Validation of a LC-MS/MS Method for Quantitative Analysis of MELK Inhibitor OTS167 in Mouse Serum: Application to a Pharmacokinetic Study

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ABSTRACT

A novel, rapid and sensitive liquid chromatography-tandem mass spectrometric (LC-MS/MS) method was developed and validated for the evaluation of OTS167 pharmacokinetics in mouse serum. SN-38 was optimized to be selected as the internal standard. Chromatographic separation was performed on a BDS Hypersil C18 column (100 x 2.1 mm, 5 μm) using gradient elution with mobile phase solvent A as water containing 0.1% formic acid and solvent B as acetonitrile containing 0.1% formic acid. The analysis was carried out using multiple reaction monitoring (MRM) with a triple-quadrupole mass spectrometer operated in the positive electrospray ionization mode. Mass transitions of 487.2 > 348.0 and 393.2 > 349.2 were monitored for OTS167 and SN-38 respectively. The standard calibration curve demonstrated high linearity at a range of 5-1000 ng/mL, with a coefficient of determination (r^2) ≥ 0.996. The accuracy for OTS167 ranged from 92.3% to 102.2% and the precision was ≤ 12.7%. Recovery was consistent at about 83% to 89%. No significant matrix effect was observed. This method was successfully applied to monitor the pharmacokinetic profiles in mice over 24 h after ingestion of 5 mg/kg of OTS167. Maximum serum concentrations (3197 ng/mL) of OTS167 was observed at 1.67 hour after OTS167 ingestion via garage at 5 mg/kg and area under the curve (AUC) was 28579 h*ng/mL, suggesting that OTS167 can be quickly absorbed after oral administration.

1. Introduction

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OTS167 is a potent MELK (maternal embryonic leucine zipper kinase) inhibitor with selective antitumor activity[1,2]. MELK plays essential roles in the proliferation[1], anti-apoptosis and metastasis of cancer cells, including the maintenance of stem cell-like properties[3,4,5]. MELK is also associated with therapeutic resistance[6] and poor prognosis[7,8,9]. Therefore, by targeting MELK, OTS167 offers the possibility to overcome therapeutic resistance by suppressing cancer stem cells and cancer proliferation[10,11]. Studies have shown that OTS167 demonstrates significant tumour growth suppression in xenograft studies using breast, lung, prostate and pancreas cancer cell lines in mice[12]. To date, only one analytical method (HPLC-UV) was published for the identification and measurement of OTS167-glucuronide, a Phase II metabolite of OTS167 in human microsomes, without any report of OTS167 quantification[13]. To the best of our knowledge, no liquid chromatography-tandem mass spectrometric (LC-MS/MS) method has been reported for OTS167 quantification in serum/plasma for preclinical/clinical studies. No data regarding its pharmacokinetic is available, thus making it hard to justify a suitable drug formulation, administration route and dosing strategy for patients in subsequent clinical trials. Since preclinical pharmacokinetic studies in mice are currently ongoing, the need for a robust and sensitive method development arises. In this study, we developed a novel, robust and sensitive LC-MS/MS method to investigate the pharmacokinetic properties of OTS167 in mice. The method was well validated via a suitable internal standard SN-38, the best among three selected candidates

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(Figure 1). This LC-MS/MS method has been successfully applied to pharmacokinetic study of OTS167 in mice.

2. Materials and Methods

2.1 Chemicals and Reagents

OTS167 was obtained from OncoTherapy Science Inc., Japan. Exemestane and SN-38 were purchased from Toronto Research Chemicals Inc., Canada. KPT-330 was purchased from Selleckchem, USA. Methanol, acetonitrile and formic acid (100%) were purchased from Merck, Germany. Milli-Q water was obtained from Milli-Q Plus system, USA. Drug-free blank mouse serum was obtained from untreated female NOD-SCID-γ (NSG) mice (NOD-scid IL2Rgammanull).

2.2 Chromatographic and mass Spectrometric conditions

Chromatographic analysis was performed on a high-performance liquid chromatographic (HPLC) system, consisting of an Agilent 1100 binary pump, degasser, auto-sampler and column oven (Agilent Technologies, Germany). BDS Hypersil C18 column (100 x 2.1 mm, 5 μm, Thermo Fisher Scientific, USA), coupled with a SecurityGuard cartridge (4.0 × 3.0 mm, Phenomenex, USA) was used for chromatographic separation. Mobile phase solvent A was water containing 0.1% formic acid and solvent B was acetonitrile containing 0.1% formic acid.

LC-MS/MS analysis was performed using multiple reaction monitoring (MRM) with an API 4000 triple-quadrupole mass spectrometer (Applied Biosystems, MDS SCIEX, Ontario, Canada) operated in the positive electrospray ionization (ESI+) mode. The

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data was analysed using Analyst software (Version 1.5.2, Applied Biosystems, MDS SCIEX, Ontario, Canada).

2.3 Standards and quality control solutions

OTS167 stock solution was prepared in methanol at 1 mg/mL. OTS167 standard solutions were prepared by serial dilution with methanol at seven concentrations: 0, 5, 10, 50, 200, 500 & 1000 ng/mL. SN-38, KPT-330 and exemestane solutions were each prepared at 100 ng/mL. QC samples of OTS167 were prepared and analysed at low (LQC), medium (MQC) and high (HQC) concentrations (30, 100 and 800 ng/mL). All solutions were stored at 4°C.

2.4 Construction of calibration curves

Five µL of OTS167, SN-38 (Internal Standard, IS) and mouse serum were each added into a 1.5 mL polypropylene (PP) centrifuge tube. 120 µL of 100% acetonitrile was added for protein precipitation. The PP tubes were vortexed for 30 sec and centrifuged at 14,000 rpm for 10 min at 4°C. 100 µL of the supernatant was transferred to a second 1.5 mL PP tube and dried for 30 min at 45°C. The dried residue was reconstituted with 70 µL of acetonitrile containing 0.1% formic acid – water containing 0.1% formic acid mixture (15:85, v/v), vortexed for 30 sec and centrifuged again as described previously. 60 µL of the supernatant was transferred to a glass insert (250 µL) in an auto-sampler vial. 20 µL was injected for quantitative

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analysis. The injection needle was washed with 100% acetonitrile after each injection. The standard calibration curve was constructed using seven concentrations of OTS167 at 0, 5, 10, 50, 200, 500 & 1000 ng/mL. The standard calibration curve was generated using peak area ratios of OTS167 to SN-38 against OTS167 concentrations.

2.5 Bioanalytical method validation

Bioanalytical method validation was carried out according to FDA guidelines[13]. Blank wash samples were analysed after the highest concentrations in the carry-over test. Coefficients of determination (r^2) of the standard curves were employed in the linearity test. Accuracy and precision (coefficient of variation) were determined by analysing OTS167 QC samples at nominal concentrations. Intra-day accuracy and precision were established by performing a standard calibration curve and analysing the QCs in quintuplicate in a single run. Inter-day accuracy and precision were established by performing standard calibration curves and analysing the QCs in quintuplicate on 5 different days. Accuracy was expressed as a percentage of the mean value measured over the nominal value at each concentration whereas precision was expressed in terms of coefficient of variation (CV), defined as a percentage of standard deviation divided by the mean.

2.6 Matrix effect and recovery

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Matrix effect (ME) is a common and detrimental phenomenon in LC–MS or LC–MS/MS procedures and can be evaluated accurately according to the following formula:

ME (%) = [peak area in matrix ÷ peak area in neat standard] × 100 …… (1)

When ME (%) is equal to 100, this indicates no matrix effect for current LC–MS/MS conditions and sample preparation procedures. Deviations of ME (%) below or above 100 indicate ion suppression or ion enhancement, respectively.[14]

Matrix effect of OTS167 and SN-38 was investigated by analysing the peak area ratios of OTS167 and SN-38 in the matrix-based tubes to those in the reference tubes performed in quintuplicate at 3 QC concentrations for OTSS167 and one concentration for SN-38. In the matrix-based tube, 5 μL of blank mouse serum and 120 μL of 100% acetonitrile were added into a 1.5 mL PP tube. The tube was vortexed for 30 sec and centrifuged at 14,000 rpm for 10 min at 4°C. 100 μL of the supernatant was transferred to a second 1.5 mL PP tube. 5 μL of QC solution and SN-38 were each added, and the PP tube was vortexed for 30 sec before being dried. The dried residue was reconstituted with 70 μL of acetonitrile containing 0.1% formic acid – water containing 0.1% formic acid mixture (15:85, v/v) and vortexed for 30 sec before being centrifuged as described previously. 60 μL of the supernatant was transferred to a glass insert (250 μL) in an auto-sampler vial for analysis. The procedure was repeated for the reference tube using 5 μL of Milli-Q water instead of blank mouse serum.

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Recovery was determined by analysing the peak area ratios of OTS167 and SN-38 in the tube spiked before extraction to those in the tube spiked after extraction. QC samples in quintuplicate were performed at each concentration. For the tube spiked before extraction, steps were performed as per Section 2.4.

2.7 Dilution procedure

In the quantification of serum samples, a dilution procedure was needed for those samples exceeded the maximum calibrated concentration of OTS167 (1000 ng/mL). 20 or 40 times dilutions were performed using blank mouse serum before sample preparation. Actual concentrations were then back-calculated by multiplying the quantified concentrations by 20 or 40. The dilution procedure was validated using a serum test sample spiked at 4000 ng/mL and then analysed with both dilution factors in quadruplicate. No extrapolation of the calibration was performed to quantify OTS167 at concentrations above 1000 ng/mL.

2.8 Stability

OTS167 stability in mouse serum was determined using triplicate of QC samples at each concentration.

a) Short-term Temperature Stability

Stability testing was performed at intervals of 4, 8 and 24 hours. 9 aliquots of each QC concentration were prepared in mouse serum and placed on the bench top. 3 aliquots of each QC concentration were taken at each time interval and the samples

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were prepared as per Section 2.4, without adding OTS167 solution and mouse serum, followed by analysis.

b) Freeze-thaw Stability

9 aliquots of each QC concentration were prepared in mouse serum, stored at -80°C until completely frozen, and thawed unassisted at room temperature. After thawing, the samples were refrozen at -80°C and the cycle was repeated to complete 3, 6 and 9 freeze-thaw cycles. Upon the completion of each set of cycles, 3 aliquots of each QC concentration were prepared as per Section 2.4, without adding OTS167 solution and mouse serum, followed by analysis.

2.9 Pharmacokinetic study of OTS167 in mice

The animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (iORC Reference Number: 2015-00787) and approved by the NUS Institutional Animal Care and Use Committee (IACUC). Female NOD-SCID-γ (NSG) mice (NOD-scid IL2Rgammanull) were purchased from INVIVOS and housed at NUS Comparative Medicine Unit, MD1. The mice were 6-8 weeks old and had an average weight of 20 g on the day of dosing. No fasting was involved prior to dosing.

OTS167 dose formulation was prepared with 50% DMSO in PBS to contain 0.5 mg/ml for dosing OTS167 at 5 mg/kg. Blood sampling was performed before dosing, and at 5, 15 and 30 min, 1, 2, 4, 6, 8 and 24 hours after oral administration by oral

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gavage. Approximately 100 µL of blood was collected from each mouse by facial bleeding and harvested in 1.5 ml Eppendorf tubes. The blood samples were allowed to clot by leaving it undisturbed for at least 30 minutes at room temperature. The clot was removed and serum was collected by centrifuging at 5000 x g for 5 min at 4°C. The serum samples were then stored frozen at -80°C and thawed unassisted at room temperature before analysis. Sample preparation was carried out as per Section 2.4., except that 5 µL of methanol was added instead of OTS167 solution. Non-compartmental analysis of the area under concentration (AUC) in a plot of concentration of OTS167 against time was calculated using Phoenix 6.4 pharmacokinetic software.

3. Results and Discussion

A sensitive and robust LC-MS/MS method validated through our experiment has been successfully applied to preclinical pharmacokinetic investigation of oral OTS167 at 5 mg/kg in mice. To our knowledge, this is the first LC-MS/MS method for quantitative analysis of OTS167 in biological samples. The optimized conditions for LC-MS/MS analysis, robustness of the developed method and pharmacokinetics properties were presented and discussed as follows.

3.1. LC-MS/MS Optimization

The mass spectrometer was operated in multiple reaction monitoring (MRM) scan mode for identification of an ideal ionization state for fragmentation of OTS167 as

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well as the internal standard. Mass transitions 487.2 > 348.0 m/z and 393.2 > 349.2 m/z were monitored for OTS167 ($C_{25}H_{28}Cl_2N_4O_2$, molecular weight: 487.4) and SN-38 ($C_{22}H_{20}N_2O_5$, molecular weight: 392.4) respectively (Figure 2). Parameters were set as in Table 1. For OTS167 and SN-38, the ion spray voltage, source temperature/auxiliary gas temperature and entrance potential were set at 5500 V, 300°C and 8 V. The declustering potential, collision cell entrance potential and collision cell exit potential are 121 V, 45 V and 18 V for OTS167 and 70 V, 35 V and 10 V for SN-38 respectively.

Three reversed-phase HPLC columns [BDS Hypersil C18 column (100 x 2.1 mm, 5 µm, Thermo Fisher Scientific, USA); Eclipse XDB-C8 column (2.1 x 50 mm, 5 µm, Agilent Technologies, USA) & Luna C18 column (50 x 3.0 mm, 3 µm, Phenomenex, USA)] were investigated for their chromatographic separation of analytes. BDS Hypersil C18 column was selected as it gave the highest intensity and peak area and the most symmetrical chromatographic peak.

Acetonitrile was selected as the mobile phase organic solvent due to a shorter run-time and an improved baseline separation from endogenous substances as compared to methanol[15]. Formic acid 0.1% was chosen as the aqueous solvent due to increased sensitivity and better peak shape obtained, and to provide a source of protons[16] for positive charge enhancement.

Gradient elution was used during chromatographic separation owing to its more efficient removal of matrix components and other impurities present in the mouse serum compared to isocratic elution. The initial mobile-phase composition of 85%

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solvent A was maintained for 0.2 min and then decreased linearly to 5% from 0.2 to 0.4 min and maintained till 2.0 min. Next, solvent A was reverted to 85% till 3.1 min. The run time was 6 min at a constant flow rate of 0.4 ml/min. A switch time table was set wherein the diverter valve directed the elution flow to the waste container from 0 min to 2 min, and then to the MS between 2 min and 5 min for sample detection before directing back to the waste container to avoid contamination of the mass spectrometer. By increasing the concentration of the organic solvent in a linear fashion, hydrophobic interference compounds can be eluted out more efficiently, thereby minimizing matrix effect which translates to better sensitivity, robustness and reliability of the analytical method. Gradient elution also helps to prolong the column lifespan as these interference components will not accumulate in the column and affect subsequent analysis[17].

3.2. Extraction Protocol Optimization

Direct protein precipitation was performed for sample extraction as it is simple, rapid and cost-effective. Since the use of this method may leave behind many matrix components which contribute to matrix effect[18], we attempted to overcome this problem by using minute volume of mouse serum (5 μL each time) and large volumes of acetonitrile (120 μL). Since an efficient protein precipitation (>99%) could be achieved with the use of acetonitrile greater than two volumes[19], acetonitrile in twenty-four volumes (120/5 =24) was employed during sample preparation in our study in order to further remove serum proteins as much as possible to reduce

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matrix effect. This was confirmed by our results, which demonstrated insignificant matrix effect (Table 2), thereby proving the effectiveness of this method in our study. In such scenario, there is no need to use solid-phase and liquid-liquid extractions even though they could produce cleaner samples than direct protein precipitation[17]. In addition, solid-phase and liquid-liquid extractions were not used as they are more costly and labour-intensive. Therefore, the absence of significant matrix effect showed that direct protein precipitation with a large volume of acetonitrile (120 μL) was effective in minimizing the amount of matrix components in minute volume of mouse serum (5 μL), thereby diminishing the need for a more tedious albeit cleaner extraction method.

3.3. Selection of internal standard

Internal standards (IS) have been a crucial integral part of LC-MS/MS analysis. During sample preparation, errors could potentially be introduced due to solvent evaporation and adsorption. Injection errors in HPLC systems are also inevitable and could result in inconsistent results[20]. Even though mass spectrometers possess high sensitivity and selectivity for the analyte, residual endogenous matrix components and exogenous materials may still be present despite adequate sample preparation, leading to deleterious impact on accuracy, sensitivity and ionization efficiency[20]. Internal standards are employed to overcome these problems since any analyte loss would be compensated by the loss of a proportional amount of IS. Even though absolute responses are affected, ratios remain consistent, thereby improving accuracy and precision[21].

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In general, IS is either a stable isotopically-labelled analogue (SIL) or a structural analogue and would hence share similar degrees of ion suppression/enhancement with the analyte[21]. SIL are preferred since they are structurally identical to the analyte. However, as OTS167 is a novel compound, both SIL and structural analogues are not commercially available. The synthesis of a compound that is structurally analogous to OTS167 is costly too. Therefore, a wider pool of readily available compounds is required to search for a suitable IS. However, no reported principle is available to guide such selection and the criteria to select a suitable IS are currently undefined. Based on our previous experience, several aspects should be considered in selection of IS during LC-MS/MS method development and validation when no SIL is commercially available. These factors include Log p value, H-bond donors and H-bond acceptors[22]. In our experiment, 3 compounds (SN-38, KPT-330 and Exemestane) with similar Log P but different H-bond donors and acceptors were investigated (Figure 1). Mass transitions of 444.1 > 333.9 m/z and 297.0 > 121.0 m/z were monitored for KPT-330 ($C_{17}H_{11}F_6N_7O$, molecular weight: 443.3) and exemestane ($C_{20}H_{24}O_2$, molecular weight: 296.4) respectively. SN-38 was found to be the best IS candidate as it gave an accuracy and precision of within ± 15% for all three nominal concentrations (Figure 4). Although KPT-330 did not pass the FDA-required ± 15% as the accuracies for its two lower nominal concentrations were less than 85%, but this stipulated requirement was only missed by a narrow margin. Exemestane however, showed an accuracy that was ≤ 72.6%, which was far off from FDA-required ± 15%. As expected, the results in table 2 demonstrated that very

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minimal signal suppression (93.8-98.1%) owing to matrix effect exited only when SN-38 was used as IS. Moreover, its relative matrix effect close to 100% for all three nominal concentrations implied that the matrix effect was quite stable, resulting in good robustness of this novel LC-MS/MS method. Hence, SN-38 was presented as the best choice and was selected as an IS candidate for method development and validation. Our results demonstrated that SN-38 which possesses the same H-bond donors (2) and acceptors (6) as those of OTS167 has been successfully used as the optimized internal standard for method development and validation. This results on selection of suitable IS for LC-ESI-MS/MS method are consistent with those discovered in our nimbolide pharmacokinetics study.[22]

3.4. Method Validation

3.4.1. Carry-over and Linearity

No carry-over effect was observed as no peaks appeared in the chromatograms when blank wash samples were injected after the highest concentration calibration standards.

Good linearity of the standard calibration curve was achieved at $r^2 \geq 0.996$, implying strong correlation between OTS167 concentrations and its peak area ratio at the linear range of 5-1000 ng/mL. LLOQ was 5 ng/mL and the signal-to-noise (S/N) ratio was 16.8, which was higher than FDA-recommended S/N of 5 (Figure 3).

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3.4.2. Accuracy and Precision

The accuracy ranged from 92.3% to 102.2% and the precision was ≤ 12.7% (Table 1). Both intra-day and inter-day accuracy and precision were within ± 15%, according to FDA guidelines.

3.4.3. Matrix Effect

Matrix effect was found to be insignificant (Table 2) as the relative matrix effect was close to 100% for all three nominal concentrations. This could be due to the minute volume of mouse serum used, which reduced the amount of biological matrix residues present in the samples that could potentially affect ionisation efficiency.

3.4.4. Recovery

Recoveries of OTS167 and SN-38 were found to be consistent and similar at about 83% to 89% (Table 3), demonstrating the efficiency of the extraction method as both compounds were extracted to a reasonable recovery.

3.4.5. Dilution Factor

As most mouse serum samples exceeded the maximum calibrated concentration of OTS167 (1000 ng/mL), 20 or 40 times dilution was performed using blank mouse serum before sample preparation. Accuracy and precision were within ± 15%, demonstrating the reliability of the dilution procedure (Table 4).

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3.4.6. Stability

OTS167 in all three nominal concentrations showed stability within ± 15%, suggesting reasonable stability according to FDA guidelines (Table 5). This indicated that OTS167 was stable in mouse serum during freeze-thaw cycles and when placed on the bench top at room temperature for a short period of time of up to 24 hours.

3.5. Application of Validated LC-MS/MS Method in pharmacokinetic study

A well-validated method for OTS167 quantification was successfully applied in a preclinical pharmacokinetic study. Pharmacokinetic profiles of OTS167 over 24 h at 5 mg/kg were plotted in Figure 5. The pharmacokinetic parameters were summarized in Table 6. The mean half-life ($t_{1/2}$) and apparent volume of distribution (V/F) of OTS167 were found to be 2.45 h and 635 mL/kg respectively, suggesting that the drug was distributed into body tissues extensively[23]. This might be attributed to relative metabolic stability, the hydrophobic and charge interactions of OTS167, which explains its potential interaction with the negatively-charged phospholipids on tissues and hence good binding to tissues.

Maximum concentrations (Cmax) of OTS167 were observed as high as 3197ng/mL at 1.67 hour (Tmax) after OTS167 ingestion of 5 mg/kg via garage. AUC were 28579 h*ng/mL. Based on our results, OTS167 offers good oral drug exposure that is more than sufficient to meet the IC_{50} of many *in vitro* cancer cell lines[24,11,25,26]. High Cmax

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detected at 1-2 h after oral ingestion as well as an appropriate $t_{1/2}$ suggests that OTS167 is easily absorbed through intestine and not extensively metabolised in mice. Based on the clinicaltrials.gov registry, most of the current clinical trials for OTS167 are conducted using the i.v. route in cancer patients. Since OTS167 offers a high oral drug exposure, clinical trials could consider dosing OTS167 orally instead, as this route of administration poses several advantages over the i.v. route. These advantages include decreased discomfort, reduced financial burden as well as a greater convenience and compliance for patients since they could take the medication themselves at home.

4. Conclusions

A novel, rapid and sensitive liquid chromatography-tandem mass spectrometric (LC-MS/MS) method was developed and validated for the evaluation of OTS167 pharmacokinetic in mouse serum according to FDA guidelines. To the best of our knowledge, this is the first LC-MS/MS method for the determination of OTS167 that has been successfully applied to monitor the pharmacokinetic profiles in mice over 24 h after oral ingestion at dose of 5 mg/kg. Our pharmacokinetics study suggested that OTS167 can be quickly absorbed after oral administration with an appropriate elimination rate.

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LEGEND

Figures:

Figure 1. The Chemical Structures of OTS167, SN-38, KPT-330 and Exemestane with their respective log P values and numbers of H-bond donors and acceptors

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Figure 2. Product ion MS/MS scan spectra of OTS167 at m/z 487.2→348.0 (left) and SN-38 at m/z 393.2→349.2 (right)

Figure 3. Representative chromatograms of OTS167 at (A) 0 ng/mL, (B) LLOQ of 5 ng/mL and (C) 24h mouse serum sample and (a), (b) and (c) as 100 ng/mL of SN-38 as internal standard

Figure 4. Accuracy of OTS167 QC samples using 3 different internal standards each at a concentration of 100 ng/mL (red dotted lines represent the range for FDA-stipulated requirement of ± 15%)

Figure 5. Mean (n = 3) serum concentration - time profile of OTS167 at 5 mg/kg administered orally in mice.

Tables:

Table 1. Intra-day and Inter-day Accuracy and Precision of OTS167 QC (ng/mL)

Table 2. Matrix Effect of OTS167 QCs and SN-38 at 100 ng/mL

Table 3. Recovery of OTS167 QC samples and SN-38

Table 4. Accuracy and Precision of Dilution Procedure for OTS167

Table 5. Short-term and Freeze-thaw Stability of OTS167 QC samples

Table 6. Pharmacokinetic parameters of OTS167 administered orally in mice

Table 1. Intra-day and Inter-day Accuracy and Precision of OTS167 QC (ng/mL)

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Nominal Conc.	*Calculated Conc.*		*Accuracy (%)*		*Precision (%)*	
QC samples	**Intra-day**	**Inter-day**	**Intra-day**	**Inter-day**	**Intra-day**	**Inter-day**
30.0	27.7 ± 2.9	28.1 ± 3.6	92.3	93.7	10.6	12.7
100.0	93.5 ± 4.8	95.1 ± 4.8	93.5	95.1	5.1	5.0
800.0	797.2 ± 60.1	817.2 ± 45.0	99.7	102.2	7.5	5.5

Table 2. Matrix Effect of OTS167 QCs and SN-38 at 100 ng/mL

Nominal Conc. (ng/mL)	*Matrix Effect of OTS167 (%)*	*Matrix Effect of IS at 100 ng/mL (%)*	*Relative Matrix Effect (%)*
30.0	88.1 ± 7.4	98.1 ± 10.4	89.8%
100.0	101.8 ± 9.9	97.4 ± 12.8	104.5%
800.0	93.1 ± 11.0	93.8 ± 5.6	99.3%

Table 3. Recovery of OTS167 QC samples and SN-38

Compound	*Nominal Conc. (ng/mL)*	*Recovery (%)*
OTS167_QC1	30.0	89.2
OTS167_QC2	100.0	83.4
OTS167_QC3	800.0	89.0
SN-38	100.0	88.8

Table 4. Accuracy and Precision of Dilution Procedure for OTS167

Nominal Conc. (ng/mL)	*Dilution Factor*	*Quantified Concentration (Mean ± S.D., ng/mL)*	*Accuracy (%)*	*Precision (%)*
4000	20	3760 ± 292.6	94.0	7.8

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	40	3451 ± 119.1	86.3	3.5

Table 5. Short-term and Freeze-thaw Stability of OTS167 QC samples

Nominal Conc. (ng/mL)	*Short-term (hours)*			*Freeze-thaw (cycles)*		
QC samples	4	8	24	3	6	9
30	96.8	86.3	85.7	90.0	91.4	89.7
100	101.5	97.3	85.8	99.5	112.5	94.0
800	95.7	93.1	89.3	114.9	97.6	108.4

Table 6. Pharmacokinetic parameters of OTS167 administered orally in mice

OTS167	*Pharmacokinetic parameters*						
Dose (mg/kg)	Cmax (ng/mL)	Tmax (h)	$T_{1/2}$ (h)	Vd/F (mL/kg)	Cl/F (mL/h)	AUC (h*ng/mL)	AUC_Extrap (%)
5	3197	1.67	2.45	635	635	28579	0.15