

Method development and validation of LC–ESI–MS/MS method for the quantification of sonidegib in healthy rabbits

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ABSTRACT

A new, specific, and precise liquid chromatography–electrospray ionization–tandem mass spectrometric (LC–ESI–MS/MS) technique was established and validated for the quantitation of sonidegib in plasma. The established procedure was applied to the pharmacokinetic study in rabbits. Chromatographic separation was achieved on Phenomenex-C₁₈ (50 × 4 mm) 5-μm column and on methanol, acetonitrile and 0.1% formic acid (25:60:15 by volume) mixture as a movable phase monitored at a flow rate of 0.70 ml/minutes. Sonidegib and gliquidone internal standard were noted at *m/z* 486.2/191.1 and *m/z* 528.5/403.4, respectively. Sonidegib and gliquidone were extracted with liquid–liquid extraction. The developed procedure was linear in the concentration range of 103–1,545 ng/ml. The method was validated with intrabatch and interbatch precision and accuracy within 1.54%–7.18%, 1.82%–6.25%, 98.56%–102.80%, and 97.62%–102.76%, respectively. The technique was applied for the successful examination of the rabbit plasma sample for application in the pharmacokinetic study. This study was carried out on rabbits; the drug showed that *T*_{max} was 3.833 hours, *C*_{max} was 677.667 ± 19.81 ng/ml, and AUC_{0–t} was 6,213.58 ± 235.4. The established technique was utilized in the regular examination of sonidegib in biological matrices with a high level of accuracy and precision.

INTRODUCTION

Sonidegib is used to treat advanced basal cell carcinoma in adult patients who have persisted post-surgery or radiation therapy. Many human cancers are due to hedgehog (Hh) pathway, but Sonidegib efficiently obstructs the controller called smoothed (SMO), inhibiting the hedgehog pathway from functioning. As a consequence, cancers that depend on the hedgehog path are incapable of growth (Einolf *et al.*, 2017; Fendrich *et al.*, 2011; Pan *et al.*, 2010). The drug prevents the transmembrane protein called SMO which plays a significant role in Hh signal transduction. This results in the prevention of Hh signaling and anti-tumor activity in several animal models. Sonidegib is chemically designated as *N*-[6-[(2*S*,6*R*)-2,6-dimethylmorpholin-4-yl]pyridin-3-yl]-2-methyl-3[4(trifluoromethoxy)phenyl] benzamide with a molecular

formula of C₂₆H₂₆F₃N₃O₃ and a molecular weight of 485.498 g/mol (Fig. 1) (Burness, 2015). Sonidegib shows low absorption, which is widely distributed and is slowly metabolized. The study of the absorbed drug was ensued largely by hydrolytic and oxidative metabolism (Zollinger *et al.*, 2014). The hedgehog pathway action (as estimated by glioma-associated oncogene-1 expression) was noted at baseline in men with localized high-risk prostate cancer. Sonidegib penetrates into the prostatic tissue and induces a >60-

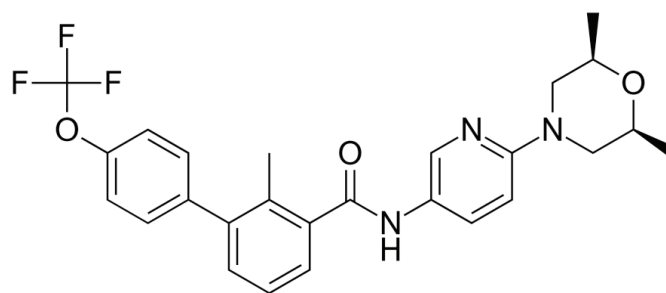


Figure 1. Sonidegib's structure.

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fold suppression of the hedgehog pathway (Ross *et al.*, 2017). A study was reported on exposure response study of the drug (Ide 225), an oral inhibitor of the hedgehog signaling path, for effectiveness and safety in patients with advanced solid tumors (Zhou *et al.*, 2016).

The literature on the drug revealed that no LC–MS/MS approaches were described for the pharmacokinetic study of Sonidegib (Saili *et al.*, 2018; Zollinger *et al.*, 2014) in healthy rabbits. An high performance liquid chromatography (HPLC) method was reported in the patent (Fredenhagen *et al.*, 2013) utilizing Waters' Symmetry C18 (3.5 μ particle, 4.6 \times 150 mm) column with a gradient program. In short, the aim of the study was not only to develop but also validate such HPLC methods which have the qualities of simple to composition mobile phase and rapid sample preparation. Different applications of the studied methods are in therapeutic drug monitoring, pharmacodynamics, and pharmacokinetic studies. The calculation and interpretation of different dosage forms of sonidegib and their *in vivo* activity in humans will also be benefited from the described method.

MATERIALS AND METHODS

Chemical and reagent materials

Sonidegib and gliquidone (reference materials) were acquired from MSN Laboratories, Telangana, India. Phenomenex-C₁₈ (50 \times 4 mm) 5- μ m columns were bought from Thermo-Fischer Pvt. Ltd, Hyderabad, India. HPLC-grade acetonitrile, methanol, and formic acid of an analytical grade were bought from SD Fine Chemicals, Chennai, India. The animal studies on rabbits were carried out at Vijaya College of Pharmacy, Hyderabad, approved by the Institutional Ethical Committee with reference no-1292/ac/09/CPCSEA/17-41/A on 8 March 2019.

Instrument

The LC–MS/MS instrument consists of a Shimadzu LCS10 chromatographic equipment combined with an MS/MS API 3,000 from Applied Biosystems Sciex, Canada, furnished with a turbo ion spray source for ionic induction. Chromatographic data were integrated and supervised by the software belonging to the Analyst version 1.4.10 of Applied Biosystems.

Liquid chromatography

Sonidegib and the internal standard (IS) (gliquidone) were separated by infusing the solution of the sample into C₁₈ Phenomenex column (50 \times 4 mm, 5 μ) utilizing a movable phase combination of ACN, CH₃OH, and 0.10% HCOOH in the proportion of 60:25:15 by volume. Degasification of the movable phase was executed by the process of filtration through 0.50 μ membrane filter, followed by sonication. Drug and IS separation was achieved with the isocratic system by infusing the movable phase through stationary phase at 0.70 ml/minutes flow. The autosampler and column temperatures were monitored at 5.0°C and 30.0°C, respectively.

Mass scanning optimization

Sonidegib stock solution was processed by solubilizing the drug in HPLC-grade CH₃OH. Serial dilutions were prepared with the solvent mixture of CH₃OH and water in the combination

of 80:20 v/v. The mass system was run in the positive ionization mode and the constituents were assessed by multiple reactions monitoring (MRM) of the transition pairs of transitions: *m/z* 486.2/191.1 and *m/z* 528.5/403.4 for Sonidegib and gliquidone, respectively.

Mass spectrometry

The source temperature was set to 500°C and curtain gas of N₂ was adjusted to a constant reading of 11.0 units. The heater at the electrospray interface was turned on and ion spray voltage was set to 4,500 V for ionization. Mass conditions were set accordingly to attain more sensitive unit resolutions. The MRM mode for sonidegib and gliquidone was noted at *m/z* 486.2/191.1 and *m/z* 528.5/403.4, respectively.

Preparation of calibration and quality control standards

Sonidegib stock solution was processed in 80% CH₃OH to get the concentration of 1 mg/ml. Calibration standards were prepared in the concentration levels of 103.0, 154.0, 240.0, 400.2, 660.0, 950.0, 1,236.0, and 1,545.0 ng/ml; these solutions were processed from sonidegib stock solution with 80% methanol. High, medium, and low quality control (HQC, MQC and LQC) lower limit of quantification (LLOQ) standards for sonidegib were (1,545.00, 1,030.00, 288.40, and 103.00 ng/ml) executed in the similar manner. The IS stock solution (1 mg/ml) was also processed with 80% CH₃OH, and further 50 ng/ml was processed by dilution method. All the prepared solutions were reserved at 2.0°C–8.0°C till the samples were analyzed (Dadhaniya *et al.*, 2013; Nageswara Rao *et al.*, 2008; Rezk *et al.*, 2016).

Sample preparation

To 400 μ l of plasma, 100 μ l of IS were mixed and to the sample 400 μ l CH₃OH was mixed and centrifugation was processed at 3,000 rpm for 15.0–20.0 minutes at 5.0°C. The organic portion was dried in a lyophilizer, and to the residue, 250 μ l of the mobile phase was added and the required amount of samples was labeled and transferred into autosampler vials, and injected into the chromatographic system (Chandrasekar *et al.*, 2009; Kumar *et al.*, 2014; Neelima *et al.*, 2014).

Pharmacokinetic study

Six male rabbits of about 2.5–3 kg were elected for the pharmacokinetic study of sonidegib. Before 12 hours of drug administration into the rabbits, food was evaded. Water was given for the rabbits for the entire study and the drug dose of 25 mg/kg was given to the rabbits and 0.60 ml of blood samples were collected from the marginal ear vein of rabbits before dosing (zero times) and at the time intervals of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0, 12.0, 16.0, 24.0, 36.0, and 48.0 hours. The resultant solution was exposed to 4,500 rpm in the centrifuge for 15.0 minutes and separated plasma was relocated into labeled polypropylene tubes at –20.0°C (Chambers *et al.*, 2014; Patel *et al.*, 2011; Wozniakiewicz *et al.*, 2014).

Validation of the analytical method

Validation of the method was carried out according to FDA guidelines on bioanalytical method validation (ICH, 1995, 1996; USFDA, 2001).

Calibration curve

For the assessment of linearity, linear plots of eight non-zero standards were utilized. Eight non-zero points of 103, 154, 240, 400, 660, 950, 1,236, and 1,545 ng/ml were analyzed. The findings from three precision and accuracy sets were taken into consideration to estimate the goodness of fit utilizing $1/x$ and $1/x^2$ weighing factors (Chandrasekar *et al.*, 2009; USFDA, 2001). Deviancy from theoretical concentration should be $\pm 20\%$ for the LLOQ level and $\pm 15\%$ for other quality control samples. Regression coefficient (r^2) should be ≥ 0.98 .

Specificity and selectivity

The interference between drug and matrix components was estimated by the study of six blank lots from six different sources. Two blanks and one LLOQ level were prepared, injected, and analyzed from each lot to estimate possible interference between IS and sonidegib. One blank spiked with the drug at the upper limit of quantification- and IS were injected into the LC system and the chromatograms were analyzed. Peak responses of constituents coeluting along with the drug should be $< 20.0\%$ of the LLOQ level peak response. Peak responses of coelutents of IS should be less than 5.0% of the average IS peak response. The estimated concentrations of LLOQ level standard should be less than 20.0% from the theoretical concentrations (European Medicines Agency, 2011).

Accuracy and precision

These parameters were assessed by analyzing five similar QC samples of sonidegib at LLOQ, HQC, MQC, and LQC concentration levels in three analytical runs. Interassay accuracy was analyzed as the relative variance between the mean calculated concentrations after three runs and the theoretical concentration (Fernandez *et al.*, 2012; Patel *et al.*, 2011). Accuracy should be $\pm 20.0\%$ for the LLOQ and $\pm 15.0\%$ for the remaining concentrations. Inter and intraassay precision results were signified by the percentage of coefficient of variation (%CV), which should be $< 20.0\%$ for the LLOQ level and $< 15.0\%$ for the other QC samples.

Matrix factor

To assess the matrix factor, plasma was processed at LQC and HQC concentration levels after extracting six dissimilar blank matrix lots (Chambers *et al.*, 2014). Simultaneously, six replicas of equivalent neat QC samples were prepared and analyzed. It was calculated with the following formula:

Matrix Factor = (Peak area in presence of matrix components / Average peak area in aqueous samples).

Recovery

The method recovery was assessed by linking the average peak responses of the six extracted HQC, MQC, and LQC samples (1,545.0, 1,030.0, and 288.4 ng/ml, respectively) to mean peak response of six spiked concentrations with the similar amounts of HQC, MQC, and LQC samples.

Autosampler stability

This constraint was evaluated by executing six QC samples (LQC and HQC) which were kept in an autosampler.

These standard samples were infused after 24.0 hours and were evaluated against freshly processed calibration controls. The resultant findings when equated with theoretical concentrations should be in the $\pm 15.0\%$.

Stability

LQC and HQC frozen samples were collected from the freezer after three freeze-thaw cycles. Samples were kept at -30.0°C in three cycles of 24.0, 48.0, and 72.0 hours. For the long-term stability of the drug, QC samples were also assessed by analysis after 4 months of storage at -25.0°C and -70.0°C . Benchtop stability was evaluated for a 7-hours period with standard concentrations. Stability samples were processed and separated along with fresh calibration standard samples. The precision and accuracy of the stability solutions should be $\pm 15.0\%$ of their original concentrations.

Dilution integrity

The concentration of the drug above the ULOQ level was processed and precision and accuracy results were assessed. The percentage of nominal concentration should be $\pm 15.0\%$.

RESULTS AND DISCUSSION

Method development and its validation

A technique for sample separation was executed for extracting the drug and IS from the plasma solutions. LC constraints were enhanced through variable trials to get improved resolution and to increase the S/N (signal to noise) proportion of drug and IS. Mass constraints were supervised by infusing the sample directly into the electro-spray ionizing source of the mass equipment.

The acetonitrile percentage was amplified to develop an accurate and specific technique after the MRM transitions were finalized. A better resolution and separation were obtained utilizing an isocratic movable phase of ACN, CH_3OH , and 0.10% HCOOH (60:25:15 v/v) at 0.70 ml/minutes flow rate.

Selectivity

Six variable batches of blank plasma samples were opted and the nosiness of interfering constituents at retaining time of analyte and IS were evaluated. The nosiness of the matrix components were not identified at the retaining time and m/z of sonidegib and IS in all batches elected (Figs. 2 and 3), simplifies the chromatograms for blank, LLOQ, and blank with IS injections. The interfering peak response should be less than 20% from the LLOQ's peak response.

Linearity

Calibration plot was made in the concentration range of 103–1,545 ng/ml in plasma with a correlation coefficient [r^2] of 0.998. Three linearity plots were linear in employed concentration range with an eight-point calibration utilized for the quantitation by linear regression. The linear curve regression equation was found to be $y = 0.9976x + 1.6425$. The precision (%CV) detected for the linear curve was found to be ≤ 4.93 for sonidegib and the findings are shown in Table 1. The r^2 value should be more than 0.99.

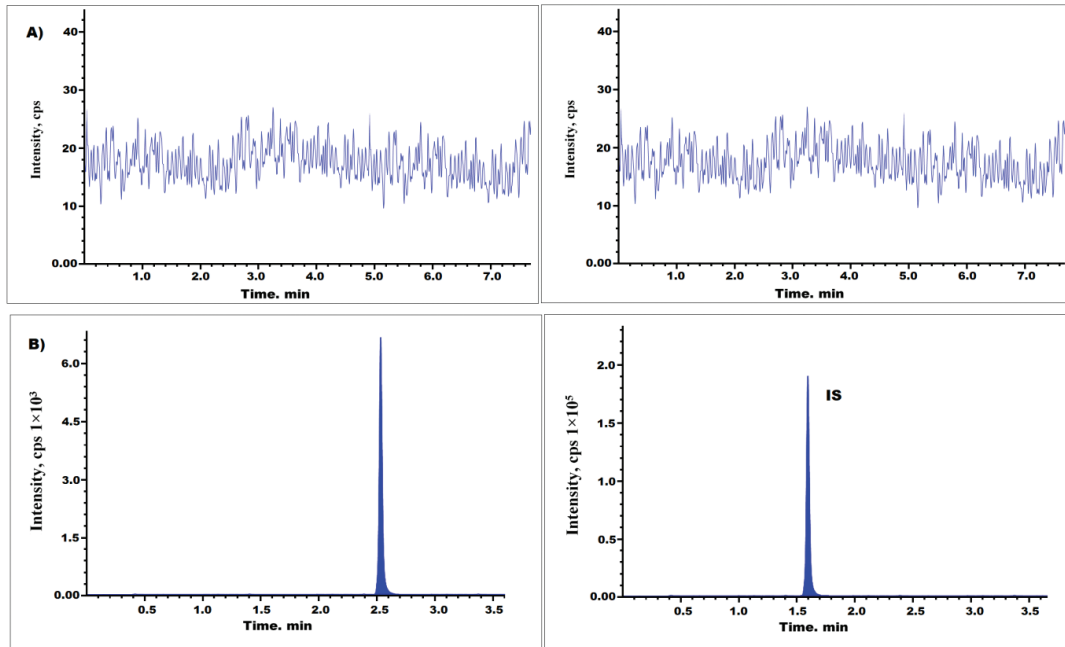


Figure 2. Typical chromatograms of (A) blank plasma (B) spiked with LLOQ and IS.

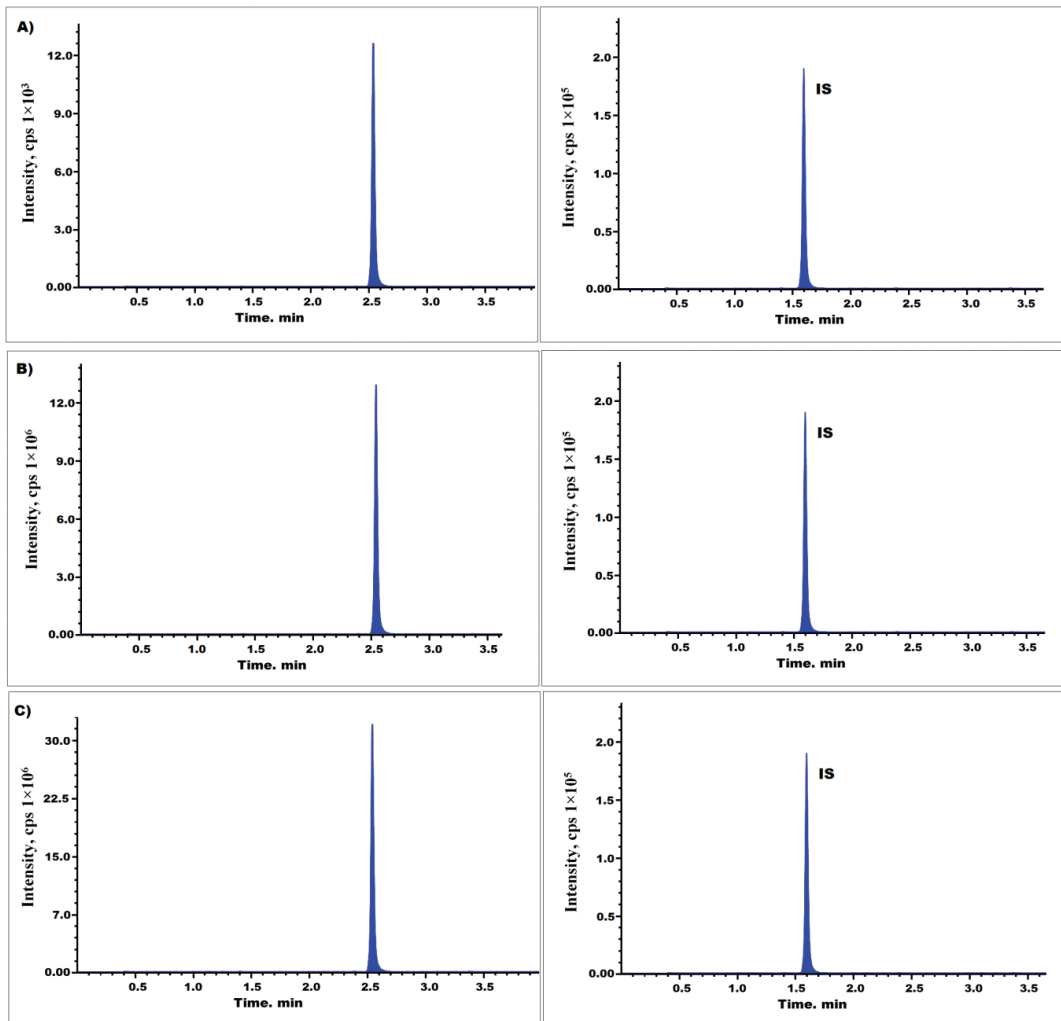


Figure 3. Sonidegib's chromatograms of (A) LQC, (B) MQC, and (C) HQC samples.

Accuracy and precision

The method's accuracy and precision were evaluated by assessing interday and intraday lot deviances of three QC standard solutions in six duplicates: 288.4, 1,030, and 1,545 ng/ml, as shown in Table 2. Intraday accuracy and precision were within 1.54%–7.18% and 98.56%–102.80%, respectively. Interday accuracy and precision were within 1.82%–6.25% and 97.62%–102.76%, respectively. These findings direct that the technique was reliable, specific, reproducible, and accurate. The %CV values were determined for the control standards at different days and the results should be $\pm 15\%$.

Matrix factor (MF)

The MF was calculated in percentage coefficient of variance and the findings for LQC and HQC levels were 1.46% and 0.28%, respectively. The calculated findings were within the acceptable limit of $\pm 15.0\%$. The calculated values are shown in Table 3.

Dilution integrity

Sonidegib was diluted up to 20-fold by blank plasma and was examined with spiked samples above the upper limit of the calibration standard and samples with the highest concentration. The percentage of original concentration was within ± 15.0 and the detected precision was within $\leq 15.0\%$. This proves that the analyte solution can be diluted up to 20 times and yet the findings can be detected.

Stability studies

Sonidegib stability data, which includes autosampler, freeze–thaw, long-term, and benchtop, were within the acceptable limit. The findings are tabulated in Table 4.

Recovery

The recovery percentages were analyzed by assessing the exact peak area of the drug and IS from the processed plasma samples as per the developed technique. The degree of recovery of

Table 1. Concentration of spiked plasma and RSD (%) for sonidegib.

Concentration in plasma (ng/ml)	Concentration assessed Mean (ng/ml) \pm SD(a)	%RSD	% Accuracy
103	98.88 \pm 0.78	3.71	96.52
154	149.38 \pm 0.201	2.77	97.26
240	248.4 \pm 0.284	4.80	103.45
400	408.84 \pm 0.213	2.37	102.21
660	716.92 \pm 6.234	2.23	108.71
950	962.47 \pm 2.90	1.53	101.31
1,236	1,275.22 \pm 9.208	0.74	103.21
1,545	1,588.13 \pm 15.562	1.52	102.81

RSD = Relative standard deviation; a = Six replicates; SD = Standard deviation.

Table 2. Sonidegib intraday and interday precision and accuracy.

Original concentration (ng/ml)	Intraday ^a			Interday ^a		
	Average identified(ng/ml)	%Precision	% Average accuracy	Average identified(ng/ml)	% Precision	% Average accuracy
288.4	281.91	1.54	99.50	279.75	7.14	97.62
1,030	1,012.23	7.18	98.56	1,012.66	6.32	98.76
1,545	1,554.72	2.65	102.80	1,552.05	1.25	102.76

a = Six replicates.

Table 3. Sonidegib results for matrix effect.

Sonidegib		
QC sample	LQC	HQC
Original concentration (ng/ml)	288.4	1,545
1	280.47	1,542.10
2	284.79	1,539.24
3	281.91	1,539.30
4	284.07	1,548.28
5	274.70	1,541.46
6	277.58	1,544.65
Mean	280.59	1,542.51
\pm SD	3.54	3.17
% CV	1.26	0.21
% Accuracy	97.29	99.83

CV = Coefficient of variation; SD = Standard deviation.

Table 4. Stability data for sonidegib.

Concentration (ng/ml)	Freeze-thaw stability		Autosampler stability		Benchtop stability		Long-term stability	
	Mean \pm SD (ng/ml)	%CV	Mean \pm SD (ng/ml)	% CV	Mean \pm SD (ng/ml)	% CV	Mean \pm SD (ng/ml)	% CV
288.4	288.4 \pm 0.19	7.6	288.4 \pm 0.14	4.8	288.4 \pm 0.21	4.85	288.4 \pm 0.14	7.1
1,545	1,545 \pm 6.12	8.1	1,545 \pm 5.86	7.25	1,545 \pm 6.41	4.94	1,545 \pm 6.32	4.9

SD = Standard deviation; CV = Coefficient of variation.

Table 5. Plasma concentrations at different time intervals.

Time in hours	Measured concentrations (ng/ml)							Mean	SD
	Animal 1	Animal 2	Animal 3	Animal 4	Animal 5	Animal 6			
0	0	0	0	0	0	0	0	0	
0.5	14	18	16	14	14	15	15.17	1.46	
1	33	32	32	31	36	33	32.83	1.53	
1.5	50	55	58	54	50	52	53.17	2.84	
2	78	81	76	75	73	70	75.5	3.5	
2.5	84	86	92	90	89	85	87.67	2.84	
3	78	85	87	81	89	92	85.33	4.75	
4	65	75	79	75	74	80	74.67	4.85	
5	66	71	73	68	71	74	70.5	2.75	
6	58	58	62	68	62	69	62.83	4.33	
8	60	59	58	51	51	50	54.83	4.21	
12	51	58	54	51	52	60	54.33	3.49	
16	41	51	49	41	34	35	41.83	6.38	
24	50	46	49	43	31	36	42.5	6.89	
36	30	29	24	26	25	20	25.67	3.29	
48	0	0	0	0	0	0	0	0	

SD = Standard deviation.

Table 6. Test animals (rabbits) PK parameters mean values.

Parameters	Animal 1	Animal 2	Animal 3	Animal 4	Animal 5	Animal 6	Mean	SD
C_{max}	659	695	676	695	645	696	677.67	19.81
$\log C_{max}$	2.89	2.85	2.85	2.86	2.79	2.38	2.77	0.18
T_{max}	3	4	4	5	3	4	3.83	0.68
$\log T_{max}$	0.4771	0.602	0.602	0.699	0.477	0.602	0.58	0.07
$t_{1/2}$	9.1414	8.113	9.563	6.7059	8.331	6.028	7.98	1.25
$\log t_{1/2}$	0.971	0.909	0.981	0.8265	0.921	0.78	0.89	0.07
Ke	0.0747	0.085	0.072	0.1033	0.083	0.115	0.09	0.01
$\log Ke$	-1.12	-1.068	-1.14	-0.986	-1.08	-0.94	-1.06	0.07
AUC_{0-t}	6,488.5	6,455	5,940	6,218	5,874	6,306	6,213.58	235.4
$\log AUC_{0-t}$	3.8121	3.81	3.774	3.7937	3.769	3.8	3.79	0.016
AUC_{0-inf_obc}	6,791.8	6,735	6,229	6,469.5	6,150	6,471	6,474.38	235.9
$\log AUC_{0-inf_obc}$	3.832	3.828	3.794	3.8109	3.789	3.811	3.81	0.015

SD = Standard deviation.

sonidegib and of IS should be constant, reproducible, and precise. The overall average recovery of drug and IS was found to be 97.26% and 98.01%, respectively.

Pharmacokinetics

The pharmacokinetic parameter of sonidegib was calculated from the graph obtained by taking plasma concentrations on Y-axis and time on X-axis using PK-Solver software. In this

study, the trapezoidal rule was opted for measuring of an area under the curve from 0.0 to 48.0 hours (AUC_{0-48}). Sonidegib has a mean T_{max} of 3.833; and mean C_{max} , AUC_{0-t} , and $AUC_{0-\infty}$ for the test dosage form was 677.667, 6,306, and 6,471, respectively. In the reported method on blood, the T_{max} , C_{max} , and AUC_{last} were 154 ± 32.6 ng/ml, 2–4 hours, and $8,680 \pm 2,510$ h.ng/ml, respectively (Fredenhagen *et al.*, 2013). The resultant values are presented in Tables 5 and 6 and Fig 4.

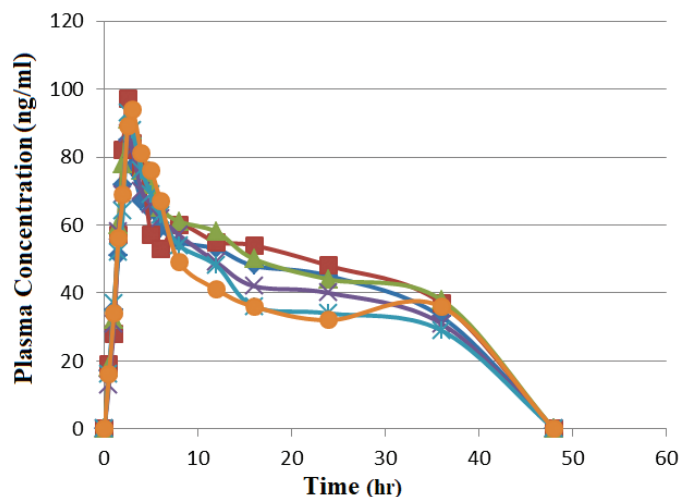


Figure 4. Plasma concentration time profile curves of six rabbits.

CONCLUSION

In the present article, an LC–ESI–MS/MS procedure for the quantitation of sonidegib in plasma was efficiently established and validated. Chromatographic elution was achieved on Phenomenex- C_{18} (50×4 mm) 5μ stationary phase with ACN, CH_3OH , and 0.1% formic acid (60:25:15 by volume) as the movable phase monitored at a 0.70 ml/minutes flow. All the validation parameters, precision, selectivity, matrix effect, accuracy, dilution integrity, stability, and recovery, were within the acceptable limit. This technique was successfully executed to study pharmacokinetic parameters in six male rabbits and the drug has shown average T_{max} of 3.833; mean C_{max} , AUC_{0-t} and $AUC_{0-\infty}$ for the sample dosage form was 677.667, 6,306, and 6,471, respectively, in the pharmacokinetic study on healthy rabbits. The applicability of the technique advises its further application for drug interaction, bioavailability, and bioequivalence studies.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

Research protocol was approved by the Institutional Ethical Committee with reference no-1292/ac/09/CPCSEA/17-41/A on 8 March 2019. .

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