RESEARCH ARTICLE

Molnupiravir Bioanalytical Method Development and Validation in Rat Plasma by LC-MS/MS Detection and Application to a Pharmacokinetic Study

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ABSTRACT

This work aims to propose a novel method for detecting the concentration of molnupiravir in rat plasma that has been developed and verified. This approach makes use of liquid chromatography-tandem mass spectrometry (LC-MS/MS) technology. Furthermore, the method's pharmacokinetic applicability in rats was assessed. Phenyl, 250 x 4.6 mm, 5 µm analytical column, operating at room temperature, was utilized to accomplish separation. The investigation employed a mobile phase consisting of a 40:60 v/v combination of 0.1% tri fluoro acetic acid and methanol at a 1.0 mL/min flow rate and an injection volume of 10 µL. The liquid chromatography (LC) procedure was carried out for four minutes. In +ESI mode, the mass spectrometer was in operation. The determination of the mass-to-charge ratio transitions for molnupiravir and D7-Molnupiravir (m/z 330.34→82.46 and 337.46→286.11, respectively) was accomplished through the utilization of multiple reaction monitoring (MRM. The concentration ranges for molnupiravir were determined to be 6.25 to 50.00 ng/mL, with a correlation coefficient 0.9996. The precision and accuracy of HQC, MQC, LQC, and LLQC were found to be 98.34, 98.83, 98.13, and 97.85%, respectively. In pharmacokinetic studies, it was observed that molnupiravir exhibited an average AUC_{0-t} value of 65 ng-hr/mL and a C_{max} value of 43.120 ng/mL in rats. In conclusion, the validated created approach has effectively demonstrated the determination of pharmacokinetic parameters subsequent to the oral administration of molnupiravir in wistar rats.

Keywords: D7-Molnupiravir, Development and validation, LC-MS/MS, Molnupiravir, Rat plasma, USFDA.

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INTRODUCTION

Bioanalysis is the study of analytes found in biological samples, including biomarkers, medications, and metabolites. This procedure encompasses several stages: data reporting, sample retrieval, and sample analysis. The initial stage entails the acquisition of samples derived from clinical or preclinical research. 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. 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.[1-9]

Molnupiravir, commercially known as lagevrio, is an antiviral pharmaceutical agent that inhibits viral replication by impeding the synthesis of viral progeny.[10-12] A prodrug of the artificial nucleoside derivative N4-hydroxycytidine 4, molnupiravir.[13] It works against viruses by causing mutations when viral RNA copies itself.[14] The chemical formula of molnupiravir $C_{13}H_{19}N_3O_7$ and its molecular weight is 329.309 $g{\cdot}mol^{-1}$ and its structure is shown in Figure 1.

MATERIALS AND METHODS

Reagents and Chemicals

Molnupiravir and D7-molnupiravir reference materials were provided as samples by Cipla Pharmaceuticals in Vijayawada. All of the chemicals, including methanol and acetonitrile of LCMS quality, were acquired from Merck's Mumbai chemical division.

Molnupiravir Stock Solution

First, to create the solution, weigh 5 mg of molnupiravir working standard and add it to a 100 mL volumetric flask.

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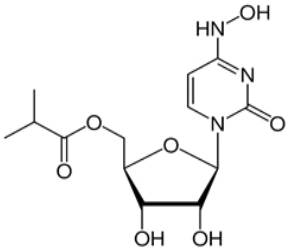

Figure 1: Molnupiravir structure

Please use a diluent to dilute the solution up to the marked level. To prepare the solution, add diluent to 0.4 mL of the solution until the final volume reaches 10 mL. To summarize, carefully transfer 1-mL of the solution into a 10 mL volumetric vial. Next, add diluent to the mark on the vial.

Preparation of D_7-Molnupiravir (IS) stock solution

A 100 mL volumetric vial containing a diluent at volume was filled with 5 mg of the D_7-molnupiravir (IS) working standard, which was weighed. Diluent was used to further dilution by 0.4 to 10 mL. Fill a volumetric flask with a capacity of 10 mL up to the designated mark by employing 1-mL of the aforementioned solution as the diluent**.**

Conditions of liquid chromatography and mass spectrometry

Phenyl, 250 x 4.6 mm, 5 µm analytical column, operating at room temperature, was utilized to accomplish separation. The investigation employed a mobile phase consisting of a 40:60 v/v combination of 0.1% tri fluoro acetic acid and methanol at a 1.0 mL/min flow rate and an injection volume of 10 µL. The liquid chromatography (LC) procedure was carried out for four minutes. In +ESI mode, the mass spectrometer was in operation. The determination of the mass-to-charge ratio transitions for molnupiravir and D_7-molnupiravir (m/z 330.34→82.46 and 337.46→286.11, respectively) was accomplished through the utilization of multiple reaction monitoring (MRM), as depicted in Figures 2 and 3.

Preparations of linearity solutions

At concentrations of 6.25, 12.50, 18.75, 25.00, 31.25, 37.50, and 50.00 ng/mL, standards for calibration curves were produced and centrifuged at 4000 rpm for 15 to 20 minutes. The supernatant solution was gathered and introduced into the chromatograph using an HPLC container**.** Molnupiravir concentrations of 1.25 ng/mL for LLoQQC, 12.50 ng/mL for LQC, 25.00 ng/mL for MQC, and 37.50 ng/mL for HQC were included in the QC samples that were made according to the aforementioned method.

Bio-analytical Method Validation

Selectivity

We use blank samples from at least six independent sources/ lots to determine selectivity. These blank samples are processed without including an analyte or internal standard (IS).

Linearity

Preparing a calibration curve using the same biological matrix as the samples is necessary. Additionally, separate calibration

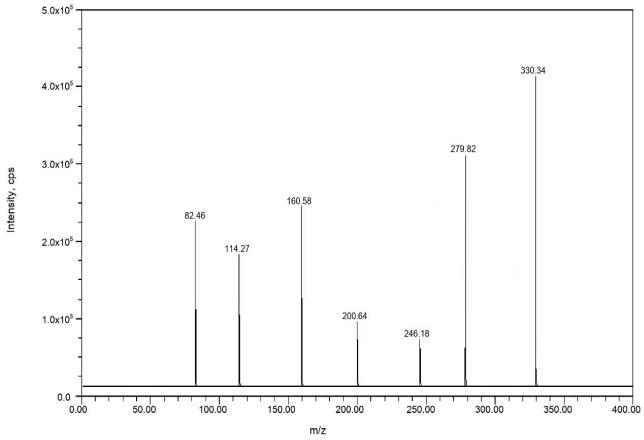

Figure 2: Mass spectrum of molnupiravir

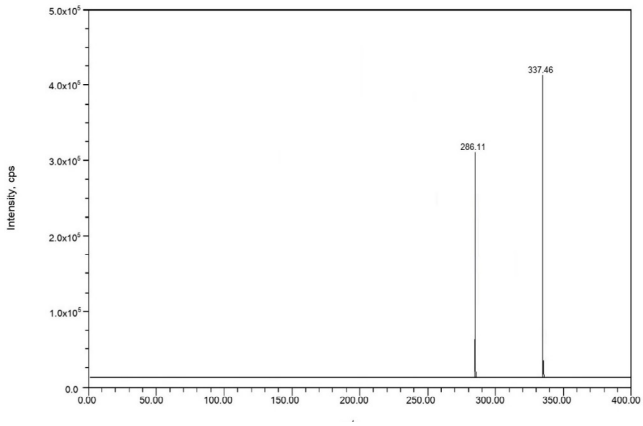

Figure 3: Mass spectrum of D_7-molnupiravir

curves are needed for each analyte that will be measured. The range of a method refers to the concentration interval within which accuracy, precision, and linearity have been demonstrated**.**

Accuracy and precision

During method development, verifying whether the technique is suitable for validation by studying replicate QCs at numerous concentrations across the assay range is crucial, and this comprises examining replicate QCs at different concentrations across the assay range. Method validation studies should include at least six independent runs, each with a calibration curve and several sample concentrations measured in repeats, in order to calculate accuracy and precision.

Recovery

Six sets of quality control samples were thawed or made fresh from the deep freezer. The internal standard was added to the quality control samples (extracted samples) before they were injected. A 100% extraction of the analyte was achieved by processing blank matrix samples screened from a single lot. These samples were then injected with six sets of each quality control dilution at low, middle, and high concentrations. Additionally, an internal standard was included in the process. At each QC level and for ISTD, the percent CV of recovery

should be less than 15.00%. The total mean recovery %CV should be less than 20.00% for all QC levels.

Matrix effects

A variation in analyte reactivity induced by intervening and sometimes undetectable components in the sample matrix is known as the matrix effect. Eight different screened plasma batches were used to make two replicates of blank plasma samples. The LQC concentration was spiked with ISTD using one set of eight independent blank matrices, whereas the HQC concentration was spiked with ISTD using another set. The analysis was carried out using spiked analyte(s) and ISTD to reconstitution solution to obtain one set of aqueous samples that were comparable to final LQC and HQC concentrations.

Stability experiments

Stability tests are essential to ensure that the concentration of the analyte remains unchanged throughout sample preparation, processing, analysis, and storage conditions. The evaluation of the analyte's stability within the matrix under investigation by utilizing quality controls for stability, including low and high concentrations. Once the storage conditions have been implemented at time zero, we proceed to analyze aliquots of the quality controls with low and high stability. Conducting and assessing at least three stability tests for every concentration level, storage condition, and time point is imperative. The Food and Drug Administration (FDA) has advised the following stability measures for biological investigations. Changing the analyte in any way can impact chromatographic behavior, making the method development process more complex.

Application of bio-analytical method to a pharmacokinetics study

A cohort of six male wistar rats weighing between 180 and 220 g was utilized to conduct the pharmacokinetic experiments. For seven days before to the start of the studies, the animals were housed in ventilated cages that were appropriately supplied with food and water. Before administering the dosage, the rats were starved overnight. A pharmacokinetic investigation was carried out on molnupiravir using a cohort of six rats. The Institute of Animal Ethics Committee has authorized the animal study protocol, with the registered number 1074/PO/Re/S/05/CPCSEA. One dosage of 200 mg/kg molnupiravir tablet powder was administered to rats. Samples were taken 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4.0 hours after dose.

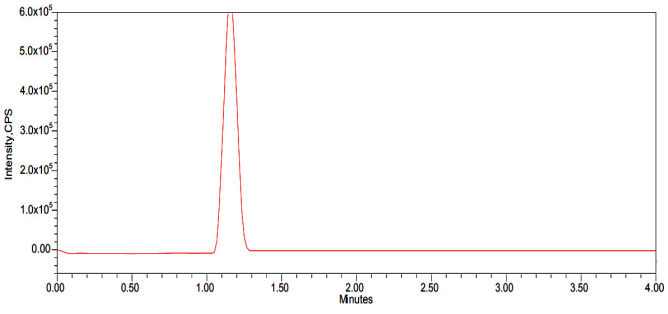

Figure 4: Chromatogram of blank

At each time point, 5 mL of blood was collected as an aliquot in K2 EDTA vacutainer containers. Furthermore, a predose sample was obtained in order to assess the potential for plasma interference. The collected samples were centrifuged to obtain plasma, which was subsequently stored at a temperature of -70°C. Four concentrations of spiked plasma and quality control (QC) samples were analyzed. Molnupiravir pharmacokinetics were determined using WinNonlin (Version 5.2).

RESULTS AND DISCUSSION

Selectivity

Compare the peak response of blank samples to spiked LLoQ samples containing IS mixtures to illustrate the selectivity of the procedure and the lack of analyte and IS interference at molnupiravir (Figure 4).

Linearity

The standard curves 'linearity was observed within the molnupiravir concentration range of 6.25 to 50.00 ng/mL. The average correlation coefficient that was observed was 0.9996. The ratio between the analyte's peak regions and the internal standard (IS) was computed in order to determine the sample amount. Table 1 visually displays the peak area ratios in proportion to the plasma concentrations (Figure 5).

Precision and Accuracy

In order to assess the intra-assay precision and accuracy, a total of six duplicates containing molnupiravir were subjected to analysis at three distinct quality control (QC) levels. Analyzing the three levels of QC samples on independent runs determined the inter-assay precision. The suggested method's %mean accuracy varied from 97.85 to 98.83%, and the precision (%CV) for LQC, MQC, and HQC was 0.33 to 2.16%. Table 2 summarises the findings

Recovery

It inferred that molnupiravir average recovery rate was 98.32% and the average IS recovery at the concentration used was 98.12%. The results showed that the extraction efficiency for molnupiravir using the liquid-liquid extraction method was satisfactory, consistent, and concentration-independent are shown in Table 3

Table 1: Molnupiravir linearity results

Final conc. in ng/mL	*Molnupiravir peak area*
0	0
6.25	0.594
12.50	1.157
18.75	1.759
25.00	2.349
31.25	2.968
37.50	3.447
50.00	4.639
Slope	0.0395
Intercept	0.00866

Table 2: Calculated concentrations obtained for precision and accuracy batches

Injections	*HQC*	*MQC*	*LQC*	*LLQC*
1	$3.182x10^5$	$2.128x10^5$	$1.385x10^5$	$0.275x10^5$
2	$3.175x10^5$	$2.754x10^5$	$1.398x10^5$	$0.284x10^5$
3	$3.156x10^5$	$2.783x10^5$	$1.384x10^5$	$0.278x10^5$
4	$3.189x10^5$	$2.784x10^5$	$1.373x10^5$	$0.267x10^5$
5	$3.191x10^5$	$2.828x10^5$	$1.401x10^5$	$0.277x10^5$
6	$3.164x10^5$	$2.824x10^5$	$1.394x10^5$	$0.282x10^5$
Mean	$3.176x10^5$	$2.798x10^5$	$1.389x10^5$	$0.277x10^5$
SD	0.01396	0. 02559	0.01046	0.00598
%CV	0.33	0.91	0.75	2.16
%Mean Accuracy (%)	98.34	98.83	98.13	97.85

Table 3: Recovery of molnupiravir

	Extracted LQC	*Un extracted LQC*	*Extracted MQC*	*Un extracted MQC*	*Extracted HQC*	*Un extracted HQC*
Mean	$1.378x10^5$	$1.394x10^5$	$2.777x10^5$	$2.798x10^5$	$3.178x10^5$	$3.195x10^5$
SD	0.01186	0.01113	0.01356	0.01346	0.01126	0.01204
%CV	0.86	0.80	0.49	0.48	0.27	0.29
%Mean Recovery	97.35	98.48	98.09	98.83	98.39	98.79
Overall Recovery	98.32					

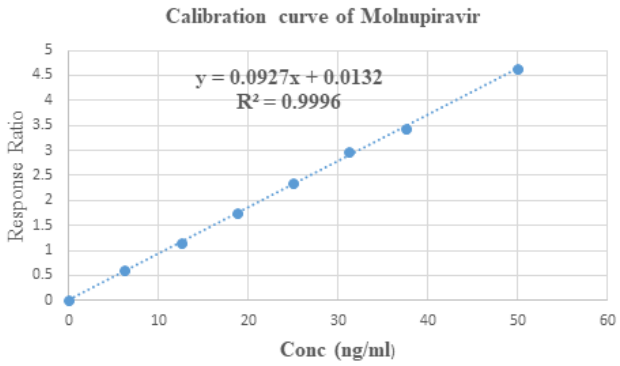

Figure 5: Molnupiravir concentration-area ratio calibration plot

Table 4: Molnupiravir QC sample stability results using LC-MS/MS

Stability	*Spiked plasma Conc. (ng/mL)*	*Mean response ± SD*	*RSD (%) (n = 6)*
Bench top stability	12.50 (ng/mL)	$1.155 x10^5 \pm 0.004$	0.86
	37.50 (ng/mL)	$3.453 x10^5 \pm 0.007$	0.20
Auto sampler stability	12.50 (ng/mL)	$1.153 x10^5 \pm 0.005$	1.6
	37.50 (ng/mL)	$3.457 x10^5 \pm 0.004$	1.39
Short term	12.50 (ng/mL)	$1.151 x10^5 \pm 0.003$	0.16
	37.50 (ng/mL)	$3.451 x10^5 \pm 0.005$	0.14
Long-term (64 days)	12.50 (ng/mL)	$1.555 x10^5 \pm 0.008$	0.43
	37.50 (ng/mL)	$3.455 x10^5 \pm 0.005$	0.20

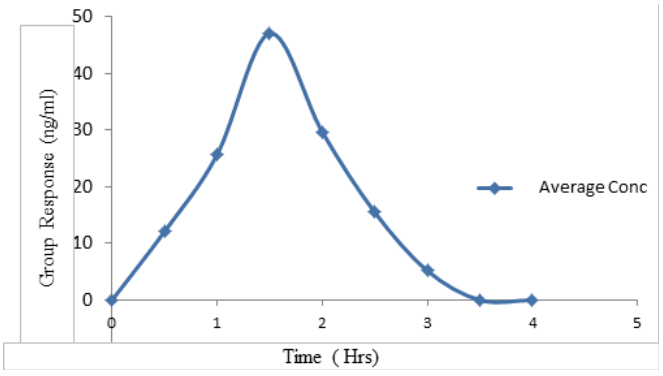

Figure 6: Rat plasma mean plasma concentration–time profile of molnupiravir

Matrix Effect

Back estimated concentrations of HQC and LQC levels had a mean %accuracy of 98.86 and 98.76%, respectively. Because the results met the acceptance criterion of 85.00 to 115.00%, the present approach did not demonstrate any ionization effects.

Stability

To assess the stability of molnupiravir in plasma, we conducted an evaluation using six replicates of quality control samples at both low and high concentrations. Molnupiravir standard solutions were added in the appropriate volumes to generate drug-free plasma samples. The findings depicted in Table 4 were determined to be within the acceptable range, suggesting that molnupiravir exhibits favorable stability.

Pharmacokinetic Application

WinNonlin (Version 5.2) identified molnupiravir pharmacokinetics. Research sample stability was assessed utilizing incurred sample reanalysis. The linear trapezoidal approach was used to estimate pharmacokinetic parameters. Table 5 and Figure 6 show the aggregated estimations.

Table 5: Pharmacokinetic parameters of Molnupiravir

Pharmacokinetic parameters	*Molnupiravir*
AUC_{0-t}	65 ng -hr/mL
C_{max}	43.12 ng/mL
$AUC_{0-\infty}$	65 ng-hr/mL
t_{max}	1.2 hour
$T_{1/2}$	3.0 hour

CONCLUSION

In LC-MS/MS, a technique for correctly and precisely identifying molnupiravir has been established. This approach is also sensitive and quick to analyze. In this procedure, D7-molnupiravir is employed as an internal standard. The total runtime for chromatography is 4.00 minutes, with the retention time for molnupiravir being 2.336 minutes and for D7-molnupiravir being 2.375 minutes. A dynamic linear range of 6.25 to 50 ng/mL has been verified for molnupiravir. The correlation coefficient (r2) is 0.9996. The new bioanalytical approach has been verified to comply with USFDA requirements. The validation parameters were all confirmed to be within the permitted range. The method successfully quantified molnupiravir in pharmacokinetics studies involving male wistar rats. Per the guidelines, the precision (%CV) for intra-batch and inter-batch measurements should be less than 15% across three levels: LQC, MQC, and HQC. Stability studies were conducted on the method, and the results consistently fell within the assay variability limits at every stage of the process. The pharmacokinetic study of molnupiravir in rat plasma was successfully conducted using this optimized method. In addition, a simpler, more efficient, and less expensive liquid-liquid extraction method was developed for pre-treating biological samples, surpassing the effectiveness of previously reported methods. Plasma concentrations of molnupiravir were measured, and the primary pharmacokinetic parameters were determined.

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