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BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF MOLNUPIRAVIR IN HUMAN PLASMA BY RP-HPLC

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ABSTRACT

A simple, accurate, isocratic stability indicating RP-HPLC in human plasma method was developed for the determination of Molnupiravir in bulk drug. This RP-HPLC was achieved on "Waters 2695 using an Phenomenex C18 (250mm×4.6mm, 5µm)" column with the mobile phase consisting of Acetonitrile and 0.01 Potassium Dihydrogen phosphate in the ratio of 65:35 %v/v. Quantification of Molnupiravir was based on measuring the peak area at 240 nm. Molnupiravir peak eluted at retention time 3.19 ±0.02 min. The developed HPLC procedure was carefully validated in terms of system suitability, linearity and range, precision, accuracy, specificity, robustness, detection and quantification limits. The suggested approach was validated according to ICH (International Council on Harmonisation) principles, and all of the validation parameters' findings were within acceptable limits.

Keywords: Human plasma, Molnupiravir, ICH, Phenomenex

INTRODUCTION

Molnupiravir is chemically described as ((2*R*,3*S*, 4*R*,5*R*)-3,4-dihydroxy-5-(4-(hydroxyimino)- 2-oxo- 3,4-dihydropyrimidin-1(2*H*)-yl) tetrahydrofuran-2- yl) methyl isobutyrate and its empirical formula is C13H19N3O7, its molecular weight is 329.31 **(Figure 1)**.

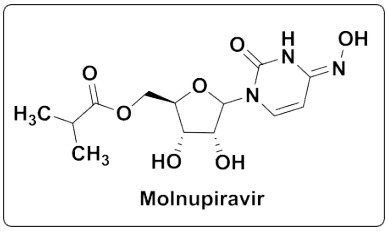

Figure 1: Molnupiravir structure

Molnupiravir is an antiviral medicine used to treat COVID-19, and it has shown good antiviral effectiveness against SARS-CoV-2 in mice, resulting in reduced virus transmission **[1]**. Molnupiravir prevent the SARS-CoV-2 transmission in ferrets within 24 hr **[2]**. When comparatively remdesivir, it has structurally simple, no considerable complicity in manufacturing process and orally available in the market. Now clinical trials are going on related to safety of the drug **[3-5]**. As part of release, all the drugs must be analysed with a stability indicating assay method in current GMP practice. Based on the stress results we can estimate and identified the degradation products in drug substance and this can help to establishment of the degradation trend and the drug substance stability. The performance of the force degradation testing will be depending on the type of drug product and the individual drug substance.

In this view the analysis of molnupiravir done in under variety of conditions, develop the LC method for separation the drug substance from the degradation products formed during forced degradation studies under ICH suggested conditions (thermal stress, hydrolysis, photolysis, and oxidation) **[6- 11]**.

These stress study experiments to provide good information about drug substance stability and which can help to establishment of method validation of drug substance. A literature reveals that one method was reported for the determination of molnupiravir in biological fluids and marketed formulations. The earlier literature reported that to developed liquid chromatographic method for estimation and separation of molnupiravir from process impurities and degradation impurities formed during stress studies.

Any one not studied retention time of the method, in this present method the analysis of molnupiravir was achieved before 15 min. So, the aim of present work was to develop economic, selective, simple and stability-indicating liquid chromatographic method that can be used to determine the assay of molnupiravir and its related substances in bulk drug sample. This work separated the degradation impurities from drug substance generated from stress studies.

MATERIALS

Analytically pure Molnupiravir was received as gift sample from Hetero Labs, Hyderabad, India. HPLC grade Methanol was purchased from Merck, India.

Analytical reagent grade ammonium phosphate monobasic was purchased from Sigma Aldrich, India. High pure water was prepared by using Millipore Milli Q plus purification system

Instrumentation

To develop a liquid chromatographic

method for the determination of Molnupiravir using a Waters HPLC alliance 2695 PDA detector, with an automated sample injector. The output signal was monitored and integrated using Empower 2 software. The chromatographic analysis was carried out in an isocratic mode using Phenomenex C18 reverse phase column (250mm×4.6mm, 5µm). The detection of the compounds was monitored at 240 nm. The details of the instruments employed in the study are as follows:

2.3.1 Preparation of solutions

a) Preparation of Molnupiravir stock solution (0.1 mg/mL)

Weighed about 10mg of Molnupiravir working standard and transferred into 100 mL volumetric flask, dissolved in Acetonitrile and then volume was made up to the mark with a further quantity of diluent. The stock solution was sonicated for 5 min, and the concentration obtained was 0.1 mg/mL.

Preparation of internal standard solution (100µg/mL)

Weighed about 10 mg of Hydrochlorothiazide in 100 mL volumetric flask, dissolved in Acetonitrile and then volume was made up to the mark with a further quantity of diluent. The stock solution was sonicated for 5 min, and the concentration obtained was 1 µg/mL.

Preparation of 0.01N Potassium dihydrogen orthophosphate

Accurately weighed 1.36 gm of Potassium dihydrogen Orthophosphate in a 1000 mL volumetric flask and 900 mL of milli-Q water was added and sonicated. Finally, the volume was made up to the volume with water and then pH was adjusted to 3.0 with dil. ortho phosphoric acid.

Preparation of Molnupiravir spiking solutions

The calibration curve dilutions were prepared from Molnupiravir stock solution in the concentration range of 0.05µg/mL to 2 µg/mL using Acetonitrile: Water as diluents. These dilutions (CC spiking solutions) were subsequently used for spiking the blank plasma.

2.3.3. Methodology Selection of wavelength

An UV spectrum of 10µg/mL Molnupiravir in Acetonitrile: Water was recorded by scanning in the range of 200-400 nm. From the UV spectrum, wavelength of 240 nm was selected, as the drug showed good absorbance at this wavelength.

Selection of Chromatographic method

Selection of Chromatographic method depends on the nature of sample properties like ionic/ionizable /neutral

character, its molecular weight and solubility. The drug selected for the study was polar in nature. Hence reverse phase HPLC or ion- pair or exchange chromatography must be used. The RP-HPLC method was selected for the initial separations because of its simplicity and suitability.

Selection of internal standard

Based upon the polarity and solubility hydrochlorothiazide was selected and chromatographed along with the standard drug. The elution time of Hydrochlorothiazide was 2.28 min. The peak of hydrochlorothiazide was symmetric and well resolved from the peak of the Molnupiravir. Hence, for the present study hydrochlorothiazide was selected as the internal standard

Extraction process of Plasma samples

250µl of plasma, 500µl of internal standard and 250µl of Molnupiravir from the spiked solutions were transferred to a set of pre-labeled polypropylene tubes containing 1.5 mL of Acetonitrile. The tubes were vortexes for 2 min and finally centrifuged for 5 min at 3200 rpm speed. After the centrifugation the organic layer is collected and 20 µl was directly injected into HPLC.

RESULTS AND DISCUSSION

2.3.4. Analytical Method Development of Molnupiravir by RP-HPLC Method in Human plasma

2.3.5. Optimization of the method

The optimization of the method development trails are summarized in **Table 2.1** and **Figures 2.2-2.5**. Optimized chromatographic conditions were given in **Table 2.2**.

Table 2.1: Optimization of RP-HPLC method

Trail No.	Column	Mobile phase composition	Injection volume	λmax (nm)	Flow rate (mL/min)	Observation
1	Phenomenex C18 (250×4.6mm,5µ)	nitrile:Na_2HPO_4 (65:35)	20µl	240nm	1.0mL/min	eak shape was not good.
2	Phenomenex C18 (250×4.6mm,5µ)	etonitrile: OPA (70:30)	20µl	240nm	1.0mL/min	ow plate count was observed.
3	Agilent C18 (250×4.6mm,5µ)	etonitrile: OPA (60:40)	50µl	240nm	1.0mL/min	ow plate count was observed.
4	Phenomenex C18 (250×4.6mm,5µ)	nitrile: KH_2PO_4 (65:35)	20µl	240nm	1.0mL/min	Good peak shape, tailing and resolution were observed.

2.3.5.1 Determination of λmax for Molnupiravir

Isobestic point (λmax) of Molnupiravir was found to be at 240 nm. It indicates that detection at 240nm would be the most sensitive wavelength for HPLC work. This λmax was selected for HPLC method development of Molnupiravir.

Table 2.2: Optimized chromatographic conditions

Parameters	Chromatographic conditions
Column	Phenomenex C18 (250mm×4.6mm, 5µm)
Mobile phase	Acetonitrile :0.01 N KH_2Po_4 Buffer (65:35)
Flow rate	1mL/min
Run time	10 min
Injection volume	20 µl
Detection wavelength	240 nm
Column temperature	30 °C
Diluent	Acetonitrile: Water (50:50)

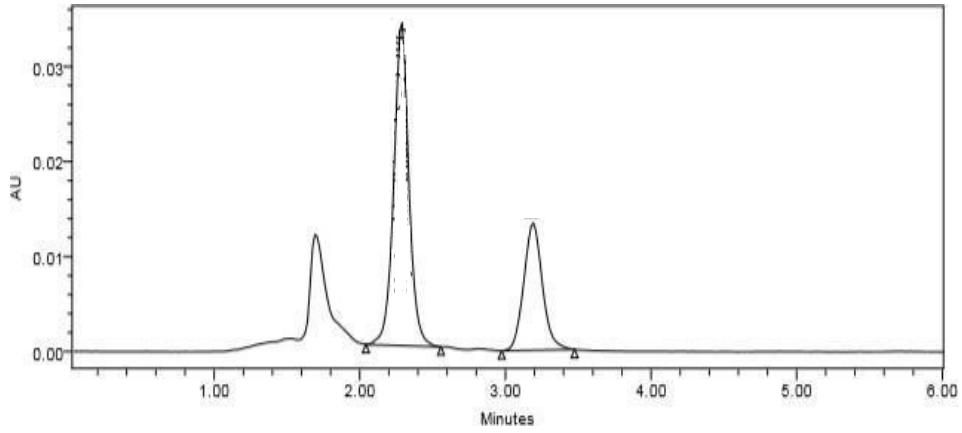

Figure 2.6: Typical optimized chromatogram

Table 2.3: System suitability parameters

S. No.	Name	Retention time	Area	USP Resolution	USP Tailing	USP Plate count
1.	Hydrochlorothiazide	2.20	61503	-	1.03	2421
2.	Molnupiravir	3.189	33328	4.2	1.10	2984

2.3.5 Method validation for bio analytical studies of Molnupiravir

The method was validated to meet the acceptance criteria of industrial guidance for the bioanalytical method validation.

2.3.5.1 Specificity and Selectivity

No interfering peaks were found in six different random blank human plasma samples at the retention times of either Molnupiravir or ISTD.

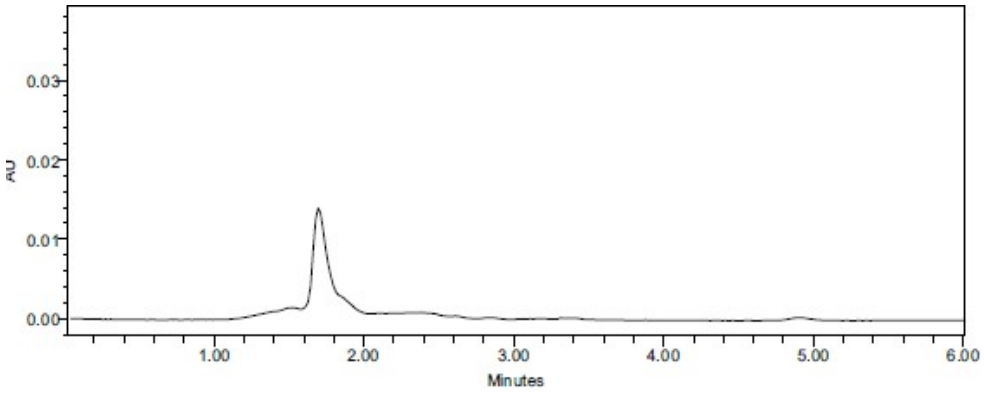

Figure 2.7: Blank human plasma

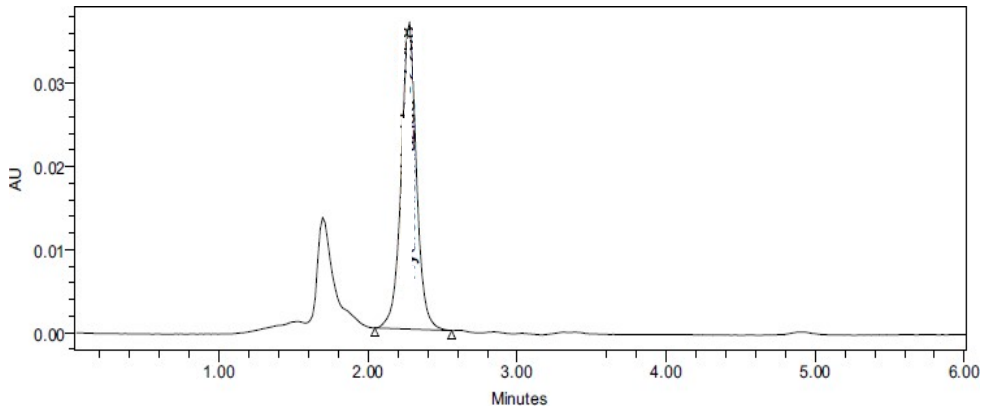

Figure 2.8: Blank human plasma spiked with ISTD

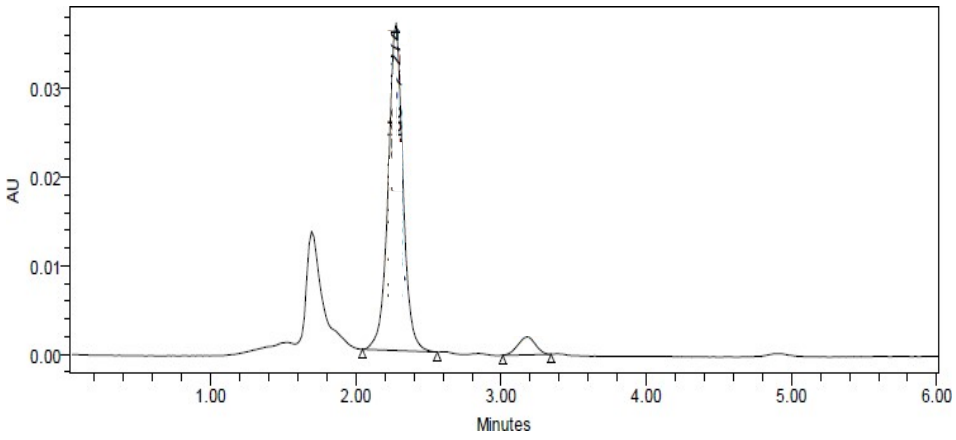

Figure 2.9: Blank human plasma spiked with analyte and ISTD at LLOQ

As observed from the above chromatogram, total run time was 6 min and the retention time of Analyte and ISTD were at 3.1 and 2.2 min. For blank plasma chromatogram there was no interfering peaks near the peaks for Molnupiravir and internal standard. Same is observed in case of the chromatogram of blank plasma spiked with ISTD.

2.3.5.2 System suitability

The % CV for Molnupiravir and ISTD area ratio was found to be 0.57%. Hence, it passed the system suitability.

Acceptance Criteria: The %CV of the retention time should be ≤ 2.00%. The % CV of the area ratio should be ≤ 5.00%.

2.3.5.3 Sensitivity

The %CV of Molnupiravir was found to be 3.06% and the % Mean accuracy was found to be 100.08%. Hence, it passed the sensitivity.

Table 2.4: System suitability Results of Molnupiravir

Sample Name	Analyte Area	Analyte Rt (min)	ISTD Area	ISTD Rt (min)	Area Ratio
MQC	33994	3.189	61813	2.285	0.5499
MQC	34079	3.199	61503	2.286	0.5541
MQC	34132	3.199	61934	2.286	0.5511
MQC	33980	3.203	61947	2.286	0.5485
MQC	33759	3.208	61734	2.291	0.5468
MQC	33663	3.208	61733	2.291	0.5453
Mean		0.321		2.288	0.54931
SD		0.0071		0.0027	0.003142
%CV		0.22		0.12	0.57

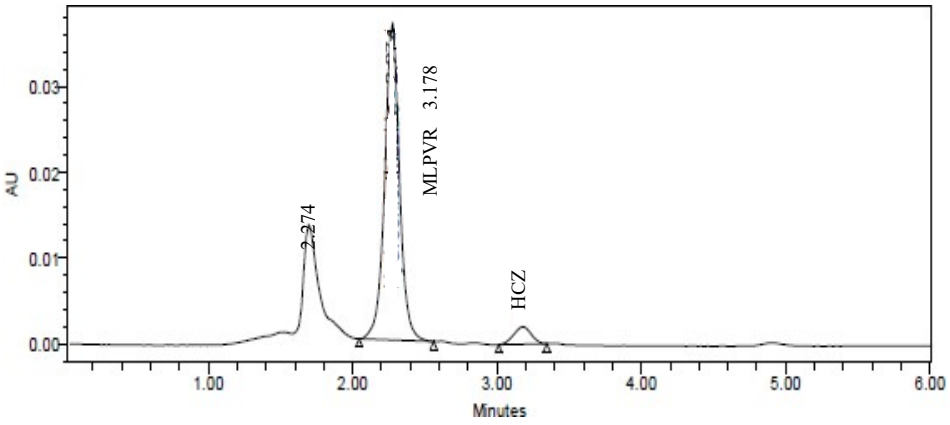

Figure2.10: Sensitivity chromatogram of LLOQ

Table 2.5: Sensitivity Results of Molnupiravir

Replicate Number	LLOQ
	Nominal Concentration (ng/mL)
	50.000
	Nominal concentration Range (ng/mL)
	(40.000-60.000)
	Calculated Concentration (ng/mL)
1	48.202
2	49.610
3	48.980
4	50.990
5	49.940
6	52.512
N	6
Mean	50.0390
SD	1.53036
%CV	3.06
% Mean Accuracy	100.08

Acceptance Criteria: At least 67% (4 out of 6) samples should be within 80.00-120.00%.

% Mean accuracy should be within 80.00-120.00%.

%CV should be ≤ 20.00%.

2.3.5.4 Matrix effect

The matrix of plasma constituents over the ionization of analyte was determined by comparing the response of post-extracted plasma standard QC samples (n=6) with the response of analyte from neat samples at equivalent concentrations. The matrix effect was assessed by using chromatographically screened human plasma.

Precision (%CV) is 1.38% and 2.00% for Molnupiravir at HQC and LQC, respectively.

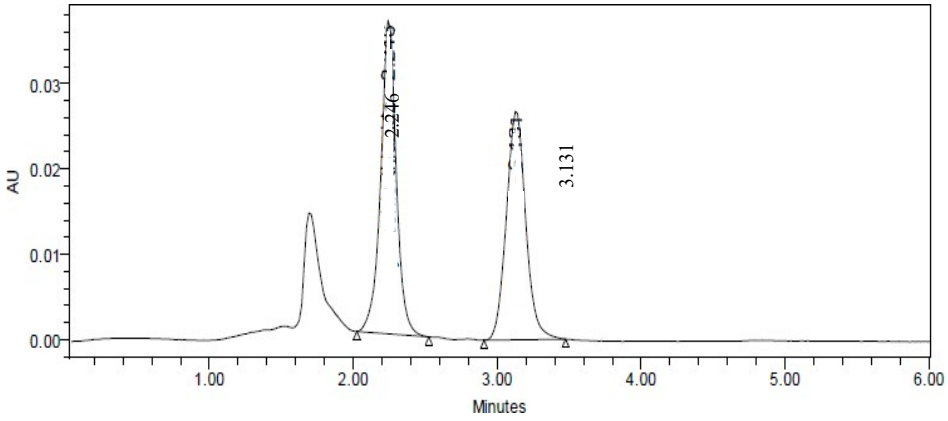

Figure 2.11: Matrix Effect chromatogram of HQC

Table 2.6: Matrix effect Results of Molnupiravir

S. No.	Plasma Lot No.	HQC	LQC
		Nominal Concentration (ng/mL)	
		1600.000	150.000
		Nominal concentration Range (ng/mL)	
		(1360.000-1840.000)	(127.500-172.500)
		Calculated Concentration (ng/mL)	
1.	Lot 1	1562	148.90
		1560	149.00
		1564	150.00
2.	Lot 2	1565	152.00
		1562	155.00
		1565	156.00
3.	Lot 3	1563	152.00
		1573	155.00
		1575	152.00
4.	Lot 4	1571	160.00
		1585	157.00
		1588	151.00
5.	Lot 5	1599	155.00
		1615	152.00
		1608	155.00
6.	Lot 6	1615	149.00
		1589	151.00
		1628	153.00
N		18	18
Mean		1582.6111	152.9389
SD		21.88779	3.06539
%CV		1.38	2.00
% Mean Accuracy		98.91	101.96
No. of QC Failed		0	0

Acceptance Criteria: At least 67% (2 out of 3) of samples at each level should be within 85.00-115.00%. At least 80% (5 out of 6) of the matrix lot should be within the acceptance criteria.

The % mean accuracy of back calculated concentration of LQC and HQC samples prepared from different biological matrix lots should be within 85.00-115.00%.

2.3.5.4 Linearity

The linearity of the method was determined by a weighted ($1/x^2$ where X is concentration) least square regression analysis of the standard plots associated with the six point standard curve of Molnupiravir. The standard curve was linear over the concentration range of 50.00-2000.00 ng/mL of Molnupiravir. The correlation coefficient was found to be 0.998. Samples were quantified using the ratio of peak area of analyte to that of ISTD. Peak area ratios were plotted against plasma concentrations.

Table 2.7: Linearity data of Molnupiravir

S. No.	Concentration (ng/mL)	Peak area ratio
1	50	0.03
2	100	0.06
3	150	0.09
4	400	0.23
5	1000	0.55
6	1200	0.68
7	1600	0.88
8	2000	1.07

Table 2.8: Back calculated concentrations of Molnupiravir

Conc. (ng/mL)	50	100	150	400	1000	1200	1600	2000
1	47.20	95.80	149.80	391.00	978.00	1181.00	1599.0	1962.00
2	56.90	98.21	153.00	395.30	982.00	1175.000	1585.00	1965.00
3	49.10	107.23	147.20	406.00	980.00	1172.000	1601.00	1976.00
N	3	3	3	3	3	3	3	3
Mean	51.06	100.41	150.00	397.43	980.00	1176.00	1595.00	1967.66
SD	5.1403	6.0251	2.9051	7.7242	2.0000	4.5825	8.7178	7.3711
%CV	10.07	6.00	1.94	1.94	0.20	0.39	0.55	0.37
%Mean Accuracy	102.13	100.41	100.00	99.36	98.00	98.00	99.69	98.38

Acceptance Criteria: The Linearity Regression coefficient should be R^2 = 0.999

2.3.5.4 Precision and Accuracy

The intra-assay precision and accuracy were estimated by analyzing six replicates containing Molnupiravir at six different QC levels. The inter-assay precision was determined by analyzing the four levels QC samples on four different runs. The criteria for acceptability of the data include accuracy within 85-115% from the actual values and a precision of within ± 15% relative standard deviation except for LLOQ QC, where it should be within 80-120% for accuracy and ≤ 20.00% of RSD.

Table 2.10: Accuracy and Precision data of Molnupiravir (Between batch n=18)

Batch ID	HQC	MQC	LQC	LLOQ QC
Mean	1589.7486	991.6739	150.2509	49.2047
SD	11.14880	26.66895	6.75293	3.01827
%CV	0.70	2.69	4.49	6.13
% Mean accuracy	99.36	99.17	100.17	98.41

Acceptance Criteria: The within and between batch precision for LQC, MQC and HQC samples should be ≤ 15.00% and for LLOQ QC, it should be ≤ 20.00%.

The % mean accuracy of back calculated concentration of LQC and HQC samples prepared from different biological matrix lots should be within 85.00-115.00%.

2.3.5.4 Recovery of Analyte

The recovery of drug and ISTD was evaluated at three concentration levels namely low, medium and high quality control. Recovery was calculated by comparing response in replicate samples with that of neat standard solution responses. Analyte recovery from a sample matrix (extraction efficiency) is a comparison of analytical response from an amount of analyte added to that determined from sample matrix. Because of basic properties of Molnupiravir, extraction was carried out using Acetonitrile solvent.

Experiments with spiked compounds resulted in recoveries of analyte 97.0%-99.80% and for ISTD 97.40%.

Table 2.11: Recovery of analyte of Molnupiravir

Replicate No.	HQC		MQC		LQC	
	Un extracted Response	Extracted Response	Un extracted Response	Extracted Response	Un extracted Response	Extracted Response
1	56162	54731	34143	33994	5655	5516
2	55831	54259	34026	34079	5662	5523
3	56165	54611	34189	34132	5686	5508
4	55806	54156	34112	33980	5628	5556
5	55910	54355	34210	33759	5640	5538
6	56059	54250	34313	33663	5700	5514
N	6	6	6	6	6	6
Mean	55989	54394	34166	33935	5662	5526
SD	161.55	227.06	97.07	184.40	27.24	18.00
%CV	0.29	0.42	0.28	0.54	0.48	0.33
%Mean Recovery	97.15		99.32		97.60	
Overall % Mean Recovery	98.024					
Overall SD	1.1475					
Overall %RSD	1.17					

Table 2.12: Recovery of Internal Standard

S. No.	Un extracted Area ratio	Extracted Area ratio
1	63585	61438
2	63258	61689
3	63125	61341
4	62984	61292
5	63152	61601
6	63028	61911
n	6	6
Mean	63188.7	61545.3
SD	216.79	234.48
%CV	0.34	0.38
%Mean Recovery	97.40	

Acceptance Criteria: The %RSD of recovery at each QC level and for ISTD should be ≤ 15.005. The overall % mean recovery and %RSD for all QC levels should be ≤ 20.00%.

Ruggedness

Ruggedness experiment was performed by different column and different analyst. To evaluate ruggedness, precision and accuracy batch was processed against calibration curve standards and analyzed by a different analyst using different column.

Table 2.13: Ruggedness Linearity of Molnupiravir

Conc. (ng/mL)	50	100	150	400	1000	1200	1600	2000
Different column	48.259	97.539	147.758	393.180	998.130	1198.120	1604.1	1998.51
Different Analyst	50.221	101.442	150.675	401.980	1002.59	1215.850	1597.5	2081.82

Table 2.14: Ruggedness Precision and Accuracy of Molnupiravir

Batch ID	QC ID	HQC	MQC	LQC	LLOQ QC
Nominal Conc. (ng/mL)		1600	1000	150	50
Different column	1	1592.790	978.210	142.751	44.263
	2	1594.380	975.520	140.730	47.220
	3	1597.980	985.320	144.680	55.260
	4	1595.750	984.580	148.699	48.221
	5	1606.890	978.900	155.780	52.251
	6	1603.390	984.260	150.810	49.289
	Mean	1598.5300	981.1317	147.2417	49.4173
	SD	5.50836	4.10431	5.60161	3.87323
	%CV	0.34	0.42	3.80	7.84
	% Mean accuracy	99.91	98.11	98.16	98.83
Different Analyst	1	1594.340	985.160	140.790	48.281
	2	1592.260	988.216	142.765	46.210
	3	1596.120	975.020	144.720	48.255
	4	1598.020	974.550	147.680	52.260
	5	1603.940	982.650	156.640	50.243
	6	1601.800	980.540	150.821	49.230
	Mean	1597.7467	981.0227	147.2360	49.0798
	SD	4.45458	5.47120	5.81989	2.05151
	%CV	0.28	0.56	3.95	4.18
	% Mean accuracy	99.86	98.10	98.16	98.16

Acceptance Criteria: The within and between batch precision for LQC, MQC and HQC samples should be ≤ 15.00% and for LLOQ QC, it should be ≤20.00%.

At least 67% (16 out of 24) of total QC samples and 50% (3 out of 6) at each level should be within 85.00-115% except LLOQ QC. LLOQ QC should be within 80.00-120.00%.

% Mean accuracy for LQC, MQC and HQC samples should be within 85.00- 115% and for the LLOQ QC sample it should be within 80.00-120.00%.

CONCLUSION

The optimized HPLC-UV method is simple, accurate, precise and reproducible. The method is linear over a wide range and utilizes a mobile phase which can be easily prepared. Simple sample preparation procedure and a relatively short chromatographic run time make this method suitable for processing of multiple samples in a limited amount of time. The method developed complies with the validation criteria laid down by the US-FDA guidelines. Hence, the developed method can be applied for pharmacokinetic studies and therapeutic drug monitoring in humans. As a result of the current work , a new, more sensitive and faster method for quantitating Molnupiravir in human plasma was developed.

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