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Quantification of Leniolisib in Rat Plasma Using LC-MS/MS: Method Development, Validation, and Pharmacokinetic Study

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ABSTRACT:-Leniolisib, a selective phosphoinositide 3-kinase δ (PI3Kδ) inhibitor, was recently approved for the treatment of activated PI3Kδ syndrome (APDS). A robust bioanalytical method is essential for pharmacokinetic (PK) studies and therapeutic drug monitoring. This study aimed to develop and validate a sensitive, selective, and reproducible liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for quantifying leniolisib in rat plasma. The method involved protein precipitation followed by LC-MS/MS analysis using gefitinib as an internal standard. Chromatographic separation was achieved on an Inertsil ODS column (150 mm × 4.6 mm, 3.5 µm) with a mobile phase of methanol and water (50:50, 0.1% formic acid). The method was validated per FDA guidelines for selectivity, linearity, precision, accuracy, recovery, matrix effects, and stability. The assay exhibited linearity over 10–80 ng/mL ($R^2 = 0.9998$). Intra- and inter-day precision (%CV) was <1.7%, and accuracy ranged from 94.98% to 98.03%. Recovery was consistent (~97%), with no significant matrix effects. Stability studies confirmed leniolisib's robustness under various storage conditions. Pharmacokinetic analysis in rats revealed a Tmax of 2 h, Cmax of 39.366 ng/mL, and t1/2 of 8 h. The validated LC-MS/MS method is precise, accurate, and suitable for pharmacokinetic studies of leniolisib in preclinical and clinical research.Pharmacokinetics studies in rat of leniolisib demonstrated that AUC_{0-t} value is 318 ng-hr/ml while C_{max} value is 39.366 ng/ml. The successful validation of bioanalytical method demonstrated that the PK parameters could be extrapolated from rats to human for administration of leniolisib.**Keywords:** Leniolisib, LC-MS/MS, Bioanalytical method validation, Pharmacokinetics, PI3 inhibitor, and Rat plasma

Introduction

Bioanalysis is the study of analytes found in biological samples, including biomarkers, medications, and metabolites which is an integral part of the pharmacokinetic (PK)/pharmacodynamic (PD) evaluation of a novel new chemical entity [1-3]. This procedure encompasses several stages, which comprise data reporting, sample retrieval, and sample analysis. The initial stage entails the acquisition of samples derived from clinical or preclinical research [4-6]. Subsequently, the aforementioned samples are dispatched to a laboratory for the purpose of examination. The subsequent stage in bioanalysis involves sample clean-up, which is alternatively referred to as sample preparation. This step is crucial for ensuring accurate and reliable results [7-9]. To obtain accurate results, it is crucial to utilize a sample preparation method that is both robust and stable. Eliminating any impurities that may be present in the sample matrix and optimizing the performance of the analytical system are the objectives of sample preparation [10-13].

Leniolisib is a potent and selective inhibitor of phosphoinositide 3-kinase δ (PI3Kδ). On March 24, 2023, the FDA approved leniolisib as the first treatment for activated phosphoinositide 3-kinase delta syndrome (APDS). Activated PI3Kδ syndrome is classified as a primary immunodeficiency disorder. It is caused by mutations in genes that encode the PI3Kδ enzyme. These mutations lead to increased activity of PI3Kδ, which in turn causes dysfunction in the immune system[14-16]. As a result, individuals with APDS have a higher susceptibility to infections. Leniolisib is effective in inhibiting the hyperactivity of PI3Kδ. Ongoing investigations are being conducted to explore the use of leniolisib in primary Sjogren's syndrome [17, 18].

In March 2023, leniolisib received approval for medical use in the United States. This medication is the first to be approved for a treatment of activated PI3K delta syndrome. The chemical formula of leniolisib $C_{21}H_{25}F_3N_6O_2$ and its Molecular weight is 450.466 g/mole and its structure is shown in **Figure 1** [19, 20].

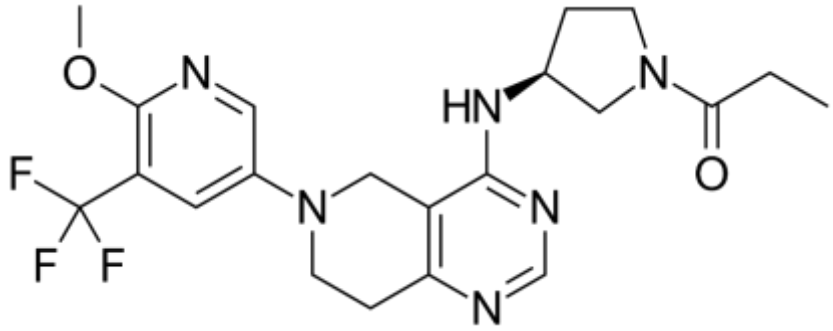

Figure 1: Structure of Leniolisib

The bioanalytical method development is a paramount step for the new molecules available to the public to develop new formulations and perform more research regarding the more appealing dosage forms. This will help with the easy availability of validated methods for the analysis of leniolisib in blood plasma. Additionally, the method can be used in human blood with slight modifications. So, this attempt has been made to develop the bioanalytical method for the leniolisib in rat plasma. The method development and validation for the analysis of leniolisib in rat plasma has been attempted using the rat model in this article. The developed method has been challenged for the validation parameter to verify the suitability of the method by using the rat plasma.

1. Materials

1.1. Chemical and Reagents

Samples of leniolisib and gefitinib were supplied as the reference material by Cipla Pharmaceuticals in Vijayawada. All compounds, including LCMS-grade acetonitrile and methanol, were procured from the chemical division of Merck located in Mumbai. Throughout the investigation, water purified using the Milli-Q water purification system and of HPLC quality was utilized.

1.2. Instrumentation

Chromatography was carried out using a Waters 2695 HPLC equipped with a high-speed autosampler, column oven, degasser, and a SCIEX QTRAP 5500 mass spectrometer with class ABSCIEX software.

1.3. Preparation of Stock and working solutions

1.3.1. Leniolisib Stock Solution (160ng/ml):

The working standard of leniolisib accurately weighed 8 mg is transferred in a volumetric flask of 100ml and diluted to volume with a diluent composed of methanol and purified in a ratio of 50:50 containing 0.1% Formic acid. Pipette out 0.2 ml of the above solution and add a diluent composed of methanol and purified in a ratio of 50:50 containing 0.1% Formic acid up to 10 ml. Also, accurately transfer the above solution of about 1 ml into a volumetric flask of 10 ml and make their marks with diluents [21-23].

1.3.2. Preparation of Gefitinib Internal Standard Stock Solution (100ng/ml)

Gefitinib working standard was weighed out in the quantity of 5mg and transferred into a 100 ml volumetric flask, then a diluent composed of methanol and purified in a ratio of 50:50 containing 0.1% Formic acid was used to bring the volume up to the mark. Furthermore, transfer 0.2ml in a 10 ml volumetric flask and the solution was diluted to 10ml with a diluent composed of methanol and purified in a ratio of 50:50 containing 0.1% Formic acid. To prepare the stock solution, accurately pipette out 1 ml from the above solution into a 10 mL volumetric flask and make up to volume with diluent [24-29].

1.3.3. Preparation of Standard Solution

A volume of 500µl of standard stock solution was transferred into a 2ml centrifuge tube. Incorporate 500µl of diluent, 200µl of plasma, and 300µl of acetonitrile into this mixture. Centrifuge for twenty minutes. The supernatant liquid is filtered and transferred to an HPLC vial[30-32].

1.4. Conditions of liquid chromatography and mass spectrometry

Analytical column employed as Inertsil ODS, 150mm x 4.6mm, 3.5µm.at ambient temperature condition was employed to secure effective separation. The methanol and purified in a ratio of 50:50 containing 0.1% Formic acid was used as the mobile phase in this study with a flow rate of 1.0 mL/min, and 10 µL was injected finally The liquid chromatography (LC) process was conducted for a duration of 5 min. In +ESI mode, the MS was operated using parameters mentioned in **Table 1** [33-35]. The determination of the mass-to-charge ratio transitions for leniolisib and gefitinib (m/z 451.2154→ 3401853 and 447.9029 →108.1534, respectively) was accomplished through the utilization of multiple reaction monitoring (MRM), as depicted in **Figure *2***.

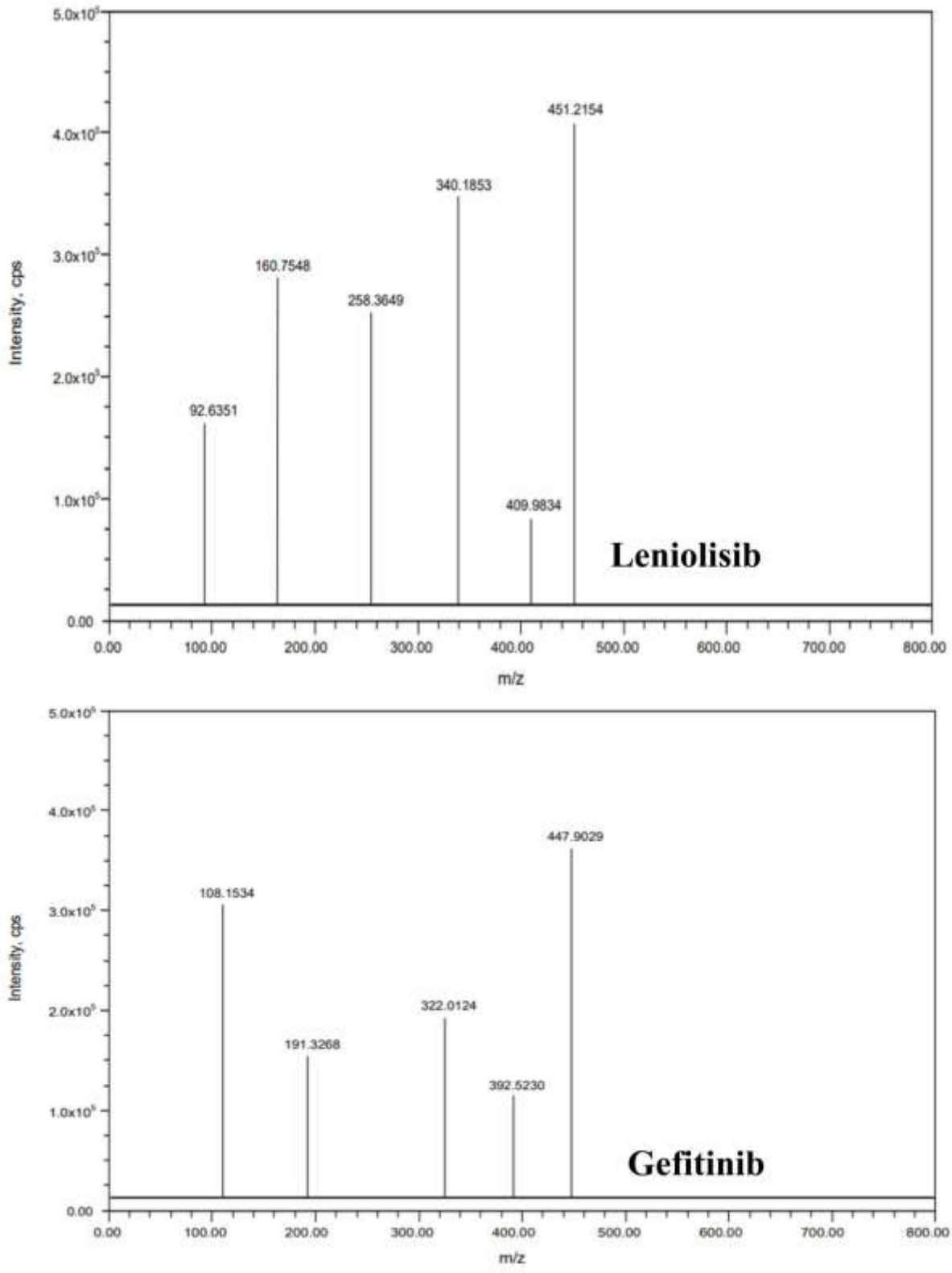

Figure 2: Mass spectrum of Leniolisib and Gefitinib

Table 1: Test Parameter for +ESI mode mass spectrometer

Drug Name	Leniolisib	Gefitinib [36-38]
Molecular Weight (g/mol)	450.5 g/mol	446.9 g/mol
Vaporizer temperature	350 °C	350 °C
Capillary temperature	350 °C	350 °C
Spray voltage positive ionization	3500 V	3500 V
Spray voltage negative ionization	2800 V	2800 V
Sheath gas	42 AU	42 AU
Sweep gas	1 AU	1 AU
Auxiliary gas	12 AU	12 AU
Retention Time	4.54	4.35
Precursor Ion (m/z)	701.3308	528.1205
Product Ion (m/z)	629.2165	383.6143
Fragmentation voltage (V)	135	170
Collision Energy (V)	17	23

1.5. Preparations of Linearity solutions

A calibration curve was performed at concentrations of 10.00, 20.00, 30.00, 40.00, 50.00, 60.00, and 80.00 ng/mL by centrifuging at 4000 RPM for 20min. The supernatant solution was collected in an HPLC glass vial and introduced into the chromatograph**.** The QC samples were prepared using the above-mentioned procedure and contained leniolisib concentrations of LLQC 4.00 ng/mL, LQC 20.00 ng/mL, MQC 40.00 ng/mL, and HQC 60.00ng/mL [39-41].

1.6. Extraction protocol

Rat plasma was subjected to liquid-liquid extraction to isolate leniolisib from the rat plasma. The 600 µL plasma samples were treated by centrifuging at 4000 RPM for 20 min and then labelled based on the appropriate time intervals. A 200 µL of supernatant plasma was diluted to 300 µL with the diluent and mixed thoroughly. Added 500 µL of acetonitrile to the above solution to precipitate all proteins and the solution was subjected to centrifuging at 4000 rpm for 15-20 minutes to obtain the resulting solution. The top layer of solution was collected and injected into the chromatograph using an HPLC-specific container [42-45].

1.7. Bio-analytical method validation

In accordance with the FDA's bioanalytical method guidelines the devised method underwent comprehensive validation by computing all validation parameters as detailed below:

1.7.1. Selectivity

Selectivity refers to the ability of an analytical procedure to accurately distinguish and measure analytes even when there are interfering substances present in the biological matrix. The six blank samples from respective independent source were used to demonstrated the selectivity and processed without indulging internal standard (IS) [46-49].

1.7.2. Linearity

The demonstrate the relationship between concentration of the analyte and response was shown on a calibration curve prepared employing the same bioanalytical mixture as the samples and utilized in differently each analyte that is to be determined. The demonstration of precision and accuracy to the respective concentration interval determines linearity [50, 51]**.**

1.7.3. Accuracy and precision

The verification of proposed method for validation performed by replicating QCs at number of aliquots of concentrations throughout the assay range. Ideally, the proposed bioanalytical method validation program advisable to indulge at minimum 6 independent runs for a proposed calibration curve and numerous analyte aliquots measured in repetitions for calculation of accuracy and precision [52, 53].

1.7.4. Recovery

Recovery determines the agreement between the amount spiked and the amount found in the sample after analysis. A set of six QC analyte preparations were brought to ambient temperature by slowly thawing or preparing a fresh from the deep freezer (LQC, MQC, and HQC). The specified amount of IS was added to the QC samples before injection into the system. A 100 % extraction of the bioanalyte was secured by making blank mixture samples that were isolated from a single aliquot. Furthermore, the 6 sets of samples were diluted low, middle, and high concentrations and then injected. Moreover, an IS was indulged in the making of samples. At each QC level and for ISTD, the percent CV of recovery should be less than 15.00 percent. For all QC levels, the complete average recovery percent CV should be less than 20.00 percent [54,55]**.**

1.7.5. Matrix effects

A matrix effect is defined by a variation in bioanalyte determination demonstrated by interfering and occasionally undetected analyte in the sample mixture. The matrix effect was measured eight times for each analyte and internal standard at LQC and HQC concentration

levels. The multiple 8 examined plasma samples were used to prepare two replicates using placebo plasma samples. Two sets individually LQC and HQC concentration were intentionally added with ISTD using blank mixtures in a separate lots. The samples were analysed using prepared employing the spiked analytes and reconstituted using ISTD to form a solution and obtain batch of aqueous samples that could be used for comparision to final LQC and HQC concentrations [56-58].

1.7.6. Stability experiments

Stability tests are paramount to make sure that the amount of bioanalyte remains in its original concentration till performing all the process involved such as sample preparation, processing, bioanalysis, and storage conditions. The stability determination of the bioanalyte within the proposed mixture during research activities is performed by employing low to high designated concentrations. The defined storage temperature and appropriate conditions were implemented at time zero and measure aliquots of low and high stability. It is imperative to conduct and assess a minimum of 3 set of stability tests for each proposed individual concentration, proposed storage condition, and defined set of time point. The change in the biological matrix in lieu of any reason makes the chromatographic techniques more challenging as per FDA guidance [59-62].

The parameters listed above can make the development and validation processes much easier to implement. The approach is not acceptable for its intended purpose if selectivity cannot be demonstrated. It will be difficult to develop the approach if recovery is uneven and the analytes are fractionated after being adjusted. Accuracy, precision, range, and other qualities would most certainly be considerably altered under such circumstances.

1.8. Application of bio analytical method to pharmacokinetics study

To conduct the pharmacokinetic experiments, a group of six male Wistar rats having same age and weighing between 180 and 220 g was utilised. The animals were accommodated in well-ventilated enclosures, and they were adequately provided with food and water for a duration of seven days prior to the initiation of the experiments. The rats were fasted overnight before being given a dose. A pharmacokinetic study was conducted on leniolisib in a group of six rats [63-65]. The animal study protocol has been approved by the Institute of Animal Ethics Committee (Registration Number: 1250/PO/RcBi/s/18/CPCSEA). Rats were given a single dose of leniolisib tablet powder (0.83 mg/kg). The samples were taken at the following times

after the dosing event: 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, and 10 hours. At each pre-defined time point, 5 ml of blood volume was withdrawn in K2 EDTA vacutainer vials as an aliquot. Subsequently, a pre-dose samples were collected to verify interference of plasma. The withdrawn samples were subjected to centrifugation to collect the plasma, and the obtained plasm were stored at a temperature of -70 °C. The spiked plasma samples were analysed alongside quality control (QC) samples at four different concentrations. The software application WinNonlin (Version 5.2) was used to determine the pharmacokinetic parameters of leniolisib [63-70].

2. Results and Discussion

2.1. Bio-analytical Method Validation

2.1.1. Selectivity

When comparing the peak response of blank samples with the response of spiked LLOQ samples containing IS mixtures, it was shown that the selectivity of the method demonstrated the absence of interference with leniolisib of both the analyte in **Figure 3**. The method can effectively and selectively differentiate between the blank and internal standard.

2.1.2. Linearity

The linearity of the standard curves was observed in the concentration range of 10.00-80.00 ng/ml leniolisib and the calibration curve is represented in **Figure *4*.** The observed average correlation coefficient was 0.9998. The determination of sample quantity was accomplished through the computation of the ratio between the peak areas of the analyte and the internal standard (IS). The peak area ratios were graphically shown concerning the plasma concentrations. The linearity with the developed method is excellent which exhibits that the method can effectively determine the concentration of the leniolisib.

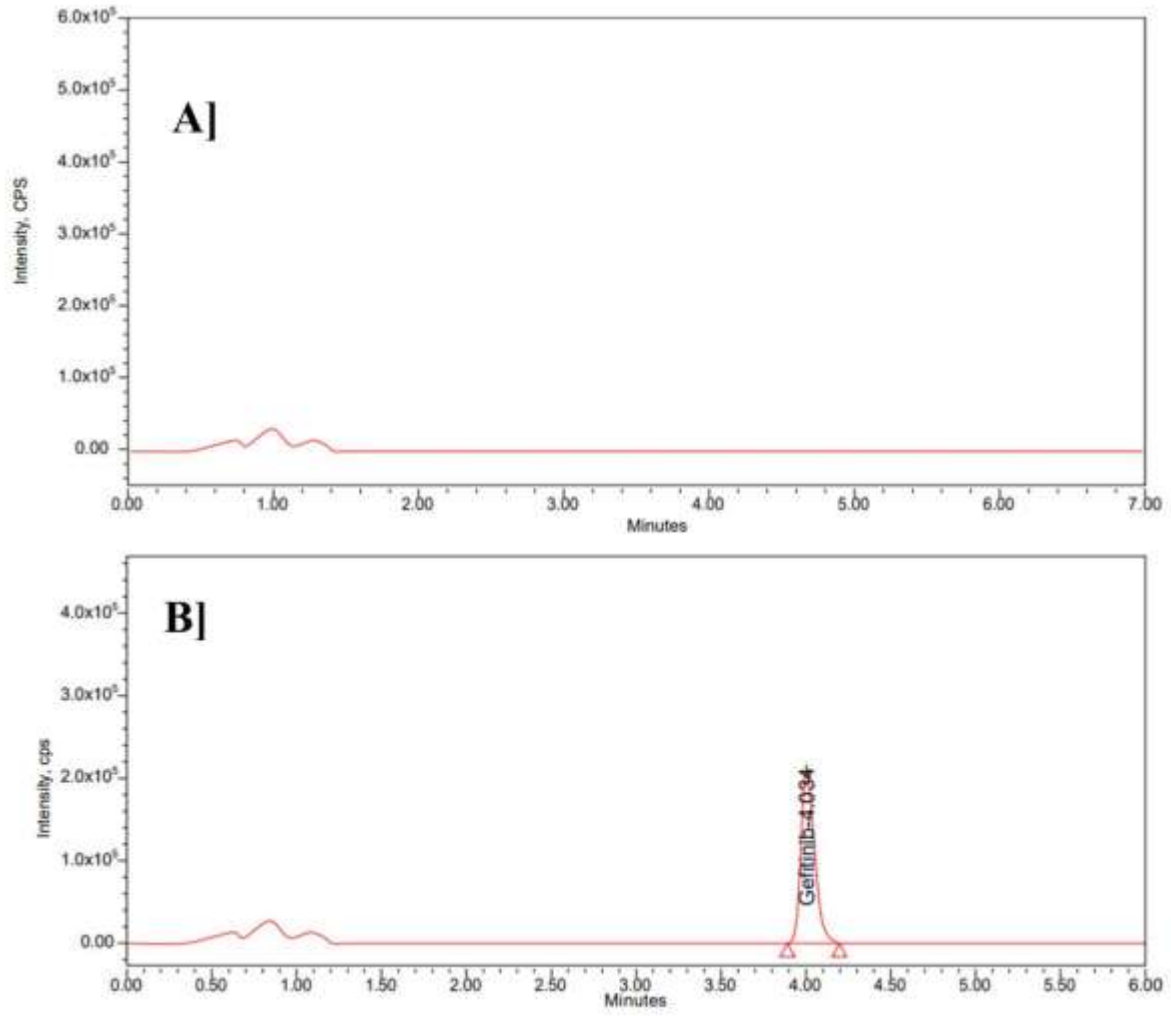

Figure 3: Chromatogram of A] Blank Samples; B]Blank Samples + IS

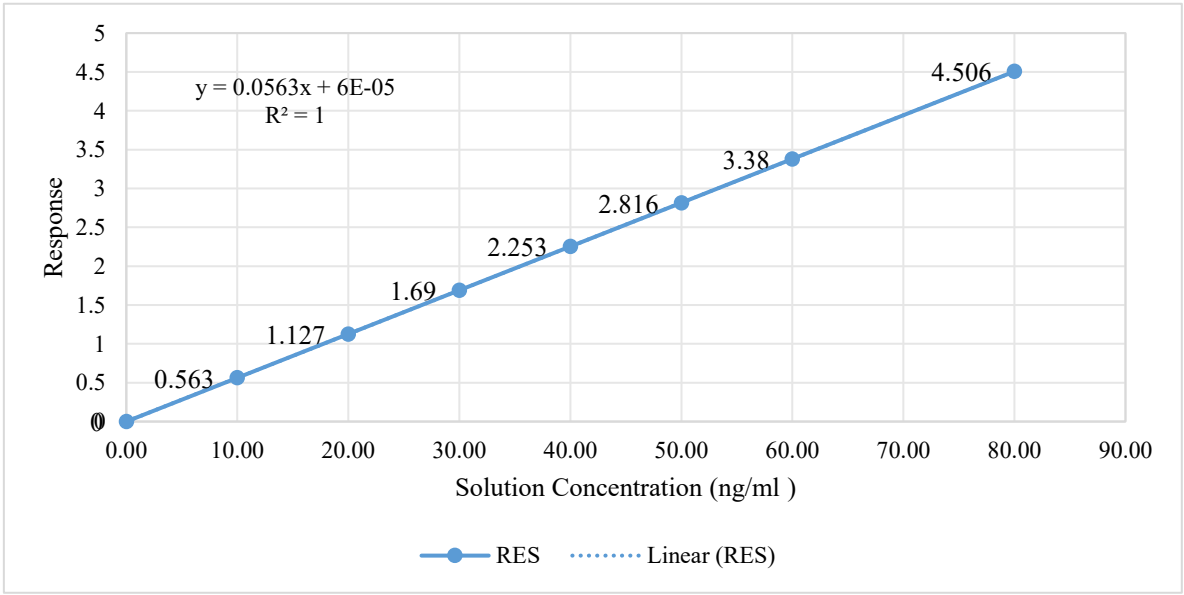

Figure 4: Calibration plot for concentration v/s Area ratio of Leniolisib

2.1.3. Precision and accuracy

To verify the intra-assay precision and accuracy, a total of 6 replicates comprising leniolisib were determined for analyte at 3 different QC levels. Analysis of 3 different concentrations of QC samples by using independent runs measured for inter-assay precision. The demonstrated method's percent average accuracy ranged from 94.98 percent to 98.03 percent, and the precision (percent CV) for low, medium, and high quality control concentrations was 0.06 to 0.73 percent. All the findings have been summarised in **Table 2**. The precision and accuracy results exhibit that the method can determine the concentration effectively by using the specified parameters.

Table 2: Calculated concentrations obtained for precision and accuracy batches

Injections	HQC	MQC	LQC	LLQC
1	3.313x105	2.187x105	1.088x105	0.214x105
2	3.309x105	2.182x105	1.091x105	0.219x105
3	3.315x105	2.189x105	1.087x105	0.216x105
4	$3.317x10^5$	$2.185x10^5$	$1.089x10^5$	$0.211x10^5$
5	$3.312x10^5$	$2.191x10^5$	$1.085x10^5$	$0.209x10^5$
6	$3.310x10^5$	$2.188x10^5$	$1.090x10^5$	$0.212x10^5$
Mean	$3.313x10^5$	$2.187x10^5$	$1.088x10^5$	$0.214x10^5$
SD	0.00301	0.0032	0.00216	0.0036
% CV	0.09	0.14	0.20	1.70
%Mean Accuracy	98.03%	97.07%	96.58%	94.98%

2.1.4. Recovery

It inferred that leniolisib's average recovery rate was 97.15 percent and the mean internal standard recovery at defined concentration employed was 97.89 percent. **Table 3** and **Table 4** represent the findings, which demonstrated that the extraction efficiency for leniolisib was good, constant, and concentration-independent when employing the liquid-liquid extraction technique. The recovery of the leniolisib with the developed method is acceptable for the method development and validation.

Table 3: Recovery of Leniolisib

	Extracted LQC	Un extracted LQC	Extracted MQC	Un extracted MQC	Extracted HQC	Un extracted HQC
Mean	$1.082x10^5$	$1.093x10^5$	$2.189x10^5$	$2.207x10^5$	$3.281x10^5$	$3.312x10^5$
SD	0.00319	0.00286	0.00306	0.00187	0.00301	0.00327
%CV	0.29	0.26	0.14	0.08	0.09	0.10
%Mean Recovery	96.05%	97.03%	97.16%	97.96%	97.09%	98.00%
Overall Recovery	97.33					

Table 4: Internal standard Results of Gefitinib (25 ng/ml)

Sr. No.	Extracted Area Ratio	Un Extracted Area Ratio
1	$2.095x10^5$	$2.111x10^5$
2	$2.091x10^5$	$2.109x10^5$
3	$2.098x10^5$	$2.113x10^5$
4	$2.096x10^5$	$2.115x10^5$
5	$2.094x10^5$	$2.108x10^5$
6	$2.097x10^5$	$2.112x10^5$
Mean	$2.095x10^5$	$2.111x10^5$
SD	0.00248	0.00258
% CV	0.12	0.12
%Mean Recovery	97.31%	98.05%

2.1.5. Matrix Effect

Back determined concentrations of high and low quality control concentrations have an average percent accuracy of 97.11 and 96.05 percent, respectively as tabulated in **Table 5**. It could be interpreted that the demonstrated method did not exhibit any ionization effects as obtained results met the pre-defined acceptance criterion of 85.00-115.00 percent.

Table 5: Matrix effect results of Leniolisib (HQC-60ng/ml, LQC-20ng/ml)

Mean	$3.282x10^5$	$1.082x10^5$
SD	0.00471	0.00536

%CV	0.14	0.50
% Mean Accuracy	97.11%	96.05%
No. of QC Failed	0	0

2.1.6. Stability

Leniolisib stability in plasma was assessed employing six duplicates of quality control (QC) samples at concentrations of high and low. To generate drug-free plasma samples, leniolisib standard concentration solutions were mixed in the defined ratio's to achieve pre-defined volumes. The results tabulated in **Table 6** demonstrate that the approvable stability as the results are acceptable range for leniolisib. The stability at benchtop for 8 hours, freeze-thaw conditions, wet extract for 18 hours, dry extract for 18 hours, autosampler at 15°C for 12 hours, short for 24 hours, and long term for 28 days is stable which exhibits that the sample can be stored for the analysis without loosing the integrity of the sample.

Table 6: Leniolisib QC sample stability results using LC-MS/MS

Stability Parameters	Spiked concentration	Mean ±SD	%RSD	% Accuracy
Bench Top (8 hours)	25(ng/ml) 75(ng/ml)	$1.082x10^5 \pm 0.00232$ $3.311x10^5 \pm 0.00237$	0.21 0.07	96.05 97.97
Freeze-Thaw		$1.083x10^5 \pm 0.00283$ $3.312\ x10^5 \pm 0.00216$	0.26 0.07	96.14 98.00
Wet extract (18 hours)		$1.085x10^5 \pm 0.00286$ $3.281\ x10^5 \pm 0.00258$	0.26 0.08	96.32 97.09
Dry Extract (18 Hours)		$1.082x10^5 \pm 0.00351$ $3.293x10^5 \pm 0.00286$	0.32 0.09	96.05 97.44
Autosampler (15°C for 12 hours)		$1.093x10^5 \pm 0.00679$ $3.278x10^5 \pm 0.00635$	0.62 0.19	97.03 97.00
Short Term (24 hours)		$1.028x10^5 \pm 0.00351$ $3.139x10^5 \pm 0.00301$	0.34 0.10	91.26 92.88
Long Term (28 Days)		$0.941x10^5 \pm 0.0264$ $2.839x10^5 \pm 0.00258$	0.28 0.09	83.53 84.01

2.2. Pharmacokinetic application

The pharmacokinetic properties of leniolisib were determined by calculation. The peak plasma concentration (C_{max}) and time to reach peak plasma concentration (T_{max}) values were extracted directly from the plasma time profile curve shown in **Figure 5**. The pharmacokinetic characteristics of leniolisib were determined using the WinNonlin software tool (Version 5.2).

The determination of the stability of the research samples was conducted using incurred sample reanalysis (ISR). The linear trapezoidal method was used to estimate several pharmacokinetic parameters such as AUC_{0-t}, $AUC_{0-\infty}$, $t_{1/2}$, C_{max}, T_{max}, and $T_{1/2}$ and the values found are 318 ng-hr/ml, 39.366 ng/ml, 318 ng-hr/ml, 2.0 Hrs, and 8.0 Hrs. $T_{1/2}$ of leniolisib is 8 hours in studied rat plasma concentration and as per the clinical trials study in the human, the $T_{1/2}$ is 7 hours which is very much comparable, so it can be concluded that the rat plasma studies for leniolisib is comparable in human subjects.

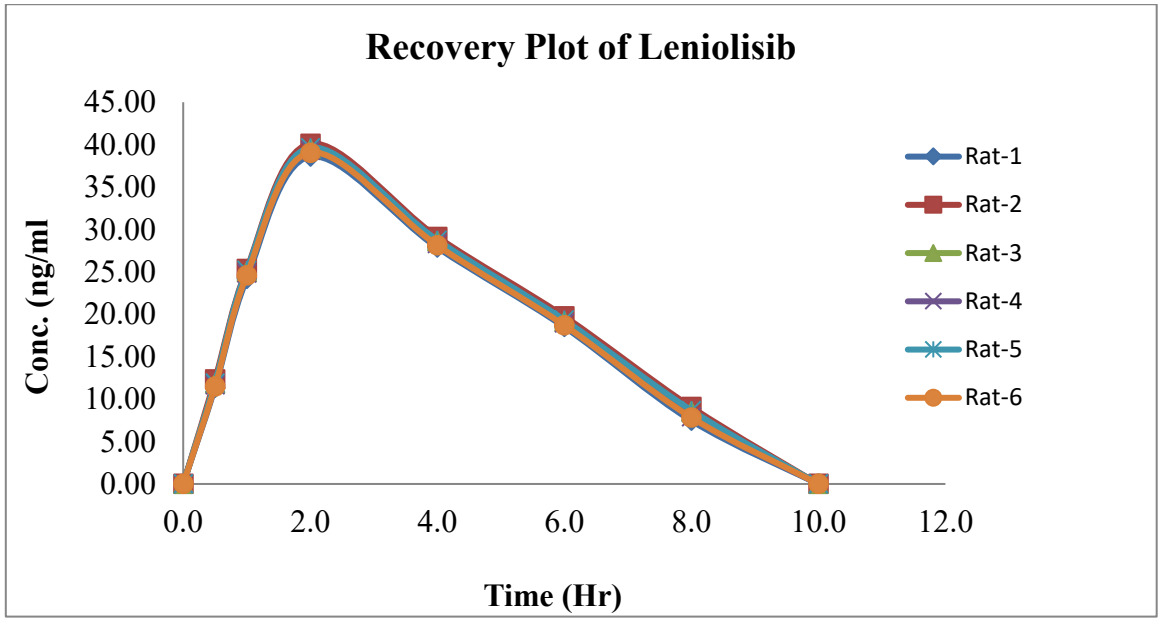

Figure 5: Mean plasma concentration–time profile Leniolisib in Rat plasma

Discussion

The developed LC-MS/MS method demonstrated excellent selectivity, with no interference from endogenous plasma components, ensuring reliable quantification of leniolisib. The linearity (10–80 ng/mL) and high correlation coefficient (R^2 = 0.9998) indicate the method's suitability for PK studies. Precision and accuracy results met regulatory criteria, with %CV <1.7% and accuracy within 95–98%. Recovery studies confirmed efficient extraction (~97%), while matrix effect assessments ruled out ionization suppression/enhancement. Stability studies under various conditions (bench-top, freeze-thaw, autosampler) support the method's robustness for routine analysis.

Pharmacokinetic findings in rats align with human clinical data (t1/2 ~7–8 h), suggesting translational relevance. The method's applicability was further confirmed by successful incurred sample reanalysis (ISR), reinforcing its reliability for preclinical and clinical studies.

Conclusion

A rapid, sensitive, and precise LC-MS/MS method for the quantification of leniolisib in rat plasma has been successfully developed and validated in accordance with USFDA guidelines. The method demonstrated excellent linearity ($r^2 = 0.9999$) over the concentration range of 10.00–80.00 ng/mL, with a short chromatographic run time of 7.00 minutes. The use of gefitinib as an internal standard ensured high accuracy, while intra- and inter-batch precision (%CV) remained within the acceptable limit of <15% across all quality control levels. A simple and cost-effective liquid-liquid extraction (LLE) technique was employed for sample preparation, offering superior efficiency compared to previous methods. The validated method complies with FDA bioanalytical guidelines and is suitable for pharmacokinetic profiling, supporting future clinical and preclinical investigations of leniolisib. The comparable PK parameters between rats and humans highlight the method's potential for translational research in APDS and other PI3Kδ-related disorders.

CRediT authorship contribution statement

Krishna Phani: Conceptualization, Resource, Methodology, Formal analysis, Data curation, Writing – review & editing, Validation. **Om Shelke:** Conceptualization, Methodology, Formal analysis, Data curation, Writing – review & editing, Validation. **Neetu Shorgar:** Conceptualization, Formal analysis, Data curation, Data Validation, Writing – review & editing,.

Declaration of competing interest

There are no conflicts of interests.

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