Original Article

Develop and validate a highly sensitive method for the estimation of Molnupiravir in rat plasma by high-performance liquid chromatography-tandem mass spectroscopy and its application to pharmacokinetic studies

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Abstract

Utilizing D6-Molnupiravir as a reference standard, a simple, rapid, accurate, effective, and repeatable LC-MS/MS approach was devised for the quantitative measurement of molnupiravir (MNR). The paper reviewed the latest developments in bioanalytical LC-MS/MS techniques employing a 150 x 4.6 mm, 3.5 column used for isocratic elution at ambient temperature. Methanol (MeOH) and acetonitrile (ACN) were employed as the mobile phase at a ratio of 60:40 with a flow rate of 1.0 ml/min. The insertion rate was 10 µl with a runtime of 4 minutes. MNR's retention time was 2.026, and the overall runtime for the separation process was 4.0 min. For MNR, the technique has been tested above a dynamically specified range of 12.50–100 ng/mL with a regression coefficient of 0.999. Outcomes for accuracy, recovery, stability, precision, matrix effect and recovery were reported to fall within acceptable limits. A quick and effective approach for observing the examined sample in bodily fluids was created in pharmacokinetic investigations. The approach indicates that every system suitability parameter, including accuracy, specificity, and linearity, is successfully utilized for pharmacokinetics research in rats and is in excellent accordance with USFDA requirements.

Keywords: Molnupiravir using D6-Molnupiravir LC-MS/MS, USFDA guidelines, Rat plasma.

INTRODUCTION

The term "coronavirus" refers to the shape of a sun's core (corona) observed through an electron microscope. A broad group of RNA viruses called coronaviruses commonly causes mild to severe upper airway infections. The severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), as well as the 3rd coronavirus, SARS-CoV-2, which is the source of the worldwide outbreak of coronavirus disease 2019 (COVID-19), were all first discovered in China in December 2019.1 Phase 3 clinical studies are currently being conducted with the antiviral drug candidate molnupiravir (MNR) for the management of COVID-19.2 The human esterases in the bloodstream transform the isopropyl ester prodrug MNR, into the bioactive ribonucleoside derivative β-D-N4-hydroxycytidine (NHC) also called EIDD-1931.3 A range of positive- as well as negative- RNA viruses can be inhibited by NHC's antiviral properties. The latest studies have focused on the synthesis of MNR, which is used to combat COVID-19 and influenza viruses.4 A pyrimidine ribonucleoside analog known as MNR has the chemical name ((2R, 3S, 4R, 5R) -3,4-dihydroxy-5-(4-(hydroxyamino)-2-oxopyrimidin1-(2H)-yl)-tetrahydrofuran-2-yl) methyl isobutyrate.5

According to a review of the literature, two analytical techniques for the drug MNR have been noted. These techniques are LCMS/MS for measuring NHC in plasma samples and NHC-triphosphate in blood mononuclear (MNC) cell homogenates,6 and an advanced LC-MS/MS technique for concurrent measurement of MNR and its breakdown product, NHC in plasma and spit samples.7 In light of this, the goal of the current study is to establish and validate a very accurate technique for determining the concentration of MNR in rat plasma using high-performance liquid chromatography-mass spectrometry (HPLC-MS) and apply it for pharmacokinetic investigations.

MATERIALS AND METHODS

Chemicals and reagents

Molnupiravir samples from Bangalore's Biocon were made available as the reference sample. All remaining compounds, including LCMS grade ACN and MeOH, were purchased from Merck Chemical Division, Mumbai. During the experiment, HPLC grade water procured from the Milli-Q water purification system was utilized.

Equipment

Apparatus included an HPLC system (Waters Alliance e2695 model) coupled with a triple quadrupole mass spectrometer (QTRAP 5500; Sciex). In addition, a software operation called Empower 2.0 was carried out.

Pharmacokinetic study

Selection of animals

In vivo pharmacokinetic studies, six healthy white New Zealand rabbits (2.0-2.5 kg) were obtained from Biological E Limited, Hyderabad, India. The institute approved the protocol of the animal study of the animal ethics committee (Reg. No: CPCSEA/IAEC/JLS16/07/21/45)

Chromatographic conditions

The chromatographic technique was carried out in isocratic elution at ambient temperature employing x-bridge phenyl (150 x 4.6 mm, 3.5 micron) columns. MeOH and ACN were used as the mobile phase in a ratio of 60:40, flowing at a rate of 1.0 ml/min. The injecting rate was 10 µl with a runtime of 4 minutes.

Preparation of standard and internal control samples

Preparation of standard stock solution

10 mg of MNR working standard was weighed and placed in a 50 ml volumetric flask, followed by dilution using a dilutant. Finally, 0.1ml to 100ml of diluent was added for further diluting.

Preparation of Internal Standard Stock Solution

10 mg of D6-MNR working standard was weighed and placed in a 50 ml volumetric flask, followed by dilution using a dilutant. Finally, 0.1ml to 100ml of diluent was added for further diluting.

Preparation of Standard Solution

500 µl of the standard solution was poured into a centrifuge tube. 200 µl of plasma, 500 µl of internal standard, 500 µl of ACN, and 300 µl of dilutant should be added to this. It was centrifuged for 20 minutes. The resulting solution was collected, filtered using a 0.45-µ nylon filter membrane, and then transferred to a vial before injecting it into the system.

Bioanalytical Method validation

The procedure was tested for selectivity, sensitivity, accuracy, linearity, and precision, as well as for matrix condition, recovery analysis, re-injection repeatability, and sustainability.

Selectivity

Selectivity was examined by assessing the six separate rat plasma samples and checking for interference at the retention time.

Matrix effect

To determine the matrix effect, compare the height-area ratios of the six different plasma samples with no drug. Six separate plasma samples were used in triplicate tests at MQC levels with a sufficient precision of ≤ 15%.

Precision and accuracy

This was demonstrated by the replication assessment of internal control samples at the low-quality control (LQC), medium-quality control (MQC), and high-quality control (HQC) categories. Except for the case of LLOQ, the accuracy must be under 15%, and the CV's half must be below 15%.

Recovery

After separating the MNR, six samples are reproduced and analyzed at every internal control concentration. Finally, recovery is assessed by comparing height areas of extracted standards with unextracted ones.

Carryover

Carryover relates to the analyte held by the separation technique throughout the matrix with a sample concentration of ULOQC

and beyond the dilution of this sample with a blank matrix

Dilution integrity

The validity of the dilution must be demonstrated by spiking the matrix with a sample concentration more than the ULOQC and dilution this sample with a blank matrix.

Stability

By contrasting the data from the processing of the new stock sample with data from the stability testing under the operation of stock solution stability. Experiments on sample stability in plasma were carried out using six repetitions for every LQC and HQC concentration level. According to US FDA rules, an analysis was deemed consistent when the variation was below 15%. For 24 hours, the accuracy and stability of spiking rat plasma kept at ambient temperature were assessed using an autosampler. By contrasting extract plasma samples that were infused right away with samples that were pumped back after being stored with wet extract at 2-8 °C and dry extract (-20° ± 3 °C) at ambient temperature at 12 h and 18 h at 2-8 °C, the autosampler stability and re-injection stability were assessed, respectively. By contrasting stability samples that were newly spiked control samples with stability samples that were frozen at-31 °C and defrosted three times, the freeze-thaw stability was determined. At 7 °C, the short-term stability experiment lasted seven days. The amounts acquired following 24 h were contrasted with the original amount for the assessment of long-term stability.

Pharmacokinetic study

Each participant received a single oral dose of the 50 mg/vial MNR tablet, and their plasma samples were taken at intervals of 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4.0 hours after the administration. K2 EDTA vacutainer tubes were used to obtain a fraction of 5 ml plasma at each interval. A sample was also taken before administration to examine any potential plasma intrusions. With four varying concentrations, plasma samples were spiked using the IS and analyzed alongside QC samples.

The findings were used to compute pharmacokinetic properties, including AUC, Cmax, Tmax, the interval during which Cmax occurs, t12, Ka, and MRT. Data were recorded from the beginning of the AUC curve to the end using the trapezoidal rule approach. The graph was used to determine Cmax and Tmax. Every value is represented as mean ± SD.

RESULTS AND DISCUSSION

Specificity

The technique's specificity for studying MNR was demonstrated. The standard and blank plasma samples' chromatograms were devoid of interference peaks, as depicted in figures 1 and 2.

Matrix Effect

For MNR LCMS/MS, the % RSD of ion inhibition or intensification within the signal was reported to be 1.0 percent, indicating that under such conditions, the matrix influence on analyte ionizing is within an appropriate limit of ionization. MNR's LQC and HQC in the matrix effect were 100.52 and 100.81, respectively. MNR's CV at the LQC level was 0.35, while at the HQC level, it was 0.14. It shows that the matrix influence on the analyte ionizing is within the acceptable range.

Linearity

Over the MNR's range of concentrations of 12.5-200 ng/mL, the calibration curves were reported to be linear. It was 0.999 for the average coefficient of correlation. The ratio of the analyte peak area: IS peak area was used to quantify samples. Peak area fractions across plasma concentrations were displayed. Table 1 below contains the linearity findings for Molnupiravir, and Figure 4 displays their calibration curves.

Precision and accuracy

Accuracy and precision were determined by adding each of the separate test findings from the various internal control samples. The data presented made it clear that the technique was accurate and efficient. Table 2 displays the Molnupiravir findings for precision and accuracy.

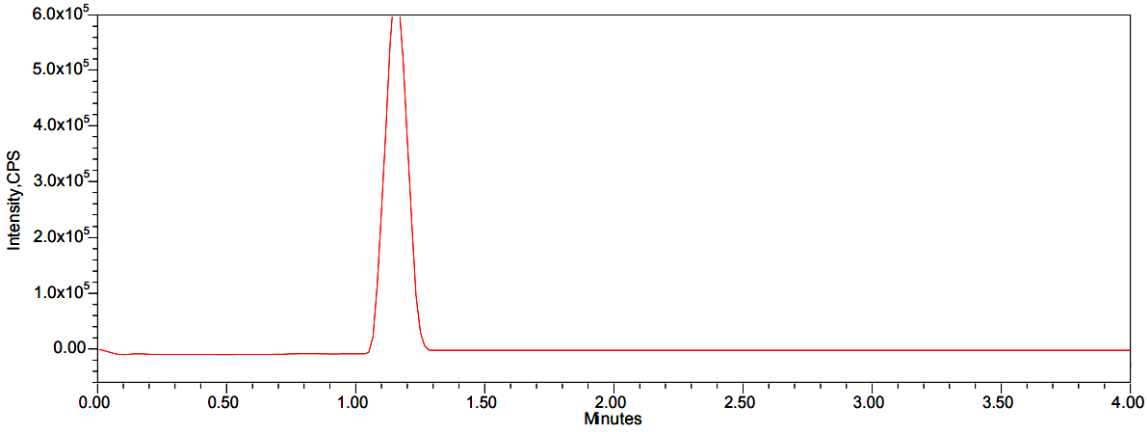

Fig-1 Chromatogram of Blank

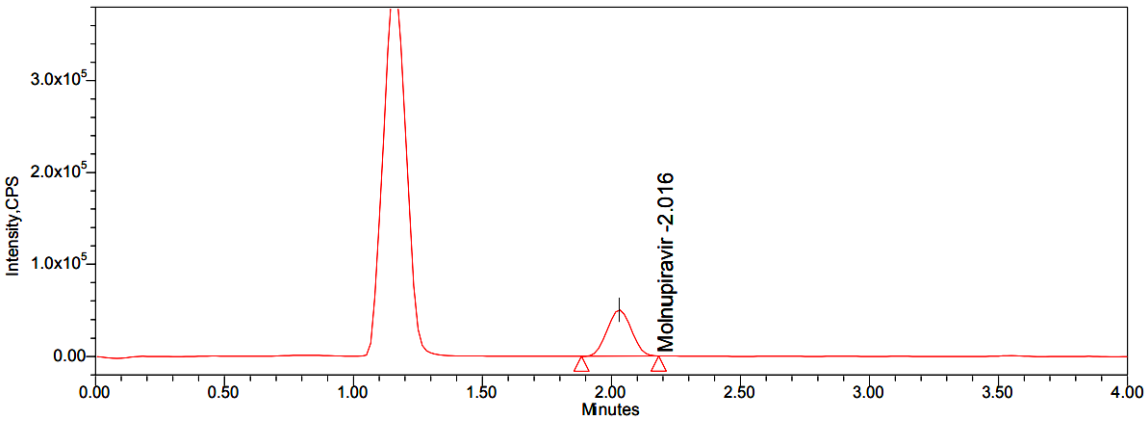

Fig-2 Chromatogram of standard

Table no: 1 Linearity Results of Molnupiravir

Final conc. In ng/ml	**RES**	**Area response ratio**
0	0	0.0
12.50	0.751	0.248
25.00	1.564	0.518
37.50	2.254	0.746
50.00	3.027	1.003
62.50	3.816	1.263
75.00	4.537	1.499
100.00	6.075	2.017
Slope	0.0201	
Intercept	0.00040	
R^2 Value	0.9999	

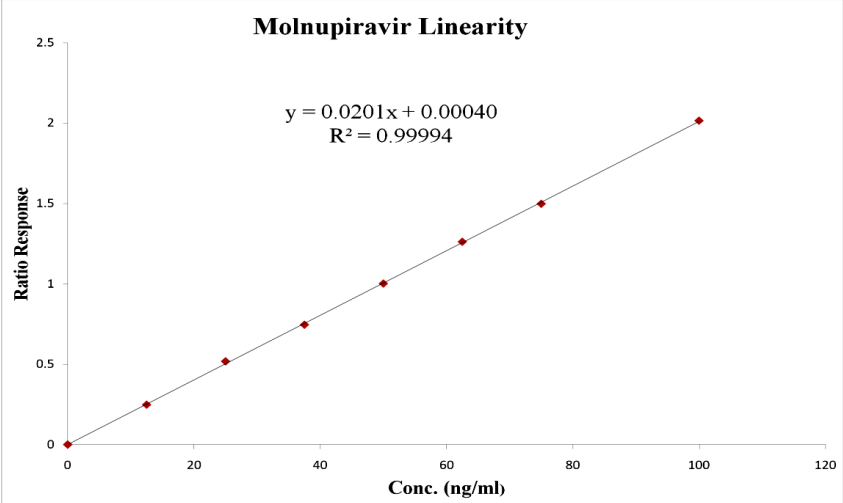

Fig-3 Calibration plot for concentration v/s Area ratio of Molnupiravir

Table No: 2 Precision and accuracy Results of Molnupiravir

Acquisition Batch ID	Date	HQC	MQC	LQC	LLQC
		Nominal Concentration (ng/ml)			
		75.0	50.0	25.0	5.0
		Analyte peak area			
		$4.611x10^5$	$3.027x10^5$	$1.525x10^5$	$0.302x10^5$
		$4.608x10^5$	$3.034x10^5$	$1.515x10^5$	$0.301x10^5$
		$4.615x10^5$	$3.045x10^5$	$1.521x10^5$	$0.296x10^5$
		$4.598x10^5$	$3.059x10^5$	$1.518x10^5$	$0.289x10^5$
		$4.592x10^5$	$3.042x10^5$	$1.511x10^5$	$0.305x10^5$
		$4.559x10^5$	$3.051x10^5$	$1.527x10^5$	$0.302x10^5$
n		6	6	6	6
Mean		$4.597x10^5$	$3.043x10^5$	$1.520x10^5$	$0.299x10^5$
SD		0.021	0.012	0.006	0.006
% CV		0.45	0.38	0.40	1.93
% Mean Accuracy		100.35%	99.64%	99.54%	97.90%

Recovery

The findings showed that MNR was recovered at LQC, MQC, and HQC levels, proving that the analytical approach had a significant extraction efficiency. The %CV varied between 0.10 and 0.50 at LQC, MQC, and HQC levels, with recoveries for MNR of around 99.25%-100.94%. The outcomes showed that the analytical approach had an effective extraction rate.

Ruggedness

In HQC, LQC, MQC, and LLQC samples, the percentages of MNR recovery and CV assessed by two distinct analyzers and using two distinct columns met the required standard. Outcomes showed that the approach is rugged. For MNR, the percentage recoveries varied between 99.48 and 100.81%. and %CV varied between 0.07 and 0.47. Findings demonstrate that the methodology is robust.

Autosampler carryover

Peak area response of MNR was not found in blank plasma samples following consecutive infusions of LLQC and ULQC at MNR retention periods. Therefore, this approach doesn't display autosampler carryover in terms of sampling.

Stability

For a solution's stability determination, MNR solutions were made with dilutants and stored at a temperature between 2 and 8 °C. Stock solutions that had been made just a day previously were compared to fresh stock solutions. For 24 hours, particularly 24hrs at 20 °C inside the autosampler, the integrity of the benchtop and the auto sampler was constant in plasma. Predictive stability showed that MNR was stable for as long as 24 hours at a temperature range of –30 °C. Table 3 below lists the findings of Molnupiravir's overall stability.

Table No:3 Stability results of Molnupiravir

Stability experiment spiked plasma	**Concentration**	**Analyte peak area**	**% cv**
Benchtop Stability	LOQ-25.0	1.525 x10^5	0.007
	MQC-50.0	3.033 x10^5	0.014
	HQC-75.0	4.625 x10^5	0.013
Freeze-Thaw stability	LOQ-25.0	1.506 x10^5	0.026
	MQC-50.0	3.015 x10^5	0.024
	HQC-75.0	4.496x10^5	0.09
Short term stability	LOQ-25.0	1.372 x10^5	1.43
	MQC-50.0	2.774 x10^5	1.37
	HQC-75.0	4.181x10^5	0.47

Application to Pharmacokinetic Studies:

Data is shown together with the computation of Cmax, Tmax, T 1/2, AUC 0-t, and AUC 0-∞. 6 distinct rats received injections of the MNR solution, and plasma samples were taken at various time points including 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4.0 hrs. Collected samples were then prepared per the test procedure, inserted into the HPLC apparatus, and recorded readings.

Molnupiravir's pharmacokinetics were investigated in rats (n = 6). The local Independent Ethics Committee gave its endorsement to the procedure. Each participant received a single oral dose of the 50 mg/vial MNR tablet, and their plasma samples were taken at intervals of 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4.0 hours after the administration. K2 EDTA vacutainer tubes were used to obtain a fraction of 5 ml plasma at each interval. A sample was also taken before administration to examine any potential plasma intrusions. With four varying concentrations, plasma samples were spiked using the IS and analyzed alongside QC samples. WinNonlin (Version 5.2) software was used to compute the pharmacokinetic parameters of MNR. Table 4 displays results for pharmacokinetic parameters, and Figure 4 displays recovery plots.

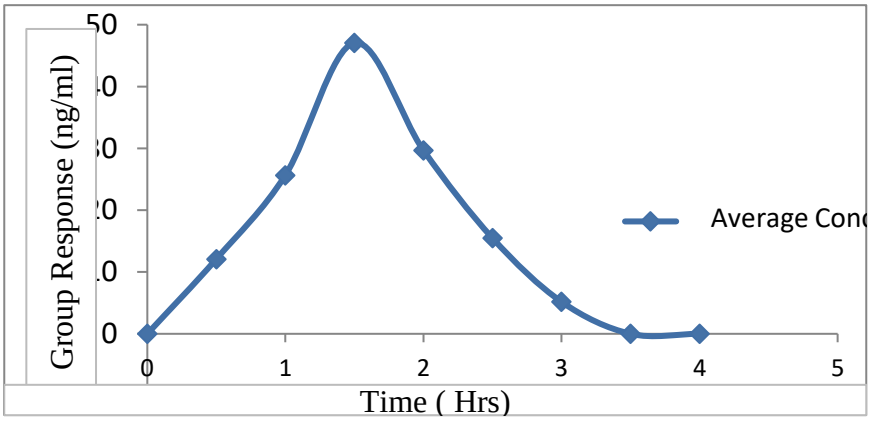

Figure 4: Recovery plot of Molnupiravir

Table no 4: Pharmacokinetic parameters of Molnupiravir

Pharmacokinetic parameters	**Molnupiravir**
AUC_{0-t}	68ng -hr/ml
C_{max}	47.1ng/ml
$AUC_{0-\infty}$	68ng-hr/ml
t_{max}	1.5 Hour
$T_{1/2}$	3.0 Hours

SUMMARY AND CONCLUSION

The purpose of this study was to provide a practical, inexpensive, robust, and reliable technique for quantifying molnupiravir in LCMS utilizing D6-molnupiravir as an internal standard. Compared to other work publications, this one has a shorter run time. MNR's retention time was 2.026, and the overall runtime for the separation process was 4.0 min. For MNR, the technique has been tested above a dynamically specified range of 12.50–100 ng/mL with a regression coefficient of 0.999. Over five categories (LLOQ, LQC, MQC, HQC, and ULOQ), the intra-batch and inter-batch precision (%CV) were below 15%. Per USFDA regulations, this could be validated.

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