetabolite profiles and distribution in urine, bile, and feces from animals were included in SI.
matrices and did not react with plasma or microsomal proteins.

The proposed metabolic pathways of ASV in humans are shown in Figure 4. ASV was metabolized mainly through hydroxylation, loss of isoquinoline and sulfonamide hydrolysis. In vitro metabolism of ASV in HLM revealed that in addition to the metabolic pathways observed in humans, O-demethylation to M6 was a prominent pathway. The absence of detectable M6 in humans was probably because of rapid conversion to downstream metabolites. Indeed, when incubated with HLM in the presence of NADPH, M6 was further metabolized to M9 and M12 suggesting that M6 was the precursor for the formation of M9. The metabolism was presumably mediated by P450s because of the NADPH-dependent nature. As illustrated in Figure 6, a plausible mechanism for the loss of isoquinoline that involves O-demethylation to give M6 followed by further oxidation of the isoquinoline moiety has been proposed. Oxidation of the isoquinoline leads to an unstable quinone intermediate that finally decomposes to give M9. A number of examples have been reported where the cleavage of aryl-ether was initiated with a quinone intermediate (Dahlin et al., 1984; Hinson et al., 1977; Laham & Potvin, 1983; Sato & Guengerich, 2000). Even though other mechanisms could also play a role in the formation of M9, this P450-mediated mechanism is likely the major contributor.

Metabolism of M6 to M9 would presumably give a quinone-like side product when the reaction was initiated by the oxidation of isoquinoline and the reactive nature of quinone raises a concern of forming covalent adducts with proteins (Bolton et al., 2000). However, no downstream products of quinone formation were detected. Instead, an acid metabolite proposed to be generated through the oxidation of quinone followed by hydrolysis was observed in the incubation mixtures (Figure 6). A GSH trapping experiment was conducted to assess whether there was any reactive intermediate generated during the loss of isoquinoline ring. The absence of GSH adducts after incubation suggested that quinone-intermediate did not react with strong nucleophiles and was not likely to form protein adducts, presumably because of rapid hydrolysis to a stable acid metabolite. Consistent with in vitro findings, no GSH adduct derived from quinone-like side product was observed in the human AMDE study.

Figure 4. Proposed biotransformation pathways of ASV in humans. Pathways were proposed based on metabolites identified in plasma, bile and fecal samples.

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Figure 5. Proposed mechanisms for the formation of M9.

Figure 6. Metabolites formation in incubations of human cDNA-expressed CYP enzymes with ASV at concentrations of 20μM. Rate of formation for the hydroxylation (■) is expressed as the sum of M3 and M7, and formation rate for the loss of isoquinoline (◆) is expressed as the sum of M9 and M12. The formation of M6 was low and is not shown in the figure.

Figure 7. Inhibition of metabolite formation relative to the control in HLM in the presence of selective chemical inhibitors at 20μM concentration of ASV: (■) hydroxylation metabolites, (◆) loss of isoquinoline.
Drug-derived radioactivity was mainly eliminated through biliary excretion followed by fecal elimination. Unchanged parent drug was the most abundant circulating drug-related component, along with numerous minor metabolites that were also observed in animal plasma. Because of low plasma concentration, these minor metabolites are not expected to contribute significantly to the pharmacology of ASV. The primary metabolic pathways of ASV were hydroxylation, sulfonamide hydrolysis and O-demethylation followed by further oxidation leading to the loss of isoquinoline. Oxidative metabolism of ASV was primarily catalyzed by CYP3A4 and 3A5.

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Declaration of interest

The authors report no declaration of interest.

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Supplementary material available online
Supplementary Information