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Bioanalytical method development and validation for determination of olutasidenib and its application to pharmacokinetic studies

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Olutasidenib is an inhibitor licensed by the FDA, indicated against mutations in isocitrate dehydrogenase-1 (IDH1). For individuals with vulnerable IDH1 mutations, it has been demonstrated to be a very effective therapy for recurrent or refractory acute myeloid leukemia (AML). After a long review procedure, olutasidenib was finally given an approval by the FDA in December of 2022. To determine the concentration of olutasidenib in rat plasma, an LC-MS/MS approach was applied. The drug ibrutinib serves as a standard for comparison. Inertsil ODS, 150 mm × 4.6 mm, 3.5 µm, mobile phase was Acetonitrile (ACN), and Ammonium formate buffer (AmF), pH 3.0 (50 : 50 v/v) at 1.0 ml min⁻¹ was used for the separation process. Liquid–liquid extraction (LLE) was adopted for both olutasidenib and the Internal standard (IS). Proton adducts of olutasidenib and ibrutinib were observed at *m/z* 354.8589 and 239.8107 and *m/z* 441.573 and 372.1236 in MRM positive mode, correspondingly. The approach was shown accurate throughout a range of 3.0–60.0 ng ml⁻¹ and correlation values of (*r*²) ≥ 0.999.6 replicates including olutasidenib at 4 distinct QC levels were analyzed to determine intra-assay precision and accuracy; the Coefficient of variations (CV) were reported to be 3.41% to 0.58% to 0.31% to 0.36, and the accuracy ranged from 97.40, 99.69, 99.4, and 99.16%, respectively, for LOQQC, LQC, MQC, and HQC. In a pharmacokinetic investigation using rat plasma, this strategy has proven effective.

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Introduction

USFDA has given approval to the use of olutasidenib, a novel medication, to treat recurring or refractory acute myeloid leukemia (AML) in adults who are sensitive to IDH1 mutation.^{1,2} Rezlidhia is the commercial name for the medicine olutasidenib, which is the active component in this medication.^{3,4} Individuals with AML who have a poor prognosis often have mutant IDH1 enzymes, which this medication works to combat. The acceptance of olutasidenib as a therapy for AML is a major step forward. The therapy choices for individuals with recurrent or refractory AML were severely restricted prior to the approval of olutasidenib. Nausea, tiredness, aches and pains, constipation, white blood cell count increase, difficulty breathing, temperature increase, rash, mucositis, diarrhoea, and transaminitis are among the most prevalent unwanted effects.⁵ Clinical studies of olutasidenib, however, have shown encouraging results, and the drug was officially approved by the FDA in December 2022. Isocitrate dehydrogenase (IDH) inhibitors, such as

olutasidenib, are a kind of medication. The mutant version of IDH1, which is prevalent in individuals with AML, is the primary target of IDH inhibitors. It is believed that 2-hydroxyglutarate buildup, caused by the IDH1 mutation, is essential in the initiation and advancement of AML. AML Patients have benefited greatly from the usage of IDH inhibitors like olutasidenib. Therapeutic studies of individuals with IDH1-mutated AML and myelodysplastic syndrome demonstrated that olutasidenib was effective and exhibited good therapeutic efficacy.

The science of pharmacokinetics investigates how medications are taken in, transported across the body, broken down, and removed. The therapeutic effectiveness, toxicity, and dosage schedule of a medicine are all heavily influenced by its pharmacokinetics.^{6,7} Thus, it is crucial to learn the pharmacokinetics of olutasidenib to create a successful treatment plan for individuals with AML. There is a lack of data about olutasidenib's pharmacokinetics.

The recent approval by the FDA for the use of olutasidenib represents a significant milestone in the treatment of AML in adults.⁸ Olutasidenib, marketed under the name Rezlidhia, is a ground-breaking medication designed to target and address a specific genetic mutation known as Isocitrate dehydrogenase-1 (IDH1). This approval marks a crucial advancement in the field of oncology.⁹

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Patients suffering from recurrent or refractory AML, particularly those with mutant IDH1 enzymes, often face limited treatment options and poor prognoses. Olutasidenib's mechanism of action, which specifically targets mutant IDH1 enzymes, offers newfound hope for these individuals. By inhibiting these aberrant enzymes, olutasidenib has the potential to disrupt the growth and proliferation of leukemia cells, improving the prognosis for patients.¹⁰

The FDA's decision to greenlight olutasidenib for AML underscores its commitment to advancing innovative therapies and improving outcomes for patients facing this challenging disease. This approval paves the way for healthcare professionals to incorporate olutasidenib into their treatment protocols, providing a more tailored and effective approach for eligible AML patients, ultimately enhancing the quality of care and patient outcomes in the fight against this devastating form of leukemia.¹¹

Before olutasidenib's approval, the therapeutic options available for individuals grappling with recurrent or refractory Acute Myeloid Leukemia (AML) were notably limited. This dearth of effective treatments left patients and healthcare providers with few choices for managing this aggressive disease. However, olutasidenib's approval represents a pivotal moment in AML treatment.¹²

This calls for an analysis of olutasidenib's pharmacokinetics and a determination of its concentration by means of LC-MS/MS in rat plasma. A reliable and accurate approach for determining drug levels in biological matrices, LC-MS/MS, was used for measuring olutasidenib. LC-MS/MS is a very effective analytical method for separating and detecting analytes in complicated matrices. Quantifying olutasidenib by means of LC-MS/MS is a crucial step in elucidating the drug's metabolism and distribution, leading to a better overall knowledge of its pharmacokinetics. Fig. 1 shows the structure of olutasidenib.

So far, LC-MS/MS techniques have not been developed for the purpose of quantifying olutasidenib. Olutasidenib quantitation using RP-HPLC has been developed,¹³ however this approach is only useful for bulk pharmaceutical formulations. A more targeted and sensitive analytical method is necessary for accurate pharmacokinetic assessment. LC-MS/MS has several benefits over RP-HPLC, such as more selectivity in intricate biological matrices, lower detection limits, and enhanced resilience for pharmacokinetic research. In order to guarantee accurate pharmacokinetic assessment as well as therapeutic monitoring, this work focuses on establishing and verifying an LC-MS/MS approach for precise evaluation of olutasidenib in plasma.

Methods

Chemicals and reagents

Moltus Research Laboratories Tarapur, M.I.D.C. Boisar, Maharashtra, kindly supplied the reference sample of olutasidenib. All chemicals, including HPLC-grade acetonitrile (ACN) and methanol (MeOH), were acquired from Merck chemical division, Mumbai. Water purification system by Milli-Q was supplied for the experiment. Manisha Analytical Laboratories in Mumbai, Maharashtra, India, was the source for the rat plasma used in this study.

Instrumentation

The Waters alliance e-2695 model HPLC system, having a column oven, auto sampler, and degasser, was utilized for the purpose of analysis. The HPLC equipment was connected to a SCIEX QTRAP 5500 mass spectrometer that was outfitted with an electrospray ionisation interface. The data from the chromatogram was analysed using SCIEX software.

Olutasidenib stock solution (120 ng ml⁻¹)

A quantity of 6 mg of olutasidenib working standard was measured and thereafter put into a 10 ml volumetric flask. The flask was then filled to its full volume with a suitable diluent. Sonication and subsequent vortexing was performed to produce a dissolved and uniform solution. The initial volume of 0.1 ml was further diluted with diluent to a final volume of 10 ml. Transfer 0.2 millilitres of the aforementioned solution into a 10 millilitres volumetric flask, and thereafter fill the flask to the desired volume with a suitable diluent.

Preparation of internal standard stock solution (120 ng ml⁻¹)

A 6 mg quantity of ibrutinib working standard was measured and thereafter put into a 10 ml volumetric flask. The flask was then filled to its full volume with a diluent. The initial volume of 0.1 ml was then diluted to a final volume of 10 ml using a diluent. Transfer 0.2 ml of the aforementioned solution into a volumetric flask (10 ml), and afterwards fill the flask to the desired volume with a suitable diluent.

Preparation of standard solution (30 ng ml⁻¹ of olutasidenib)

The standard stock solution was transferred into a 2 ml centrifuge tube, using a volume of 500 μ l. To the mixture, include 200 μ l of plasma, 500 μ l of IS, 300 μ l of ACN, and 500 μ l of diluent. Subject the sample to centrifugation for a duration of 20 minutes. The supernatant liquid should be filtered and afterwards transferred into a container suitable for High Performance Liquid Chromatography (HPLC).

Buffer preparation

The procedure involves precisely measuring and transferring 6.3 grams of ammonium formate into a 1 liter volume of HPLC water. The mixture should be thoroughly stirred and its pH should be adjusted to a value of 3.0 using formic acid. The sample was passed through a filter paper of 0.22 μ .

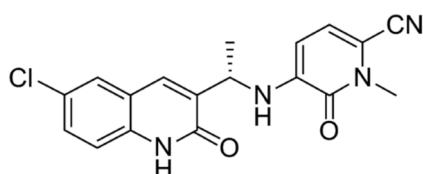


Fig. 1 Structure of olutasidenib.



Preparation of mobile phase

Combine equal volumes of acetonitrile and ammonium formate buffer in a 1:1 ratio. The solution was passed through a membrane filter paper of 0.45μ .

Detection

Olutasidenib (m/z , 239.8107) and ibrutinib (m/z , 372.1236); collision energy: 15 V; optimized mass parameters yielded product ions of m/z , 354.8589 and m/z , 441.5732; drying gas and source temperatures range from 120–250 and 550 °C, correspondingly, while the ion spray voltage is 5500 volts. Nitrogen for use as collision gas, the following parameters were determined for the drying gas; flow stream: 5 l min^{-1} , declustering potential 40 V, entrance potential 10 V, exit potential 7 V, and dwell duration 1 s.

Chromatographic conditions

At room temp., 1.0 ml min^{-1} of a mobile phase having ACN and AmF, pH 3.0 (50 : 50 v/v) was used for separation on an analytical column of Intertsil ODS, 150 mm \times 4.6 mm, 3.5 μm . As an internal reference for chromatography and extractability, ibrutinib was analyzed. With an overall run duration of 5 minutes, the drug and IS were extracted at 2.965 and 2.046 minutes, correspondingly.

Standards and QC samples preparation

Both drug and IS were produced as 30.0 ng ml^{-1} stock solutions in a solvent. Freezer settings (2 °C to 8 °C) were maintained for the storage of these solutions and internal standard spiking solutions prior to analysis. Quality control (QC) standards were prepared by adding olutasidenib standard stock solution to evaluate clean rat plasma at concentrations of 3.0, 7.5, 15.00, 22.50, 30.00, 45.00, and 60.0 ng ml^{-1} ; these were then frozen at 30 °C until investigation. ACN was used to generate aqueous standards, which were then maintained in fridge at 2–8 degrees Celsius until investigation.

Sample preparation

The drug and IS were extracted from rat plasma by LLE. Because of its better sensitivity, enhanced analyte recovery, and reduced matrix effects, Liquid-liquid extraction (LLE) was selected over Solid phase extraction (SPE) and Protein precipitation (PPT).^{14–17} In order to do this, 500 μL of dilutant, standard (appropriate concentration), and IS were introduced to marked polypropylene tubes, along with 200 μL of plasma sample, and the tubes were quickly vortexed. Then, after adding and vortexing for 10 minutes, a total of 300 μL of extracting solvent (ACN) was introduced. The materials went through a centrifuge for 15–20 minutes at 20 °C and 4000 rpm. After that, we placed the supernate from each sample into separate polypropylene tubes and gave them a quick swirling. After extraction, the material was placed in auto-sampler vials and then fed into the column.

Bioanalytical method validation^{18–33}

Selectivity and specificity. 6 Distinct blank plasma samples were prepared and examined to assess the selectivity of the procedure by testing for possible interferences of intrinsic chemicals co-eluting with the drug and IS. Retention periods and MRM responses were used to pinpoint the chromatographic peaks of olutasidenib and IS. Olutasidenib's LOQ specifies that the peak area at an appropriate retention period in these samples cannot exceed 20 percent of the typical peak area. Ibrutinib LOQ is set such that the peak area at an appropriate retention period in the samples is under or equal to 5% of the typical peak area of ibrutinib.

Recovery. Analysis of QC samples allowed us to calculate the percentage of olutasidenib and ibrutinib that were successfully extracted from rat plasma. Peak regions were compared between plasma sample and standard solution that had been spiked using blank plasma residue to calculate the percentage of recovery at 3 concentrations (15.00, 30.00, 45.00 ng ml^{-1}). Above 85% recovery was deemed sufficient to meet the criteria.

LOD and LOQ. A sample's LOD is the minimal amount at which it can be distinguished from the background yet cannot be quantified. The LOD was established by juxtaposing test outcomes from samples with an estimated amount of analytes with blank samples at S/N of 3 : 1.

LOQ is minimum detectable level of a drug in any sample that yields reliable results. The LOQ was determined by studying olutasidenib concentrations in both mobile phase and plasma standards.

Matrix effect. It was measured by assessing matrix factor, which is the ratio of the peak response ratio in the extracted matrix to that in aqueous standards, and was used for predicting the fluctuations in matrix effects within samples from each subject.

At both the low and high quality control levels, duplicate extractions of six different batches of blank biological matrices were performed and then post-spiked with the aqueous standard. The coefficient of variation (% CV) provides a measure of accuracy for the matrix factor and a value of <15% is considered acceptable.

Calibration curve, precision, and accuracy. Olutasidenib concentrations in rat plasma were used to create a calibration curve, which spans a range of 3.0–60.0 ng ml^{-1} . The calibration curve was determined using a linear model and weighted $1/\sqrt{x}$ least square. Quantitative analysis of olutasidenib and ibrutinib was performed, and the peak area ratio was compared to the olutasidenib dosage in ng mL^{-1} . Samples for the calibration curve and the QC were replicated six times ($n = 6$). Concentrations at the calibration points, when recalculated, must be ≤ 15 and $\pm 15\%$ of corresponding theoretical values, respectively, for precision and accuracy, with the exception of LLOQ samples where they must be within 20% and 20%, respectively.

Stability of olutasidenib. Following 3 freeze-thaw sessions performed in accordance with the clinical procedure, both LQC ($n = 3$) and HQC ($n = 6$) samples were removed from the refrigerator. Before being introduced into the column, samples were kept at 28 ± 5 °C for about 6 h. Olutasidenib in QC samples



was also tested for its long-term stability by analyzing the samples 7–28 days after they were stored at -30°C . Control concentrations were stored in the autosampler tray for periods ranging from one hour to twenty-four hours, and the stability was evaluated at each time point. Using constant concentrations, we analyzed the stability of a benchtop sample over 25 hours. Seven days of storage at $5 \pm 3^{\circ}\text{C}$ was preferred to analyze the short-term stability. Standard samples for use in constructing a calibration plot were prepared and extracted at the same time as stability samples. Both the precision and accuracy of these samples, expressed as a percentage of their actual dosages, are required to be within ≤ 15 and $\pm 15\%$, correspondingly.

Pharmacokinetic study. The study adhered strictly to the guidelines and regulations set forth by the CPCSEA. CPCSEA is a government body in India responsible for overseeing animal experimentation. Manisha Laboratories' Animal House Facility in Mumbai, Maharashtra, India, holds a revised CPCSEA license (registration number 1074/PO/Re/S/05/CPCSEA) specifically for educational studies involving small animals. The research, conducted under protocol No: CPCSEA/MS Lab/PK/58799, was approved by the Institute of Animal Ethics Committee (IAEC). The animals were housed in controlled laboratory conditions with access to a limited range of food, and the facility maintained at $21\text{--}24^{\circ}\text{C}$ and 50–55% RH. The animals fasted overnight prior to experimentation, had ad libitum access to water, and all necessary measures were taken to minimize any discomfort or suffering.

6 Male Sprague-Dawley rats (SD rats) were given olutasidenib (6 mg g^{-1} b.w.) orally through a BD syringe linked to an oral gavage needle (size 18), and the devised LC-MS/MS technology was effectively adopted for the pharmacokinetic investigation. Each rat was given isoflurane anesthesia, and blood samples were taken *via* retro-orbital sampling and placed into tubes carrying 10% of K2EDTA anticoagulant ($20 \mu\text{L}$) at regular intervals. Samples were taken at 0, 1, 2, 4, 8, 16, 32, 64, and 128 hours after dosing. Every rat's blood sample was between 1.9 and 2.2 ml, which is much below the 20% maximum that is advised. Approximately ten to fifteen minutes of centrifugation at a speed of three thousand revolutions per minute were required to separate plasma from whole blood. The collected samples were placed in microcentrifuge tubes with labels and frozen at 30°C . The developed approach was applied to all samples. Non-compartmental analysis was used to determine pharmacokinetic parameters with the help of the WinNonlin (version 5.2) program. The proposed approach was employed to produce concentration data (FDA 2002; FDA 2003). Incurred sample reanalysis (ISR) verified the consistency of the study's samples. Every subject had two samples taken at different times throughout the pharmacokinetic profile's Cmax and clearance phase for ISR. Not exceeding a twenty percent discrepancy was allowed between samples, at which point they were deemed stable.

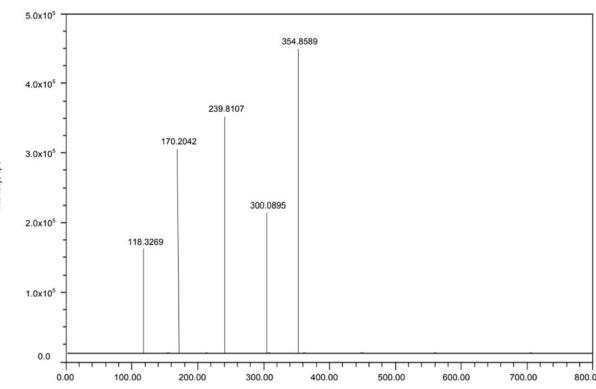


Fig. 2 MRM of olutasidenib parent and product ions.

Results and discussion

Method development and validation

Due to its specificity, sensitivity, and consistency, LC-MS/MS has proven to be among the useful analytical approaches in clinical pharmacokinetics. The purpose of this research was to design and verify a straightforward assay for the quantitative assessment of olutasidenib in rat plasma. Multiple studies were performed to find the optimal chromatographic conditions for maximizing the resolution and boosting the detection of olutasidenib and IS. These included the makeup and properties of the mobile phase, the use of distinct columns, and various techniques like SPPE and LLE for sample extraction. Directly injecting olutasidenib and IS solutions into the spectrometer's ESI pump was used to achieve optimal MS performance. Improved ionizing of the protonated ionic olutasidenib and IS was achieved by optimizing the ESI source's vital factors, including the needle and capillary voltages, source temperature, heater, nebulizer, and desolvation gases. Olutasidenib and IS both had abundant fragment ions at m/z 354.8589 and m/z 441.5732 in the corresponding product ion spectra (see Fig. 2 and 3, accordingly). To get a quick and selective LC approach, we first tuned the MS settings, and then we adjusted the chromatographic conditions, including the mobile phase, column,

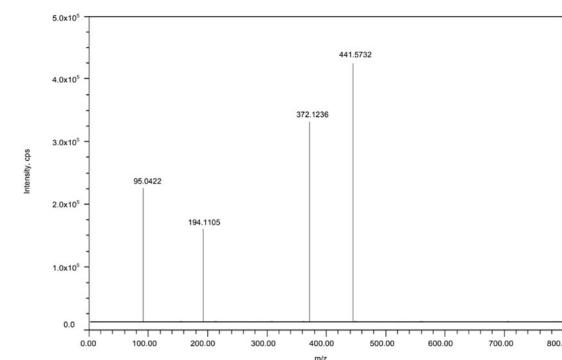


Fig. 3 MRM of ibrutinib parent ion and product ion.



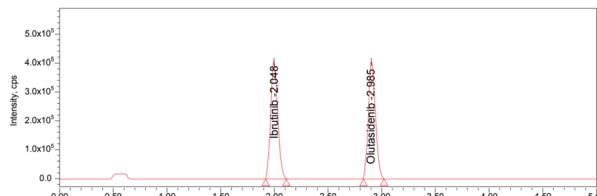


Fig. 4 Optimized chromatogram.

and extraction procedure. At 1.0 ml min^{-1} with an injection volume of $10 \mu\text{L}$, a mobile phase of ACN and AmF was found to be optimal for extraction and recovery. Fig. 4 shows the optimal chromatographic conditions: an Intertsil ODS column ($150 \text{ mm} \times 4.6 \text{ mm}$, 3.5 m) and a LLE procedure. The new approach was shown to be accurate throughout a linear range of concentrations, from 3.00 to 60.0 ng ml^{-1} . Selectivity, LOD, LOQ, matrix effect, accuracy, reliability, recovery, and stability were all assessed as part of the validation process.

Selectivity and specificity

There were no interfering chemicals in the MRM study of olutasidenib and IS. Fig. 5 and 6 displays chromatograms of olutasidenib and ibrutinib-spiked plasma. The drug's retention time of 2.965 minutes and the internal standard's retention time of 2.046 minutes demonstrate that the approach is highly selective and can efficiently separate the two compounds. When the stationary phase interacts with it, separation takes place without interference or peak overlap. This separation, along with the absence of peak overlap, confirms that the method is

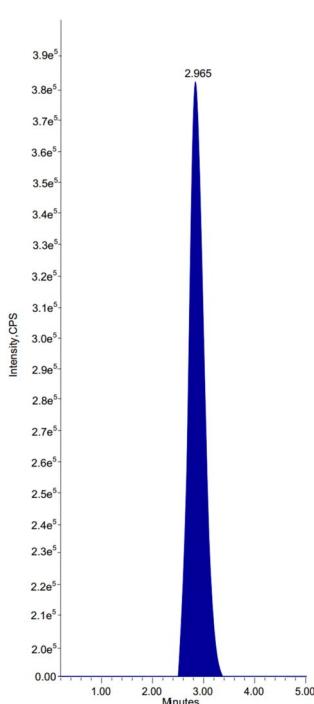


Fig. 5 Chromatogram of olutasidenib.

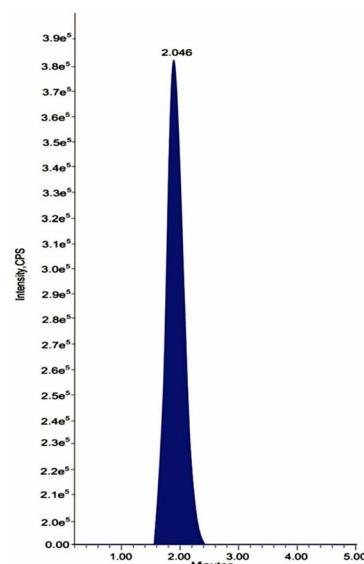


Fig. 6 Chromatogram of ibrutinib.

highly specific, ensuring interference-free identification and measurement. Therefore, the results indicate that the olutasidenib analysis using the proposed LC-MS/MS method is both highly selective and specific.

Limit of detection and quantification

Based on the response standard deviation (σ) and the calibration curve slope (S), the LOD and LOQ values were calculated (in accordance with ICH recommendations). The LOD was calculated as $3 \times \frac{\sigma}{S}$. Similarly, the LOQ was calculated as $10 \times \frac{\sigma}{S}$. Table 1 shows the instrument detection values for olutasidenib at very low doses based on the LOD. The LOD for the analyte was determined to be 3 ng ml^{-1} , demonstrating that the approach is sensitive enough to identify analyte concentrations as low as 3 ng ml^{-1} . Additionally, the analyte's LOQ was found to be 10 ng ml^{-1} , indicating that the approach can accurately and consistently measure analyte concentrations as low as 10 ng ml^{-1} .

Matrix effect

For accuracy, selectivity, and sensitivity to remain unaffected, it is necessary to assess the matrix effect. The aqueous standard was post-spiked into six batches of blank biological mediums at the intermediate QC level, and the samples were assessed against neat standards at identical levels in alternating injections. Olutasidenib has a matrix factor of 0.9973 , which indicates a high degree of accuracy. This further indicates that the matrix does not significantly impact the measurement of the

Table 1 Results of LOD and LOQ

Drug name and details	LOD (ng ml^{-1})	LOQ (ng ml^{-1})
Olutasidenib (S/B) value	3	10



analyte. Additionally, the effects of IS and the drug on ion suppression/enhancement were not seen at their expected retention durations.

Ion suppression or enhancement in LC-MS/MS analysis can be greatly affected by species-specific differences in protein binding, enzyme activity, and phospholipid composition. In particular, human plasma is known to have a higher protein content and different metabolic enzyme profiles than rat plasma, which could result in distinct matrix effects.¹⁵ Therefore, it is important for subsequent studies to evaluate and take into consideration matrix effects in human plasma to guarantee the reliability and accuracy of the technique in clinical contexts. This will allow for a smooth transition from preclinical to clinical trials.

Linearity

The linearity of the standard curves was seen across 3–60 ng ml⁻¹ of olutasidenib, this means that the approach can reliably quantify olutasidenib in biological samples across this concentration range (Table 2). The calculated average correlation coefficient was determined to be 0.999. It demonstrates that, across the measured range, the analytical response (peak area ratio) is directly proportional to the analyte concentration. The quantification of samples was performed by calculating the ratio between the peak area of the analyte and the peak area of the IS. The graphic Fig. 7 depicted the relationship between peak area ratios and plasma concentrations.

Precision and accuracy

The estimation of intra-assay precision and accuracy was conducted by analysing six duplicates consisting of olutasidenib at four distinct quality control (QC) levels. The assessment of inter-assay precision involved the analysis of four tiers of quality control (QC) samples across four distinct runs. The acceptance standards for the data encompass two key aspects: accuracy and precision. Accuracy is defined as the data falling within a range of 85–115% of the actual values. Precision, on the other hand, is determined by the relative standard deviation (RSD) being within $\pm 15\%$, except for the LLQC. For LLQC, the accuracy should be within 80–120% and the RSD should be less than

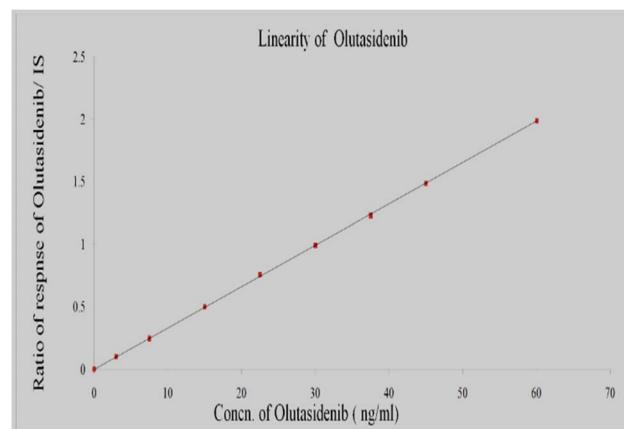


Fig. 7 Calibration plot for concentration vs. area ratio of olutasidenib.

Table 3 Precision and accuracy of olutasidenib

Parameter	HQC	MQC	LQC	LLQC
Nominal conc. (ng ml ⁻¹)				
	45.01	30.0	15.0	3.0
<i>n</i>	6	6	6	6
Mean	5.669×10^5	3.827×10^5	1.919×10^5	0.375×10^5
SD	0.021	0.012	0.011	0.013
% CV	0.36	0.31	0.58	3.41
% Mean accuracy	98.16%	99.40%	99.69%	97.40%

20%. The accuracies for LLQC, LQC, MQC, and HQC were 97.40%, 99.69%, 99.40%, and 99.16%, respectively, while the coefficient of variation (% CV) ranged from 0.31% to 3.41%. If the method's mean% accuracy is between 85% and 115%, and its mean% CV is less than 15% across all QC levels, it is considered both accurate and precise. The low % CV demonstrates the method's precision and consistency, while the accuracy is satisfactory. These results (Table 3) indicate that the approach is accurate and can be reliably used for bioanalytical or clinical analyte quantification.

Recovery of analyte

The evaluation of olutasidenib and IS recovery was conducted at three distinct concentration levels, specifically LQC, MQC, and HQC. The calculation of recovery involved the comparison of the response of duplicate samples with the response of the neat reference solution.

The recovery of an analyte from a sample matrix, also known as extraction efficiency, involves comparing the analytical response obtained from a certain analyte quantity added to the response obtained from the sample matrix. The extraction process was conducted utilizing a mobile phase solvent due to the fundamental characteristics of olutasidenib. Table 4 presents the mean recovery at each QC level. The recovery rates for LQC, MQC, and HQC are 99.38%, 99.32%, and 98.22%, respectively. With recoveries so close to 100%, these results demonstrate the efficiency of the extraction procedure. The % CV values across all QC levels are very low, ranging from 0.21%

Table 2 Linearity of olutasidenib

Final conc. (ng ml ⁻¹)	Res	Area response ratio
0	0	0.0
3.00	0.392×10^5	0.102
7.50	0.936×10^5	0.245
15.00	1.927×10^5	0.500
22.50	2.934×10^5	0.760
30.00	3.852×10^5	0.992
37.50	4.761×10^5	1.228
45.00	5.689×10^5	1.486
60.00	7.584×10^5	1.990
Slope	0.0330	
Intercept	0.00206	
<i>R</i> ² value	0.99985	



Table 4 Recovery of olutasidenib

—	Extracted LQC	Unextracted LQC	Extracted MQC	Unextracted MQC	Extracted HQC	Unextracted HQC
1	1.916×10^5	1.924×10^5	3.811×10^5	3.824×10^5	5.674×10^5	5.686×10^5
2	1.897×10^5	1.907×10^5	3.829×10^5	3.836×10^5	5.683×10^5	5.694×10^5
3	1.903×10^5	1.912×10^5	3.814×10^5	3.819×10^5	5.688×10^5	5.693×10^5
4	1.908×10^5	1.915×10^5	3.826×10^5	3.832×10^5	5.649×10^5	5.662×10^5
5	1.919×10^5	1.926×10^5	3.839×10^5	3.845×10^5	5.675×10^5	5.684×10^5
6	1.933×10^5	1.941×10^5	3.827×10^5	3.837×10^5	5.663×10^5	5.679×10^5
Mean	1.913×10^5	1.921×10^5	3.824×10^5	3.832×10^5	5.672×10^5	5.683×10^5
SD	0.013	0.012	0.010	0.009	0.014	0.012
% CV	0.67	0.64	0.27	0.25	0.25	0.21
% Mean recovery	99.38%	99.79%	99.32%	99.53%	98.22%	98.41%
Overall recovery	99.10%					

to 0.67%. This indicates that the recovery procedure is highly precise, with minimal variation across the replicates. By averaging the recoveries at the LQC, MQC, and HQC concentrations, the overall recovery is 99.10%. This confirms that the technique is capable of consistently and reliably quantifying analytes across different concentrations, without significant variation due to analyte-related factors or sample preparation.

Stability

Table 5 describe the short-term stability of olutasidenib in plasma after it has been exposed to 3 cycles of freezing/thawing (-30°C to ambient temp.), an autosampler, and benchtop. The medication passed the stability tests, as the percentage CV of the freeze-thaw, autosampler, and benchtop samples was less than 15%. The CV% results ranged from 0.20% to 0.77%, indicating excellent accuracy and negligible degradation. These stability tests demonstrate that the drug's concentration remains relatively constant, suggesting that it remains stable under varying conditions suitable for sample analysis and storage. Stability tests under these conditions are recommended by current regulatory guidelines, with variable degrees of accuracy. For example, stability on a benchtop for 4–24 hours and at least 3 freeze/thaw cycles are required. From a scientific perspective, however, it is of utmost importance to address the application-specific maximum storage duration and number of cycles.

Table 6 shows results from long-term stability experiments, demonstrating that the medication has strong plasma stability, as indicated by the low CV% in the observed concentrations over time. Although there is a slight drop in concentration over

the 28 days period, this is typical of drugs tested for long-term stability and suggests that the drug remains mostly unchanged during this time. This type of stability research may help better predict the drug's storage conditions and shelf life.

Pharmacokinetics and statistical analysis

The internal standard (IS) and olutasidenib were both extracted using liquid–liquid extraction (LLE). To measure olutasidenib concentrations in the plasma of SD rats, the optimized and validated LC-MS/MS technique was used. Six separate rats were dosed with the analyte, and blood samples were collected at various intervals: 0, 1, 2, 4, 8, 16-, 32-, 64-, and 128-hours post-dosing. Once the samples were prepared, they were injected into the chromatographic system, and the results were recorded according to the test method. Olutasidenib concentrations were effectively quantified into a single dosage (6 mg/200 g b.w.) orally through a BD syringe linked to an oral gavage needle (size 18). Maximum drug concentration (C_{\max}), the area under the curve (AUC_{0–128}) (24 hours trapezoid rule), time to achieve C_{\max} (T_{\max}), elimination rate constant (K_{el}) (established using a semi-log graph of the plasma level-time plot (by applying the least-squares method)), and half-life ($T_{1/2}$; calculated by quotient $0.693/K_{\text{el}}$) were the variables assessed. Table 7 provides information on pharmacokinetics. Fig. 8 depicts the average olutasidenib plasma concentration vs. time profile in rats.

A concentration of 9.765 ng mL^{-1} at 1 hour indicates rapid drug absorption into the circulation. After two hours, the concentration increases to $18.317 \text{ ng mL}^{-1}$, and after four hours, it rises further to $27.012 \text{ ng mL}^{-1}$. This shows that the drug is entering the bloodstream and continues to circulate. After 8

Table 5 Stability studies of olutasidenib

Stability	Spiked plasma conc. (ng mL^{-1})	Conc. (ng mL^{-1}) (mean \pm SD; $n = 6$)	(CV%) ($n = 6$)
Freeze-thaw	15.0	$1.924 \times 10^5 \pm 0.007$	0.37
	45.0	$5.670 \times 10^5 \pm 0.018$	0.32
Autosampler		$1.911 \times 10^5 \pm 0.015$	0.77
Benchtop		$5.665 \times 10^5 \pm 0.018$	0.32
Short term stability		$1.1916 \times 10^5 \pm 0.014$	0.73
		$5.657 \times 10^5 \pm 0.012$	0.22
		$1.881 \times 10^5 \pm 0.009$	0.20
		$5.626 \times 10^5 \pm 0.011$	0.5



Table 6 Long-term stability studies

Days	Spiked plasma conc. (ng mL ⁻¹)	Conc. (ng mL ⁻¹) (mean \pm SD; n = 6)	(CV%) (n = 6)
Day 1	15.0	$1.918 \times 10^5 \pm 0.013$	0.69
	45.0	$5.683 \times 10^5 \pm 0.012$	0.22
Day 7		$1.878 \times 10^5 \pm 0.007$	0.35
		$5.618 \times 10^5 \pm 0.011$	0.20
Day 14		$1.842 \times 10^5 \pm 0.010$	0.55
		$5.546 \times 10^5 \pm 0.009$	0.17
Day 21		$1.800 \times 10^5 \pm 0.011$	0.55
		$5.486 \times 10^5 \pm 0.009$	0.16
Day 28		$1.688 \times 10^5 \pm 0.008$	0.46
		$5.367 \times 10^5 \pm 0.011$	0.24

Table 7 Pharmacokinetic results of olutasidenib

Time intervals (hours)	Olutasidenib (ng mL ⁻¹)
1	9.765
2	18.317
4	27.012
8	22.190
16	15.210
32	7.298
64	2.379
128	0

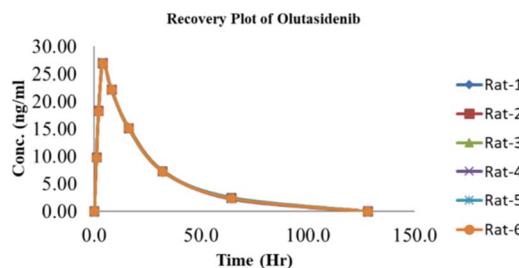


Fig. 8 Recovery plot for olutasidenib in rat plasma.

hours, the concentration begins to decrease to 22.190 ng mL⁻¹, suggesting that the medication is being metabolized or eliminated from the body. The concentration continues to fall, reaching 15.210 ng mL⁻¹ after 16 hours. It decreases further to 7.298 ng mL⁻¹ at the 32nd hour, and drops to 2.379 ng mL⁻¹ after 64 hours. The medication is almost entirely eliminated from the body by 128 hours, as the concentration reaches 0 ng mL⁻¹. Olutasidenib appears to have a half-life characterized by a slow decline in concentration until it is fully excreted from the body. When the drug's clearance rate is directly proportional to its concentration, it likely follows first-order kinetics.

Conclusion

The decision was made to employ ibrutinib as the IS. To isolate the drugs, a mobile phase consisting of 50:50 ACN and ammonium formate buffer was used, with a flow rate of 1

ml min⁻¹. The mobile phase was paired with an Inertsil ODS column measuring 150 mm \times 4.6 mm and having a 3.5 μ m particle size. The drug and the IS both exhibited proton adducts at approximately *m/z* 354.8589–239.8107 and *m/z* 441.573–372.1236–143.7695–76.7964, respectively, which could be detected *via* MRM positive modalities. The method demonstrated a linearity range of 3.00–60.00 ng mL⁻¹ with an *r*² value of 0.9996. Intraday precision and accuracy for this approach were 96.40% and 99.59%, respectively.

According to the results of the stability studies, benchtop testing, and three freeze-thaw cycles, olutasidenib remained stable. The goal of this study was to investigate the oral pharmacokinetics of olutasidenib by quantifying the drug in rat plasma using a newly developed and well-established LC-MS/MS analytical method. The pharmacokinetic (PK) properties of olutasidenib demonstrated an immediate onset of action followed by absorption. The collected data have the potential to pave the way for olutasidenib's clinical and preclinical studies while also providing benchmarks and valuable information for the continued development of new medications and pharmacology pathways.

Data availability

All pertinent data supporting the conclusions of this study are presented within the article. Certain data access is restricted due to privacy or ethical considerations.

Author contributions

All authors have made equal contributions to the research findings and writing.

Conflicts of interest

There are no conflicts to declare.

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