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A rugged and accurate liquid chromatography–tandem mass spectrometry method for the determination of asunaprevir, an NS3 protease inhibitor, in plasma

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ABSTRACT

Asunaprevir (BMS-650032) is a potent hepatitis C virus (HCV) non-structural protein protease inhibitor currently in Phase III clinical trials for the treatment of HCV infection. A rugged and accurate LC–MS/MS method was developed and validated for the quantitation of asunaprevir in rat, dog, monkey, rabbit and mouse plasma. A systematic method screening and optimization strategy was applied to achieve optimized mass spectrometry, chromatography, and sample extraction conditions. The validated method utilized stable-isotope labeled D_9-asunaprevir as the internal standard. The samples were extracted by liquid–liquid extraction using 10% ethyl acetate in hexane. Chromatographic separation was achieved with gradient elution on a Waters Atlantis dC18 analytical column. Analyte and its internal standard were detected by positive ion electrospray tandem mass spectrometry. The standard curve, which ranged from 5.00 to 2000 ng/mL for asunaprevir, was fitted to a $1/x^2$ weighted linear regression model. The intra-assay precision was within ±3.6% CV, inter-assay precision was within ±4.0% CV, and the assay accuracy was within ±8.1% of the nominal values in all the species. The method was successfully applied to support multiple pre-clinical toxicokinetic studies in different species.

1. Introduction

Hepatitis C virus (HCV) infects an estimated 170 million people worldwide, and is a major cause of chronic liver disease [1]. Most infections progress to chronic hepatitis, which can lead to cirrhosis, liver failure, and hepatocellular carcinoma. In the United States, HCV infections cause more than 10,000 deaths annually [1] and are the leading indication for liver transplantation [2]. The viral non-structural protein 3 (NS3) protease, a serine protease located in the N-terminal region of NS3, interacts with its activating cofactor NS4A to form an active proteolytic complex required for subsequent viral replication [3]. The inhibition of the NS3 serine protease activity could effectively block viral replication, which makes the protease inhibitor an attractive target for new anti-HCV drugs [4]. Two protease inhibitors, telaprevir and boceprevir, were approved in the United States and Europe in 2011 for treating chronic HCV, and multiple protease inhibitors are in clinical development [4]. Asunaprevir (BMS-650032; Fig. 1), a potent HCV NS3 protease inhibitor, is currently in Phase III clinical trials for the treatment of HCV infection. Asunaprevir demonstrated robust antiviral activity in patients with HCV genotype 1 infection in single-ascending-dose and multiple-ascending-dose clinical studies [5].

Asunaprevir was previously used as the model compound to develop a convenient strategy for quantitative bioanalytical assay in tissue samples [6]. In this manuscript, we report the method development and validation of an LC–MS/MS method for the quantification of asunaprevir in rat, dog, monkey, rabbit and mouse plasma. A systematic method screening and optimization strategy [7,8] was applied during method development to achieve optimized mass spectrometry, chromatography, and sample extraction conditions. Incurred samples were used for method development and optimization, which ensured the quality of the method and

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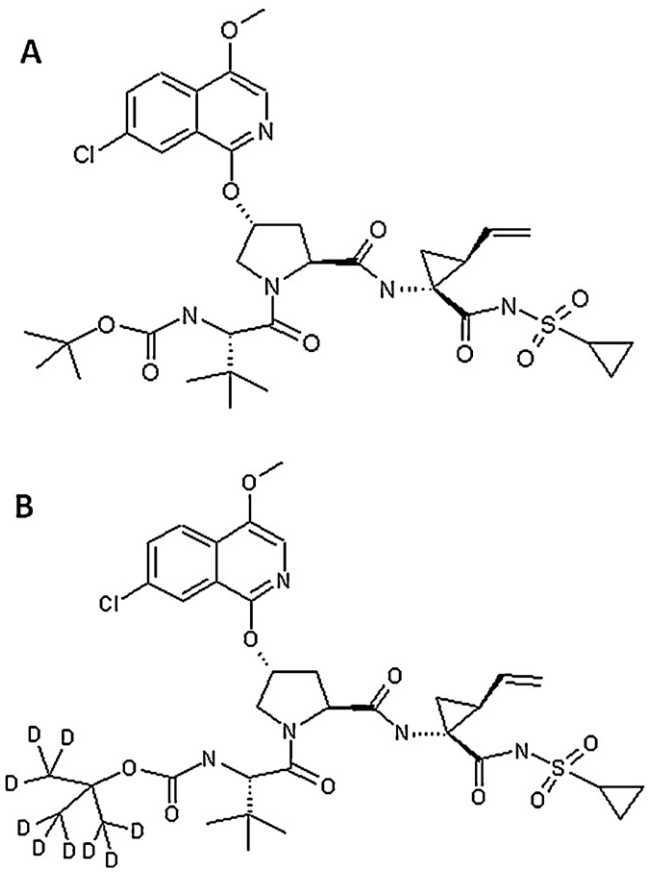


Fig. 1. Chemical structures of asunaprevir (A) and its SIL-IS, D_9-asunaprevir (B).

minimized bioanalytical risks from potential metabolite interference. The method utilized stable-isotope labeled D_9-asunaprevir as internal standard and liquid–liquid extraction (LLE) to clean up the plasma samples. The validated method has been successfully used to support pre-clinical toxicokinetic studies in different species.

2. Materials and methods

2.1. Chemicals, reagents, materials, and apparatus

Asunaprevir (BMS-650032) and its stable isotope labeled internal standard (SIL-IS) D_9-asunaprevir were obtained from Bristol-Myers Squibb (New Brunswick and Princeton, NJ, respectively). HPLC-grade acetonitrile, methanol, and isopropanol were purchased from Burdick & Jackson (Muskegon, MI). Ammonium formate, isopropanol, hexane, and ethyl acetate were obtained from J.T. Baker (Phillipsburg, NJ). Formic acid (>98%) was obtained from EMD Chemicals (Gibbstown, NJ). Ammonium bicarbonate was purchased from Mallinckrodt (Paris, KY). Deionized water was obtained from an in-house Barnstead water purification system (Dubuque, IA). Control rat, dog, monkey, rabbit, and mouse K_2EDTA plasma were obtained from Bioreclamation (Hicksville, NY). A Quadra 96 liquid handling robotic system (Tomtec, Hamden, CT) was used for the liquid transfer in sample extraction.

2.2. LC–MS/MS equipment

The HPLC system consisted of a Shimadzu System Controller (Model SCL-10A Vp), Binary Pumps (Model LC 10AD Vp), SIL-HTC autosampler, and solvent degasser (Shimadzu Scientific Instrument, Columbia, MD). Chromatographic separation was achieved on an Atlantis dC_{18} column (2.1 × 50 mm, 3 μm; Waters, Milford, MA). LC–MS/MS data was acquired on a Sciex API 4000 mass spectrometer (Sciex, Toronto, Canada) with Analyst software v 1.4.2.

2.3. LC–MS/MS conditions

A gradient solvent system consisting of mobile phase A (10.0 mM ammonium bicarbonate in water), and mobile phase B (acetonitrile) was used. The linear gradient was as follows: 0–0.1 min 30%B; 0.1–2.5 min 30–70%B; 2.5–3.0 min 70%B; 3.0–3.1 min 70–30%B; and run stopped at 4.0 min. The flow rate was 0.40 mL/min and the injection volume was 5 μL.

The mass spectrometer was operated in electrospray positive ionization mode. The optimized operating parameters were: curtain gas 30 units; ion source gas 1, 30 units; ion source gas 2, 60 units; temperature 500 °C; ion spray voltage 4500 V; dwell time 200 ms; declustering potential 60 V; and collision energy 39 eV. The selected reaction monitoring (SRM) monitored was m/z 748 → 535 for asunaprevir, and m/z 757 → 536 for D_9-asunaprevir.

2.4. Preparation of standard (STD) and quality control (QC) samples

STD and QC stock solutions of asunaprevir at 0.500 mg/mL were prepared from separate weighings by dissolving the analyte into methanol. A standard working stock solution containing 0.100 mg/mL asunaprevir was prepared by appropriate dilution of the 0.500 mg/mL stock solution with methanol. This solution was diluted appropriately with control plasma to obtain the standards with final concentrations of 5.00, 10.0, 20.0, 50.0, 100, 500, 1000, and 2000 ng/mL. Standards were prepared fresh daily. Similarly, six levels of QCs were prepared at final concentrations of 5.00, 15.0, 125, 1000, 1600, and 50,000 ng/mL and stored at −20 °C.

2.5. Sample extraction

Samples were extracted by LLE as follows: 50 μL of samples, blanks, STDs and QCs were added into wells of a 96-well plate. Then 50 μL of internal standard working solution (100 ng/mL of D_9-asunaprevir in 50:50 (v:v) methanol/water) and 100 μL of 1.0 M ammonium formate in water buffer (pH 3.0) were added into each well. After adding 600 μL of 10% ethyl acetate in hexane into each well, the plate was covered with a sealing mat and vortexed for 1 min at high speed. The plate was then centrifuged at 2000 × g for 4 min. The supernatant (480 μL) was transferred into a new 96-well plate and evaporated to dryness at 40 °C under a nitrogen flow. The samples were reconstituted with 100 μL of the reconstitution solution (5 mM ammonium bicarbonate in 50:50 (v:v) water/acetonitrile).

2.6. Application in a dog toxicology study

To demonstrate the utility of the validated LC–MS/MS assay, results from a long term toxicity study conducted in dogs are presented here. Dogs were treated with control vehicle articles or 15, 50, and 100 mg/kg asunaprevir test articles once daily via oral administration (capsule dosing). Blood samples were collected at 1, 2, 3, 4, 8, and 24 h from a peripheral vessel following dosing on day 1 and after a daily dose during weeks 13, 26, and 39. Blood were processed for plasma within 1 h and stored at -20 °C until analysis. Plasma samples were analyzed for asunaprevir. Toxicokinetic (TK) parameters were calculated from plasma concentration and time data using non-compartmental methods by Kinetica in eToolbox.

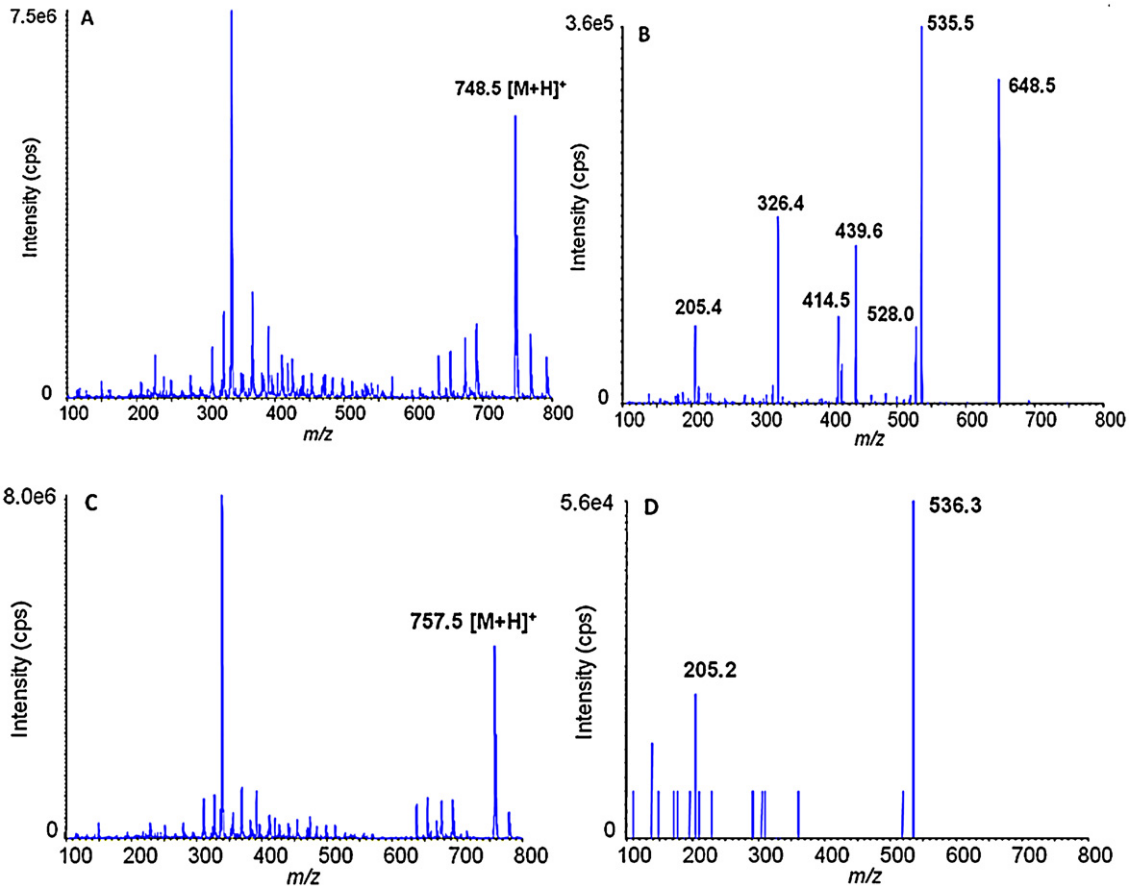


Fig. 2. Electrospray positive ion MS scan ion spectra and electrospray positive ion product ion spectra of asunaprevir (A and B) and D_9-asunaprevir (C and D).

3. Results and discussion

3.1. Mass spectrometry, chromatography, and sample extraction

Using the systematic method screening and optimization strategy previously described [8], we achieved optimized chromatography, mass spectrometry, and sample extraction conditions during method development, which also ensured the quality and robustness of the method for subsequent validation and sample analysis. As shown in Fig. 2A and B, m/z 748 is the protonated ion of asunaprevir under positive electrospray and the major product ion of m/z 748 is m/z 535. Therefore, the SRM transition m/z 748 → 535 was chosen for the monitoring of asunaprevir. Similarly, the SRM transition m/z 757 → 536 was chosen for D_9-asunaprevir (Fig. 2C and D).

Seven aqueous mobile phases from acidic to basic (see details in Fig. 3) and two organic mobile phases (acetonitrile and methanol) were screened. As presented in Fig. 3, the mass spectrometric response of asunaprevir was much higher in basic mobile phases than in acidic mobile phases. Better peak shape (sharper and symmetric) was also achieved in basic mobile phases. After further optimization, ammonium bicarbonate in water and acetonitrile were selected as the mobile phases, since they provided the best mass spectrometric response, chromatographic peak shape, and suitable retention time.

Six different extraction solvents (10% ethyl acetate in hexane, 20% ethyl acetate in hexane, n-butyl chloride, methyl tertiary-butyl ether (MTBE), ethyl acetate, and toluene) and three different extraction buffers (acidic, neutral, and basic) were screened. As shown in Fig. 4, both n-butyl chloride and 10% ethyl acetate in hexane achieved good recoveries (>70%). However, the matrix effect was

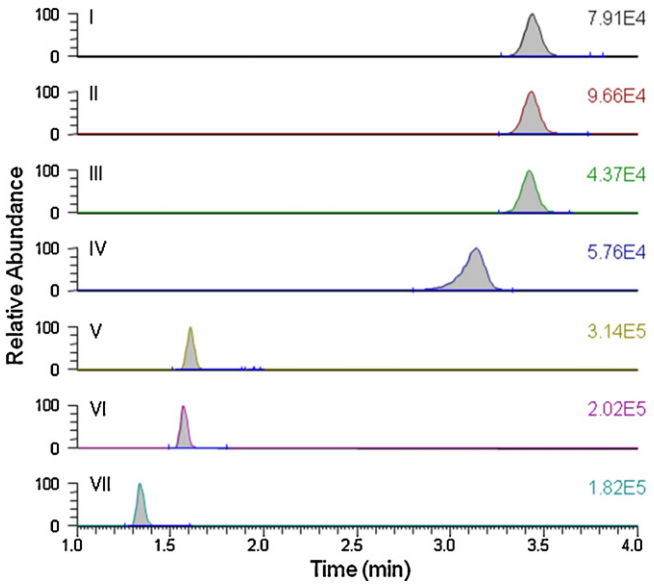


Fig. 3. LC–MS/MS chromatograms of asunaprevir using different mobile phases (mobile phase A: I – 0.05% formic acid at pH 2.7, II – 5 mM ammonium formate with 0.04% formic acid at pH 3.0, III – 0.05% acetic acid at pH 3.6, IV: 5 mM ammonium acetate with 0.04% acetic acid at pH 4.2, V – 5 mM ammonium bicarbonate at pH 7.8, VI – 5 mM ammonium carbonate at pH 10, and VII – 0.1% ammonium hydroxide at pH 11; mobile phase B: acetonitrile).

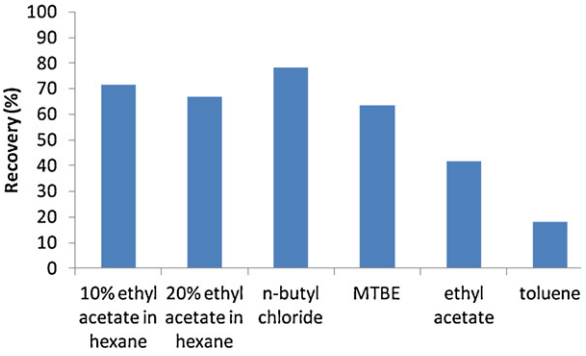


Fig. 4. Recovery of asunaprevir after LLE using different extraction solvents (extraction buffer: 1 M ammonium formate in water, pH 3.0).

higher using n-butyl chloride and was minimal using 10% ethyl acetate in hexane. Therefore, 10% ethyl acetate in hexane under acidic condition (1 M ammonium formate in water, pH 3.0) was chosen as the extraction method. We also screened solid-phase extraction (SPE) methods for the sample cleanup. From our results, the SPE methods did not achieve better recovery and matrix effect for asunaprevir compared to the LLE method (data not shown). Therefore, we chose the optimized LLE method based on its good recovery and matrix effect, simpler operation and reduced cost.

3.2. Evaluation of interference from phospholipids, polyethylene glycol 400, and metabolites

Matrix effect, the ionization suppression or enhancement of analyte due to co-eluting matrix constituents, may affect the accuracy, precision and sensitivity of LC–MS/MS bioanalytical assays [9,10]. One major source of matrix effects is the abundant phospholipids in plasma samples [7]. We evaluated phospholipids profiles of incurred samples under the optimized chromatographic condition using positive precursor ion scan of *m/z* 184 and negative precursor ion scan of *m/z* 153 [11]. Polyethylene glycol 400 (PEG 400) is one of the most commonly used dosing excipients and is one major component in asunaprevir test vehicle. PEG 400 has been demonstrated to be present in blood after intravenous or oral administration, and may lead to significant matrix effect to analyte in LC–MS/MS analysis [12–14]. We also evaluated the potential interference from PEG 400 using positive precursor ion scan of *m/z* 133. As presented in Fig. 5, the asunaprevir peak was well separated from the major phospholipids peaks and PEG 400, which demonstrated minimum risks for matrix effect from phospholipids or PEG 400.

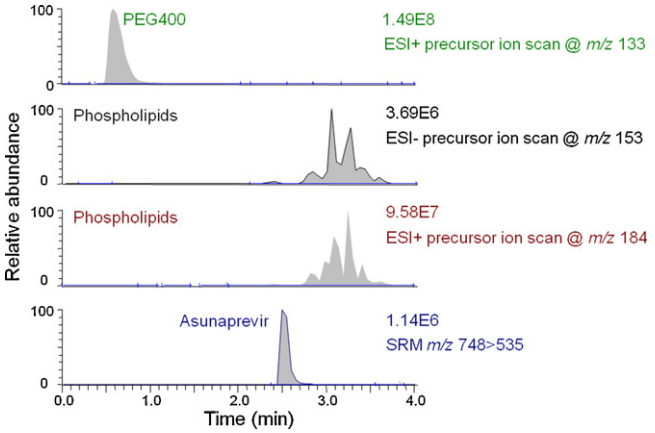


Fig. 5. Chromatograms of PEG 400, phospholipids and asunaprevir in a pooled incurred sample.

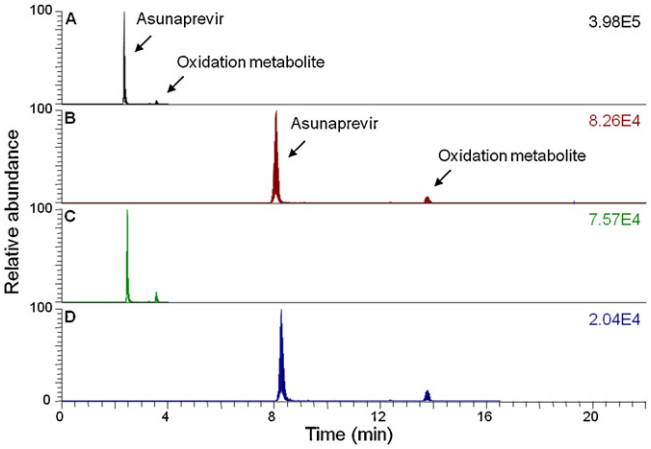


Fig. 6. SRM total ion chromatograms of a pooled rat (A, B) and dog (C, D) incurred sample using a short (4 min for A and C) or long (22 min for B and D) gradient. The long gradient method was as follows: 0–0.5 min 30%B, 0.5–18.5 min 30–90%B, 18.5–21 min 90%B, 21–22 min 30%B.

To evaluate potential interference from asunaprevir metabolites, the SRM channels of all known and predicted metabolites (21 SRM channels in total) were monitored under the optimized chromatographic condition using pooled incurred samples. To minimize the potential co-elution of metabolites with analyte, the experiments were also done using a much longer gradient (22 min). As shown in Fig. 6, no metabolite peak interfering with asunaprevir was observed in pooled incurred rat and dog plasma samples using either the short or long gradient. These evaluations using incurred samples ensured the quality of the method for subsequent validation and study sample analysis, and minimized the risk of redoing the method development and validation due to unexpected metabolite interference or matrix effect.

3.3. Assay validation

Assay validation was conducted following the FDA Guidance for Industry: Bioanalytical Method Validation (2001) [15] and internal standard operation procedures (SOPs), and was fully compliant with Good Laboratory Practices (GLP). Standard curve linearity, accuracy and precision, specificity, matrix effect, recovery, and stability were evaluated. The method was first fully validated in rat plasma and followed by partial validations in dog, rabbit, monkey, and mouse plasma. At least three accuracy and precision runs were conducted for a full validation, and at least one accuracy and precision run was conducted for partial validations.

3.4. Accuracy, precision and standard curve linearity

The accuracy and precision data of asunaprevir in rat plasma are listed in Table 1. Similar performance was obtained in all other species (intra-assay precisions within 3.6% CV, inter-assay precisions within 4.0% CV, accuracy within ±8.1% Dev of the nominal concentration). A linear $1/x^2$ weighted regression model provided the best fit for asunaprevir over the range of 5.00–2000 ng/mL for all the species with coefficient of determination (R^2) > 0.996 in all validation runs. The results of the STD curves and QCs demonstrated that the method was accurate and precise for the analysis of asunaprevir in plasma from all the species.

3.5. Specificity and lower limit of quantification

Six different lots of blank plasma from each species were analyzed with and without internal standards. No significant

Table 1
Accuracy and precision for asunaprevir in rat plasma.

QC Type (nominal conc. in ng/mL)	LLOQ (5.00)	Low (15.0)	GM (125)	Mid (1000)	High (1600)	Dilution (50,000)
Mean observed conc.	5.26	15.58	129.13	1029.50	1619.39	50,019.27
%Dev	5.2	3.9	3.3	3.0	1.2	0.0
Between run precision (%CV)	3.4	0.0	1.7	1.5	1.2	3.1
Within run precision (%CV)	2.4	2.4	1.7	1.3	1.7	3.1
Total variation (%CV)	4.2	2.3	2.4	2.0	2.1	4.4
n	18	18	18	18	18	18
Number of runs	3	3	3	3	3	3

GM: geometric mean.

Table 2
Recovery and matrix effect of asunaprevir and its SIL-IS in plasma of different species.

Compound	Rat		Dog		Monkey		Rabbit		Mouse	
	Recovery %	Matrix factor	Recovery %	Matrix factor	Recovery %	Matrix factor	Recovery %	Matrix factor	Recovery %	Matrix factor
Asunaprevir	46.2–46.8	0.97–1.12	52.4–64.2	1.01–1.06	43.8–47.5	1.09–1.15	50.6–51.3	0.97–1.00	70.7–84.6	1.22–1.25
D_9-asunaprevir	52.1–56.7	0.95–1.14	59.7–71.0	0.99–1.01	35.7–37.0	1.12–1.13	43.9–44.4	0.99–1.04	62.2–65.4	1.16–1.18

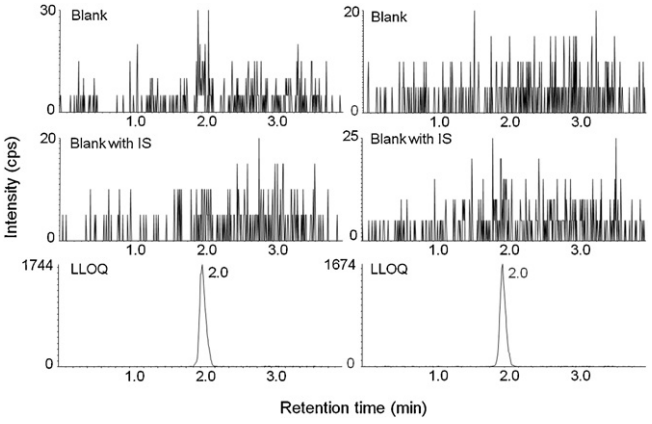


Fig. 7. Representative SRM chromatograms of asunaprevir in blank, blank spiked with IS, and LLOQ sample in monkey plasma (left) and mouse plasma (right).

interfering peaks from the plasma were found at the retention time of either the analyte or its IS, which demonstrated the good specificity of the assay. Representative SRM chromatograms of blank, blank spiked with IS, and lower limit of quantification (LLOQ) samples of asunaprevir in monkey and mouse plasma are presented in Fig. 7. The LLOQ of the assay (5.00 ng/mL of asunaprevir) was assessed using six different lots of plasma for each species. The deviations of the measured concentrations from the nominal LLOQ values were within ±14.4% for at least five of the six lots in all the species.

3.6. Extraction recovery and matrix effect

The recovery of the analyte was determined at 15.0 and 1600 ng/mL by comparing the response ratios in plasma samples, which were spiked with the analyte prior to extraction, with those spiked post-extraction. The matrix effect, expressed as matrix factor (MF), was determined by dividing the analyte response in plasma spiked post-extraction by the analyte response of those spiked in reconstitution solution. The recovery and matrix factor of the IS were determined similarly. The MFs of asunaprevir were within 0.95–1.25 and the IS normalized MFs were within 0.97–1.06, indicating minimum matrix effect on the measurement of the analyte. The recovery and matrix effect data of asunaprevir and its SIL-IS in plasma of different species were listed in Table 2.

3.7. Stability

The room temperature, freeze–thaw, and frozen storage stabilities of asunaprevir in plasma from different species were evaluated in triplicate. The re-injection integrity was evaluated by re-injecting an entire run. To establish the stability of the analyte, the deviations of the mean measured concentrations of the test samples have to be within 15% of the nominal concentrations. The established stabilities for asunaprevir in plasma from different species are summarized in Table 3. For long term stability, the duration listed for each species reflect the longest period tested to date and do not indicate any difference in stability between species. The stability of asunaprevir stock solution was evaluated in replicates of six. The analyte and its SIL-IS were stable in methanol for at least 6 h at room temperature, and at least 143 days at 4 °C.

3.8. Incurred sample reproducibility

The incurred sample reproducibility (ISR) for plasma samples were evaluated for each species by re-analyzing incurred study samples selected across different dose groups, dosing period, and collection times. To demonstrate the assay reproducibility, the initial and repeat value have to be within 10.0% of the mean for at least two-thirds (2/3) of the samples tested. Representative testing results for the rat plasma assay are presented in Table 4. All ISR

Table 3
Stability data of asunaprevir in plasma of different species.

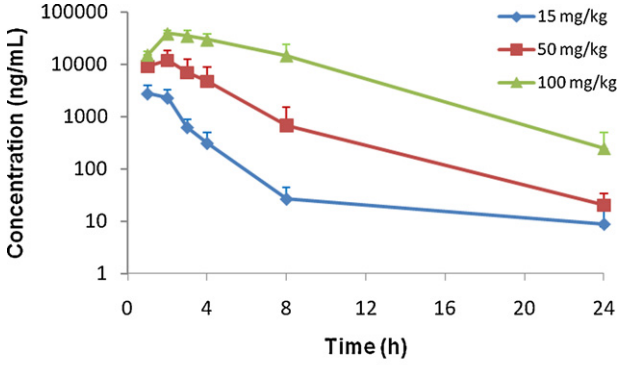
Stability Type	Rat	Dog	Monkey	Rabbit	Mouse
Room temperature stability	24 h	24 h	24 h	24 h	24 h
Freeze–thaw stability at −20 °C	5 cycles	NA	5 cycles	5 cycles	NA
Frozen stability at −20 °C	263 days	171 days	95 days	108 days	100 days
Reinjection Integrity at 5 °C	72 h	NA	NA	NA	NA

NA: not available.

Table 4
Result of incurred sample reproducibility testing for asunaprevir in a rat toxicokinetic study.

Sample Identification	Initial value (ng/mL)	Incurred repeat (ng/mL)	Mean (ng/mL)	% Dev from mean[a]
Sample 1	8186.86	8657.79	8422.33	2.8
Sample 2	6857.84	6936.36	6897.10	0.6
Sample 3	4570.91	4837.19	4704.05	2.8
Sample 4	26,952.95	26,919.77	26,936.36	−0.1
Sample 5	28,306.34	27,586.56	27,946.45	−1.3
Sample 6	33,024.35	34,064.39	33,544.37	1.6
Sample 7	30,480.00	30,090.63	30,285.32	−0.6
Sample 8	40,391.49	39,240.55	39,816.02	−1.4
Sample 9	22,880.27	23,469.62	23,174.95	1.3
Sample 10	47,488.80	47,201.6	47,345.20	−0.3
Sample 11	15,831.08	15,617.72	15,724.40	−0.7
Sample 12	48,750.76	46,722.05	47,736.41	−2.1
Sample 13	580.90	580.12	580.51	−0.1
Sample 14	930.49	909.14	919.82	−1.2
Sample 15	532.01	517.40	524.71	−1.4

[a] All samples met acceptance criteria (within 10% of mean value).



Parameter	Asunaprevir dose (mg/kg/day)		
	15	50	100
Cmax (ng/mL)	3400	12400	39100
AUC (ng·h/mL)	6670	46300	312000
Tmax (h)	1.3	1.7	2.2

Fig. 8. Plasma concentration (mean + SD) vs. time profiles of asunaprevir in male dogs after oral administration (dose as indicated).

results were within 2.8% from the mean value demonstrating good assay reproducibility. The ISR tests passed in all species tested.

3.9. Application to toxicokinetic studies

The validated method has been successfully applied to the sample analysis for multiple TK studies in rats, dogs, monkeys, rabbits, and mice. A representative concentration vs. time profile of asunaprevir in male dogs after day 1 dosing and the corresponding TK parameters are shown in Fig. 8.

4. Conclusions

A rugged, accurate, and sensitive LC–MS/MS method for the quantitation of asunaprevir in 50 μL plasma was developed and validated over the concentration range of 5.00–2000 ng/mL. The method was successfully applied to support TK studies in different species. The use of incurred samples during method development minimized the bioanalytical risks due to the interferences from drug-related components and ensured the quality and robustness of the method.

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