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Determination of SK7324, a new class of hepatitis C virus inhibitor, in rat plasma by UPLC-MS/MS and its application to the pharmacokinetic study of SK7324

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In this study, a sensitive method for quantitation of the unique small-molecule inhibitor of hepatitis C virus SK7324 in rat plasma has been established using ultra performance liquid chromatography-electrospray ionization tandem mass spectrometry (UPLC-ESI/MS/MS). SK7324 and internal standard (tramadol) in plasma sample was extracted using acetonitrile. A centrifuged upper layer was then evaporated and reconstituted with the mobile phase of 0.5% formic acid-acetonitrile (35:65, v/v). The reconstituted samples were injected into a C_{18} reversed-phase column. Using MS/MS in the multiple reaction monitoring (MRM) mode, SK7324 and tramadol were detected without severe interference from rat plasma matrix. Detection of SK7324 in rat plasma by the UPLC-ESI/MS/MS method was accurate and precise with a quantitation limit of 1.0 ng/mL. The validation, reproducibility, stability, and recovery of the method were evaluated. The method has been successfully applied to pharmacokinetic studies of SK7324 in rat plasma. Pharmacokinetic parameters of SK7324 were evaluated after intravenous (i.v.; at doses of 5 mg/kg) and oral (p.o.; at doses of 10 mg/kg) administration of SK7324 in rats. After p.o. administration (10 mg/kg) of SK7324, *F* (Fraction absorbed) value was approximately 87.1%.

1. Introduction

Approximately 200 million people worldwide are chronically infected with hepatitis C virus (HCV). This pathogen is the major cause of acute hepatitis and chronic liver disease, including cirrhosis and liver cancer; therefore, HCV is the leading indication for liver transplantation (Lavanchy 2009). HCV is an enveloped, positive-stranded RNA virus and a member of the Flaviviridae family in the hepacivirus genus. HCV is closely related to the flavivirus genus, which includes a number of viruses implicated in human disease, such as dengue virus and yellow fever virus (Lindenbach and Rice 2005). Seven major HCV genotypes and numerous subtypes have been described and differ as much as 30% in nucleotide sequence (Van Regenmortel 2007; Simmonds et al. 2005)

Despite increasing efforts to develop novel drugs effective against HCV, patients are mainly being treated with a virus-unspecific combination therapy of pegylated interferon alpha (PEG-IFN) and ribavirin (RBV). This treatment is expensive and can be associated with severe side effects; in addition, it is effective in only 50–60% of patients infected with HCV genotype 1 (ElHefnawi et al. 2010). Since early 2011, two direct acting antivirals (DAAs) targeting the viral NS3 protease have been approved by the United States Food and Drug Administration. Unfortunately, both drugs induce severe side effects and have a low resistance barrier as well as an inconvenient administration regime (Aghemo et al. 2013). Viral resistance may eventually become an issue; due to potential HCV genotype specificity, it is unclear whether all seven HCV genotypes and their subtypes are covered (Hofmann et al. 2011). Therefore, it is a primary goal to identify targets with a significantly higher genetic barrier of resistance that cover all HCV genotypes. Other challenges include appearance of escape mutants, high costs of current therapy regimens, and negative side effects. The mechanisms of resistance to interferon (IFN)-based therapy through immune system interception (Chou 2006) and also to DAAs like telaprevir and boceprevir through resistance-conferring mutations could be avoided by combinatorial treatment. Although drugs that target the virus are currently in clinical trials, a medical need remains for new HCV therapeutic agents. There is a particular need for HCV therapeutic agents that have broad activity against the majority of HCV genotypes and their subtypes (e.g., 1a/b, 2a/b 3a/b, 4a/b, etc.). The entry process of HCV into hepatocytes has been recognized as one of the potential targets for therapeutic intervention to treat or prevent HCV infection. To develop new types of small-molecule inhibitors for HCV, we first sought to screen HCV entry inhibitory molecules using an infectious cell culture system (HCVcc). During the preliminary screening, our derivatives were found to possess useful activity against all major HCV genotypes. Accordingly, we further tested 296 compounds to determine the lead optimization process. Herein, we present a new class of compound, SK7324 (Fig. 1), that exert antiviral activity against HCV. The protein binding of SK7324 to 4% HSA was independent of SK7324 concentrations ranging from 0.5 to 100 mg/mL; the mean binding value was 96.5%. The present article reports a quantification method for SK7324 in rat plasma using UPLC-MS/MS with protein precipitation which is faster and simpler methods compared to previous reported methods (Masnatta 2009).

The purpose of this study was to develop and validate a sensitive and reliable ultra performance liquid chromatographic-electrospray ionization tandem mass spectrometry (UPLC-MS/MS) method. Based on the developed method, we determined the pharmacokinetic properties of SK7324 after intravenous (i.v.) injection of SK7324 at a dose of 5 mg/kg or oral (p.o.) administration of SK7324 at a dose of 10 mg/kg to rats to evaluate the absolute p.o. bioavailability (*F*) of SK7324 in rats.

2. Investigations, results, and discussion

2.1. Development and validation of the UPLC-MS/MS method

We screened 296 derivatives for anti-viral activity against genotypes 1/2 using the infectious HCVcc system and selected compounds,

SK7324, with potent activities for further *in vivo* ADME/toxicity studies (Fig. 1). Accordingly, *in vitro* ADME and cytotoxicity evaluations were also conducted to confirm the best potential drug candidate. We have developed a reliable method for detecting SK7324 in rat plasma, using PP and UPLC-ESI/MS/MS for pharmacokinetic studies. Under electrospray ionization condition, SK7324 and tramadol (internal standard) exhibited a fairly high sensitivity in positive ion detection mode rather than in negative ion detection mode. The analysis for compounds with basic sites, as for SK7324 and tramadol, should be performed at a low pH using positive ion detection. SK7324 produced a protonated precursor ion ($[M+H]^+$) at m/z 432.3 with a major product ion at m/z 114.4. On the other hand, tramadol (internal standard) produced a protonated precursor ion ($[M+H]^+$) at m/z 264.4, with a major product ion at 58.1. The most abundant product ions (m/z 114.4 for SK7324 and m/z 58.1 for tramadol) were selected for MRM analysis.

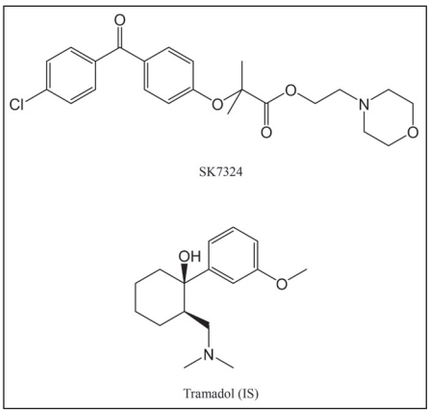

Fig. 1: Chemical structures of SK7324 and tramadol (an IS).

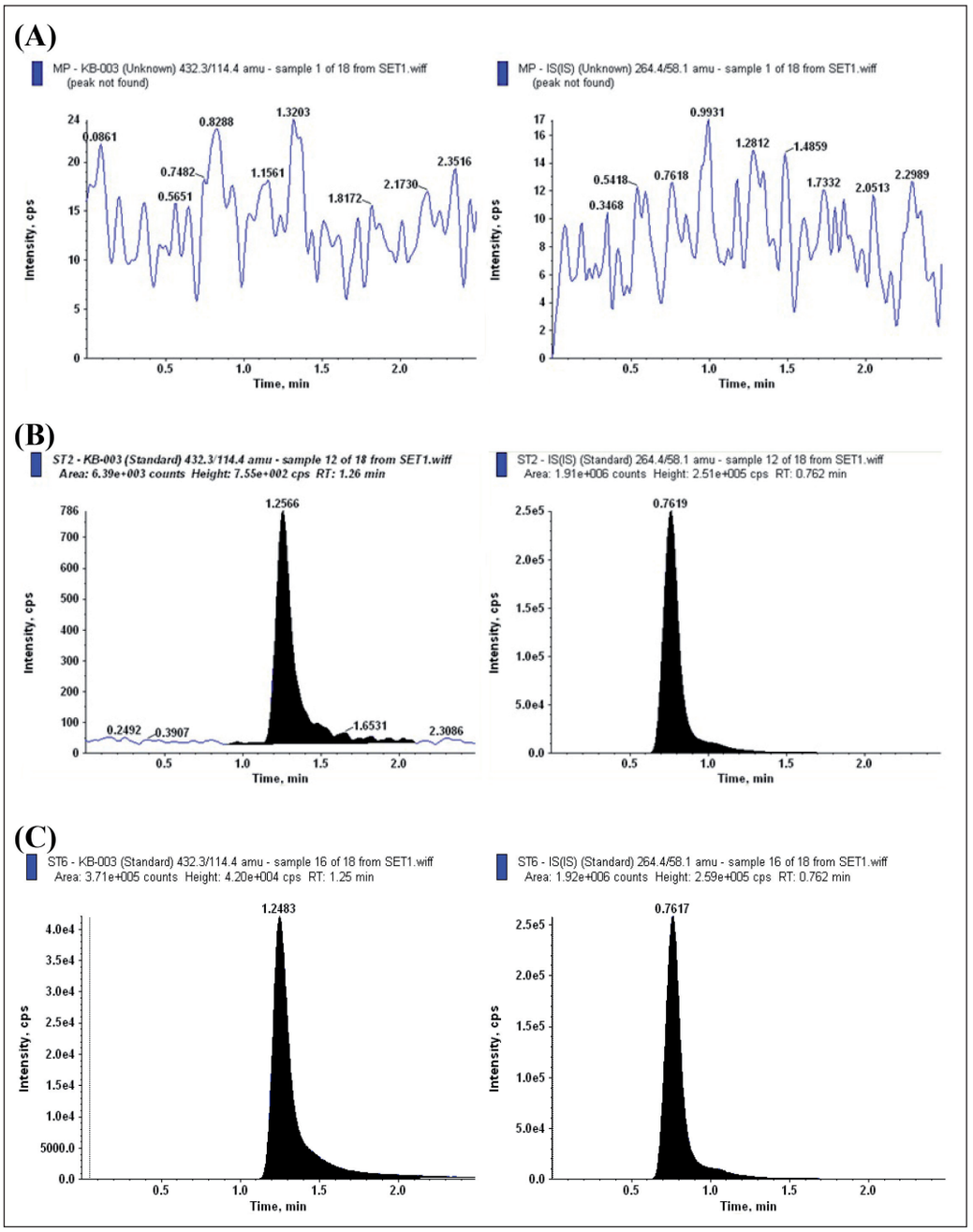

Fig. 2: Representative LC-MS/MS chromatograms after deproteinization of drug-free rat plasma (A), drug-free rat plasma spiked with 1 ng/mL (LLOQ) of SK7324 (left column) and 120 ng/mL of IS (right column) (B), plasma collected 4 hr after oral administration of 10 mg/kg of SK7324 to a male Sprague–Dawley rat (C).

Multiple reaction monitoring (MRM) mode was used for quantitation and achieved very high sensitivity and selectivity. By using MRM mode in MS/MS, SK7324 and internal standard were detected without severe interference from the rat plasma matrixes. Figure 2 shows the UPLC-MS/MS chromatogram of SK7324 in rat blank plasma without internal standard. From Fig. 2, no interference was observed in drug-free rat plasma samples at the retention times of SK7324 and tramadol. Ultra performance liquid chromatography-tandem mass spectrometry is still limited to conditions that are suitable for mass spectrometry operations. There are restrictions on pH, solvent choice, solvent additives, and flow rates for UPLC to achieve optimal ESI-MS/MS sensitivity. For the chromatographic analysis and electrospray ionization of SK7324 and tramadol, we initially attempted to develop a reversed phase chromatographic method with methanol or acetonitrile as mobile phase. Acetonitrile was used instead of methanol, because acetonitrile affords better sensitivity and resolution in the analysis of SK7324 and internal standard. It is reported that the extent of ionization suppression seen is much more severe with electrospray ionization than with atmospheric pressure chemical ionization (King et al. 2000). Therefore, analysts need to use a post-extraction spiked matrix blank and compare the results with an analytical standard in solution to determine the influence of the matrix on the analysis (Henion et al. 1998). The intraday precision, expressed as RSD (%), was 4.7–11.4% for 1.0, 10, 100 and 750 ng/mL standard concentrations, based on six replicate analyses at each concentration level. The intraday accuracy, expressed as a percentage of relative error was measured as 0.0–7.2% for four standard concentrations, based on six replicate analyses at each concentration level. Table 1 shows the measured intraday precision and accuracy of SK7324 in rat plasma. The interday precision was measured as 3.7–9.3% for four standard concentrations, based on six replicate analyses at each concentration level. The interday accuracy was measured as 3.0–10.0% for four standard concentrations, based on six replicate analyses at each concentration level. Table 1 shows the measured interday precision and accuracy of SK7324 in rat plasma.

Table 1: Intra- and inter-day precision and accuracy for SK7324 in rat plasma QC samples

Nominal conc. (ng/mL)	Measured conc. (ng/mL)	Coefficient of variation (%)	Relative error (%)
Intra-day (*n*=6)			
1	1.0 ± 0.1	10.0	0.0
10	10.5 ± 1.1	10.5	5.0
100	107.1 ± 8.2	7.7	7.1
750	731.3 ± 31.5	4.3	-2.5
Inter-day (*n*=18)			
1	1.1 ± 0.1	9.1	10.0
*****10	9.6 ± 0.9	9.4	-4.0
100	104.8 ± 5.8	5.5	4.8
750	722.1 ± 25.3	3.5	-3.7

Data represent mean ± SD.

Coefficient of variation (%) = (SD/mean) □ 100

Relative error (%) = ((Measured conc. - Nominal conc.) / Nominal conc.) □ 100

Standard calibration curves (reproducibility) were constructed on different working days (three days) using the rat plasma. The response was linear throughout the concentration range of the study, with the coefficient of determination (r^2) always greater than 0.9999. The %recovery of PP was measured as 87.5–95.0% for 1.0, 10, 100 and 750 ng/mL standard concentrations, with five replicates at each concentration level (Table 2). The stability of SK7324 and internal standard was evaluated in the dissolution solvent and in rat plasma. It was found that SK7324 and internal standard were stable for the duration of the experiment (Table 3). On the basis of a signal-to-noise ratio (S/N) of 10, the lower limit of quantitation (LLOQ) for SK7324 was found to be 1.0 ng/mL. Determining the concentration of SK7324 in rat plasma has been applied to pharmacokinetic studies by use of PP with UPLC-MS/MS.

Table 2: Matrix effect, recovery, and process efficiency data for SK7324 and tramadol in rat plasma

Concentration (ng/mL)	Matrix effect (%) (B/A×100)	Recovery (%) (C/B×100)	Process efficiency (%) (C/A×100)
JW5473			
10	71.5 ± 11.9	95.0 ± 3.0	69.5 ± 11.8
100	72.6 ± 4.2	92.9 ± 5.3	68.3 ± 6.2
750	81.6 ± 3.9	87.5 ± 2.8	71.3 ± 3.2
Tramadol			
200	74.8 ± 1.8	92.6 ± 2.8	68.6 ± 2.3

A, Peak area of analytes in mobile phase

B, Peak area of analytes spiked after extraction

C, Peak area of analytes spiked before extraction

2.2. Pharmacokinetics of SK7324 after intravenous (i.v.) or oral (p.o.) administration of SK7324 to rats

For the i.v. administration of SK7324 at doses of 5 mg/kg to rats, the mean arterial plasma concentration–time profiles of SK7324 are shown in Fig. 3, and relevant pharmacokinetic parameters are listed in Table 4. After i.v. administration of SK7324, the plasma concentrations of SK7324 declined in polyexponential manners with a half-life of 3.11±0.62 h. The estimated total body clearance was 17.5±4.8 mL/min/kg which was considerably smaller than the reported cardiac output of 295 mL/min/kg based on blood data (Davies and Morris 1993; Chiou 1978) in rats. This suggests that the first-pass effect of SK7324 in the lung and heart could be almost negligible in rats.

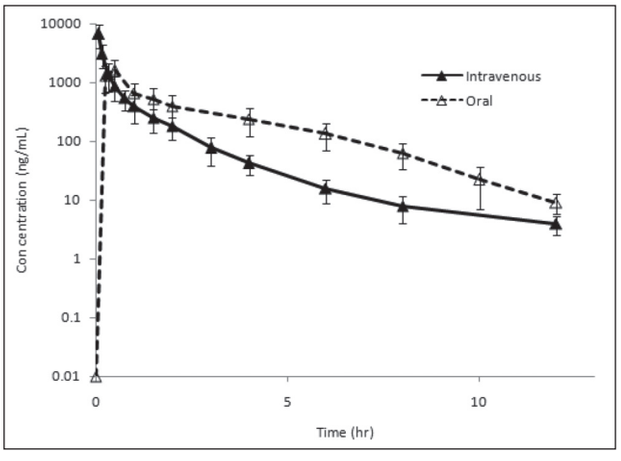

Fig. 3: Mean arterial plasma concentration–time profiles of SK7324 after i.v. (▲) injection of SK7324 at a dose of 5 mg/kg to rats and mean arterial plasma concentration–time profiles of SK7324 after oral (Δ) administration of SK7324 at a dose of 10 mg/kg to rats. Bars represent S.D.

For the p.o. administration of SK7324 at a dose of 10 mg/kg to rats, the mean arterial plasma concentration–time profiles of SK7324 are shown in Fig. 3, and the relevant pharmacokinetic parameters are listed in Table 4. After p.o. administration of SK7324, absorption of SK7324 from the rat gastrointestinal tract was rapid; SK7324 was detected in plasma from the first or second blood sampling time (15 or 30 min) and rapidly reached T_{max} (at 30 min). SK7324 was well absorbed, and showed that C_{max} was 1,128±765 ng/mL and AUC_{0-12} was 2,664±1,258 ng·h/mL. Hence, the *F* value at a SK7324 dose of 10 mg/kg was calculated to be about 87.1%. After intraportal and i.v. administration of SK7324 at a dose of 5 mg/kg to rats, the AUC of SK7324 after its i.p. administration was significantly smaller than that after its i.v. administration

(unpresented data), suggesting that the hepatic first-pass effect of SK7324 after its absorption into the portal was major in rats. The considerable hepatic first-pass effect of SK7324 is being tested based on the liver homogenate studies. In conclusion, after p.o. administration of SK7324 at a dose of 10 mg/kg to rats, the *F* value was 87.1% showing good bioavailability for entering preclinical studies. The major elimination pathway is thought to be hepatic and gastrointestinal first-pass effects.

Table 3: Stability of SK7324 in rat plasma and stock solutions (n=3)

Nominal conc. (ng/mL)	Duration	Measured conc. (ng/mL)	Relative error (%)
Short- term stability (at room temperature, RT)			
10	4 h	10.3 ± 0.8	3.0
100		102.5 ± 3.4	2.5
750		773.4 ± 32.1	3.1
Long-term stability (at -80°C)			
10	7 days	10.5 ± 1.1	5.0
100		107.2 ± 4.7	7.2
750		745.2 ± 42.8	-0.6
Freeze and thaw stability			
10	3 cycles	10.4 ± 0.8	4.0
100		106.8 ± 4.9	6.8
750		793.1 ± 37.6	5.7
Auto-sampler stability (at 4°C)			
10	24 h	10.5 ± 0.9	5.0
100		106.3 ± 5.2	6.3
750		742.6 ± 64.6	-1.0
Stock solution			
50	2 h at RT	51.7 ± 0.6	3.4
	11 days at 4°C	48.1 ± 0.4	-3.8

Data represent mean ± SD

Relative error (%) = ((Measured conc. - Nominal conc.) / Nominal conc.) x 100

Table 4: Pharmacokinetic parameters of SK7324 after a single i.v. administration of SK7324 at a dose of 5 mg/kg and a single oral administration of SK7324 at a dose of 10 mg/kg to male rats

Parameters	Intravenous (n=5)	Oral (n=5)
dose (mg/kg)	5	10
AUC_{0-12} (mg×hr/mL)	1529 ± 893	2664 ± 1258
C_{max} (mg/mL)	6221 ± 3092	1128 ± 765
T_{max} (hr)	0.08 ± 0.00	0.50 ± 0.24
CL (mL/min/kg)	17.5 ± 4.83	-
$T_{1/2}$ (hr)	-	1.43 ± 0.58
F (%)	-	87.1

The values of kinetic parameters represent the mean ± SD (n=5).
AUC: Area under the curve to the collected time point (ng·hr/mL).
C_{max}: Peak plasma concentration (ng/mL)
T_{max}: Time to reach peak plasma concentration (hr)
CL: Total body clearance (mL/min/kg)
$T_{1/2}$: Elimination half life (hr)
F: Fraction absorbed (%)

3. Experimental

3.1. Materials

SK7324 were supplied from Jungwon University (Geosan, South Korea). Tramadol, the internal standard, was purchased from Sigma–Aldrich Corporation (St. Louis, MO). Acetonitrile and methanol were purchased from Burdick & Jackson (Muskegon, MI, USA). HSA (human serum albumin, 20%) was obtained from SK Chemical Pharmaceutical Company (Seongnam, South Korea). Polyethylene glycol 400 (PEG 400) was a product from Showa Chemical Company (Tokyo, Japan). Other chemicals were of reagent grade or HPLC grade.

3.2. Animal experiments

The protocols for these animal studies were approved by the Institute of Laboratory Animal Resources of the Jungwon University, Geosan, South Korea. Male Sprague–Dawley rats, 6–8 weeks old and weighing 220–300 g, were purchased from the Samtako Bio Korea (Osan, South Korea). Rats were maintained in a clean room at a temperature of 23±2 °C with 12-h light (07:00–19:00) and dark (19:00–07:00) cycles, and a relative humidity of 55±5%. Rats were housed in metabolic cages (Tecniplast, Varese, Italy) under filtered pathogen-free air and with food (Sam Yang Company, Pyeongtaek, South Korea) and water available ad libitum. The rats were fasted overnight before drug administration and for 4 h after dosing. SK7324 was dissolved in PEG400:distilled water=1:1 (v/v) to make a concentration of 5 mg/mL. The rats were placed in a restrainer and were intravenously and orally administered a dose of 5 and 10 mg/kg with a catheter, respectively. A 150-mL of blood was collected in a heparinized tube at the pre-dose stage, and at 5, 10, 20, 30, 45 m, 1, 2, 3, 4, 6, 8, and 12 h after i.v. administration and at 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 10, and 12 h after p.o. administration. Plasma was harvested after centrifugation at 3,000 rpm and 4 °C for 10 min and stored frozen at -70 °C until it was analyzed.

3.3. Preparation of calibration standards and quality control samples

Stock solutions of SK7324 (1 mg/mL) were prepared in methanol. Appropriate dilutions of the stock solutions of SK7324 were made with methanol (0.01, 0.03, 0.1, 0.2, 0.5, 1, 2, 5, or 10 mg/mL). Standard solutions of SK7324 in rat plasma were prepared by spiking with an appropriate volume (100 mL/mL of plasma) of the diluted stock solutions, giving final concentrations of 1, 3, 10, 20, 50, 100, 200, 500, or 1000 ng/mL for plasma. The internal standard working solution was prepared by dissolving tramadol in acetonitrile to give a final concentration of 200 ng/mL.

3.4. Preparation of plasma samples

A 50-mL aliquot of sample was deproteinized with a 75-mL of acetonitrile containing 200 ng/mL tramadol (an IS). After vortex-mixing and centrifugation at 3,000 rpm for 10 min, the supernatant was transferred into a vial and a 5-mL aliquot was injected directly onto the HPLC column.

3.5. UPLC-MS/MS analysis

Tandem mass spectrometry (MS/MS) was performed with a Xevo TQ triple quadruple mass spectrometer (Micromass Co., Manchester, UK) equipped with an electrospray ion source. The sample (5 mL) was delivered into the ESI source by UPLC (Model Acquity UPLC, Waters Co., Milford, MA, USA) with C18 Fortis column (2.0×50 mm, 1.7 μm particle size). The mobile phase was composed of 0.5% formic acid and acetonitrile (35:65, v/v) and was used after degassing. The flow rate was 300 μL/min and the total run time was 2.5 min. The electrospray interface was maintained at 600 °C. Nitrogen nebulization was performed with a nitrogen flow of 800 L/h. Argon was used as collision gas. SK7324 and the internal standard were detected by the MRM scan mode with positive ion detection; the parameter settings were: capillary voltage at 2.0kV, cone voltage at 10 V, extractor at 3 V, source temperature at 150°C, collision cell entrance potential at 0.5 V, collision energy at 10 eV, collision cell exit potential at 0.5 V, multiplier at 529 V, and dwell time of 0.20 s. Mass calibration was performed by infusion of a 10^{-4} M polyethylene glycol 1000 (PEG 1000) solution into the ionspray source. The peak widths of precursor and product ions were maintained at 0.7 mass unit at half-height in the MRM mode.

3.6. Analytical method validation

The analytical method was validated with regards to its specificity, linearity, intra- and interday precision and accuracy, matrix effect, and stability according to the US Food and Drug Administration's "Guidance for Industry, Bioanalytical Method Validation, 2018"

3.7. Pharmacokinetic and statistical analyses

The total area under the plasma concentration-time curve to the last time (AUC_{last}), the maximum plasma concentration (C_{max}), the time to reach C_{max} (T_{max}), and the half-life ($T_{1/2}$) were estimated using noncompartmental calculations carried out within WinNonlin™ 5.2 (Pharsight, Sunnyvale, CA,USA). All data are expressed as the mean±standard deviation (n=5).

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Conflicts of interest: None declared.

References

Aghemo A, Degasperi E, Colombo M (2013) Directly acting antivirals for the treatment of chronic hepatitis C: unresolved topics from registration trials. Dig Liver Dis 45: 1–7.

Chiou WL (1978) Critical evaluation of potential error in pharmacokinetic studies using the linear trapezoidal rule method for the calculation of the area under the plasma level–time curve. J Pharmacokinet Biopharm 6: 539–546.

Chou TC (2006) Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev 58: 621–681.

Davies B, Morris T (1993) Physiological parameters in laboratory animals and humans. Pharm Res 10: 1009-1095.

ElHefnawi MM, Zada S, El-Azab IA (2010) Prediction of prognostic biomarkers for interferon-based therapy to hepatitis C virus patients: a meta-analysis of the NS5A protein in subtypes 1a, 1b, and 3a. Virol J 7: 130.

Henion J, Brewer E, Rule G (1998) Sample preparation for LC/MS/MS: Analyzing biological and environmental samples. Anal Chem 70: 650A–656A.

Hofmann WP, Chung T, L Osbahr C, Herrmann E (2011) Impact of ribavirin on HCV replicon RNA decline during treatment with interferon-alpha and the protease inhibitors boceprevir or telaprevir. Antivir Ther 16: 695–704.
http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation / Guidances/Bioanalytical Method Validation Guidance for Industry.pdf: 6-10.
King R, Bonfiglio R, Fernandez-Merzler C, Miller-Stein C, Olah TJ (2000) Mechanistic investigation of ionization suppression in electrospray ionization. Amer Soc Mass Spectrom 11: 942–950.
Lavanchy D (2009) The global burden of hepatitis C. Liver Int 29 Suppl 1: 74–81.
Lindenbach BD, Rice CM (2005) Unravelling hepatitis C virus replication from genome to function. Nature 436: 933–938.
Masnatta LD, Cuniberti LA, Rey RH, Werba JP (2009) Determination of bezafibrate, ciprofibrate and fenofibric acid in human plasma by high-performance liquid chromatography. Biomed Chromatogr 23: 922–928.
Simmonds P, Bukh J, Combet C (2005) Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatology 42: 962–973.
Van Regenmortel MH (2007) Virus species and virus identification: past and current controversies. Infect Genet Evol 7: 133–144.