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RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE QUANTITATIVE ESTIMATION OF MOLNUPIRAVIR IN BULK FORM AND MARKETED PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

Objective: The current investigation was pointed at developing and progressively validating novel, simple, responsive and stable RP-HPLC method for the measurement of active pharmaceutical ingredient and Marketed Pharmaceutical Dosage form of Molnupiravir. **Methods:** A simple, selective, validated and well-defined stability that shows isocratic RP-HPLC methodology for the quantitative determination of Molnupiravir. The chromatographic strategy utilized Symmetry ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm, using isocratic elution with a mobile phase of Phosphate Buffer (0.02M) and Acetonitrile were consists of 48:52% v/v (pH-2.80). A flow rate of 1.0 ml/min and a detector wavelength of 248 nm utilizing the UV detector were given in the instrumental settings. Validation of the proposed method was carried out according to an international conference on harmonization (ICH) guidelines. **Results:** LOD and LOQ for the two active ingredients were established with respect to test concentration. The calibration charts plotted were linear with a regression coefficient of R2>0.999, means the linearity was within the limit. Recovery, specificity, linearity, accuracy, robustness, ruggedness were determined as a part of method validation and the results were found to be within the acceptable range. **Conclusion:** The proposed method to be fast, simple, feasible and affordable in assay condition. During stability tests, it can be used for routine analysis of the selected drug.

KEYWORDS: Molnupiravir, RP-HPLC, Method Development, Validation, Accuracy, Robustness.

INTRODUCTION

Molnupiravir is an orally bioavailable prodrug of EIDD-1931, the synthetic ribonucleoside derivative N4-hydroxycytidine and ribonucleoside analog, with potential antiviral activity against a variety of RNA viruses. Upon oral administration, Molnupiravir, being a prodrug, is metabolized into its active form EIDD-1931 and converted into its triphosphate (TP) form. The TP form of EIDD-1931 is incorporated into RNA and inhibits the action of viral RNA-dependent RNA polymerase. This results in the termination of RNA transcription and decreases viral RNA production, and viral RNA replication. N4-hydroxycytidine and its prodrug Molnupiravir are being studied for its activity against a number of viral infections including influenza, MERS-CoV, and SARS-CoV-2. Molnupiravir[1] is approved in the UK for reducing the risk of hospitalization and death in mild to moderate COVID-19 cases for patients at increased risk of severe disease (eg. with obesity, diabetes mellitus, heart disease, or are over 60 years old). In the US, Molnupiravir[2] is authorized for emergency use for the treatment of high-risk adults with mild to moderate COVID-19. Molnupiravir is hydrolyzed in vivo to N4-hydroxycytidine, which is phosphorylated in tissue to the active 5'-triphosphate form, and incorporated into the genome of new virions, resulting in the accumulation of inactivating mutations, known as viral error catastrophe. A Remdesivir resistant mutant mouse hepatitis virus has also been shown to have increased sensitivity to N4-hydroxycytidine.

Molnupiravir[3] is indicated for treatment of mild to moderate coronavirus disease (COVID-19) in adults with a positive SARS-COV-2 diagnostic test and who have at least one risk factor for developing severe illness. The IUPAC name of Molnupiravir is [(2R, 3S, 4R, 5R)-3, 4-dihydroxy-5-[4-(hydroxy amino)-2-oxopyrimidin-1-yl] oxolan-2-yl] methyl 2-methyl propanoate. The Chemical Structure of Molnupiravir is follows.

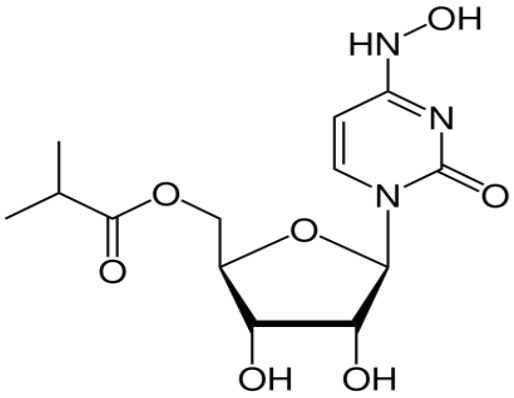

Fig-1: Chemical Structure of Molnupiravir.

EXPERIMENTAL

1. INSTRUMENTS USED

Table-1: List of Instrument used.

S. No.	Instruments/Equipments/Apparatus
1.	**Waters HPLC** with Empower2 Software with Isocratic with UV-Visible Detector.
2.	**ELICO SL-159** UV – Vis spectrophotometer
3.	Electronic Balance **(SHIMADZU ATY224)**
4.	Ultra Sonicator **(Wensar wuc-2L)**
5.	Thermal Oven
6.	Symmetry RP C_{18}, 5µm, 250mm x 4.6mm i.d.
7.	P^H Analyzer **(ELICO)**
8.	Vacuum filtration kit **(BOROSIL)**

2. CHEMICALS / REAGENTS USED

Table-2: List of Chemicals used.

S.N.	Name	Specifications		Manufacturer/Supplier
		Purity	Grade	
1.	Doubled distilled water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
2.	Methanol	99.9%	HPLC	Loba Chem; Mumbai.
3.	Dipotassium hydrogen orthophosphate	96%	L.R.	Sd fine-Chem ltd; Mumbai
4.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
5.	Potassium dihydrogen orthophosphate	99.9%	L.R.	Sd fine-Chem ltd; Mumbai
6.	Sodium hydroxide	99.9%	L.R.	Sd fine-Chem ltd; Mumbai
7.	Hydrochloric acid	96%	A.R.	Sd fine-Chem ltd; Mumbai
8.	3% Hydrogen Peroxide	96%	A.R.	Sd fine-Chem ltd; Mumbai

METHOD DEVELOPMENT AND ITS VALIDATION FOR MOLNUPIRAVIR BY RP-HPLC

Selection of Wavelength

The standard & sample stock solutions were prepared separately by dissolving standard & sample in a solvent in mobile phase diluting with the same solvent. (After optimization[4] of all conditions) for UV analysis. It scanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Molnupiravir, so that the same wave number can be utilized in HPLC UV detector[5] for estimating the Molnupiravir. While scanning the Molnupiravir solution we observed the maxima at 248 nm. The UV spectrum has been recorded on ELICO SL-159 make UV – Vis spectrophotometer model UV-2450. The scanned UV spectrum is attached in the following page.

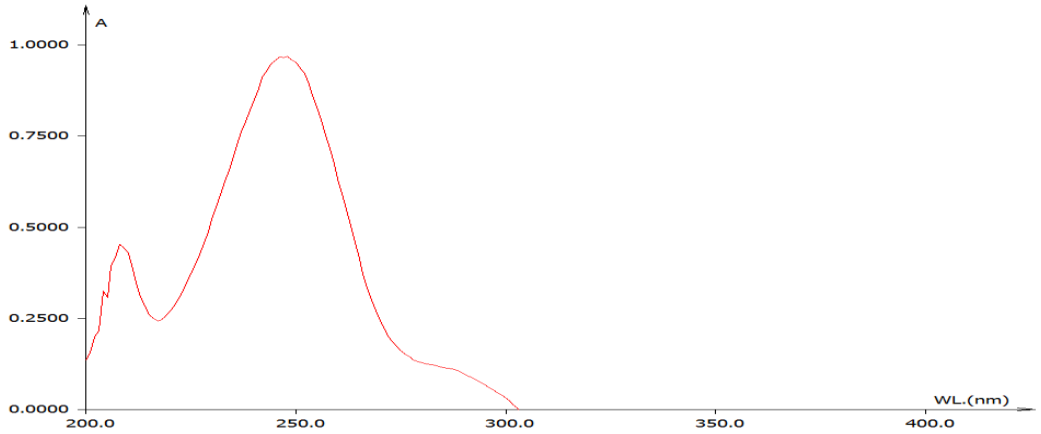

Fig-2: UV Spectrum for Molnupiravir (248nm).

Preparation of Standard Solution

Accurately weigh and transfer 10 mg of Molnupiravir working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol. Further pipette 0.5ml of the above Molnupiravir stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure

Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.[25,30]

Preparation of Sample Solution

Twenty tablets were taken and the average weight was calculated as per the method prescribed in I.P. The weighed tablets were finally powdered and triturated well. A quantity of powder of Molnupiravir equivalent to 10mg were transferred to clean and dry 10 ml volumetric flask and 7 ml of HPLC grade methanol was added and the resulting solution was sonicated for 15 minutes. Make up the volume up to 10 ml with same solvent. Then 1 ml of the above solution was diluted to 10 ml with HPLC grade methanol. One ml (0.5 ml) of the prepared stock solution diluted to 10 ml and was filtered through membrane filter (0.45µm) and finally sonicated to degas.[6]

Optimization of Chromatographic Conditions

The chromatographic conditions[7] were optimized by different means. (Using different column, different mobile phase, different flow rate, different detection wavelength & different diluents for sample preparation etc.

Preparation of 0.02M Potassium Dihydrogen Orthophosphate Solution

About 2.72172grams of Potassium dihydrogen orthophosphate was weighed and transferred into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC Grade water. The pH was adjusted to 2.80 with diluted orthophosphoric acid Solution.

Preparation of Mobile Phase

480mL (48%) of above Phosphate buffer solution and 520mL of HPLC Grade Acetonitrile (52%) were mixed well and degassed in ultrasonic water bath for 15 minutes. The resulted solution was filtered through 0.45 µm filter under vacuum filtration.

RESULTS AND DISCUSSION

Method Development

Summary of Optimized Chromatographic Conditions

The Optimum conditions[8] obtained from experiments can be summarized as below.

Table 3: Summary of Optimised Chromatographic Conditions.

Mobile phase	Phosphate Buffer (0.02M): Acetonitrile = 48:52 (pH-2.80)
Column	Symmetry ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm
Column Temperature	Ambient
Detection Wavelength	248 nm
Flow rate	1.0 ml/ min.
Run time	08 min.
Temperature of Auto sampler	Ambient
Diluent	Mobile Phase
Injection Volume	20µl
Mode of Elution	Isocratic
Retention time	3.649 minutes

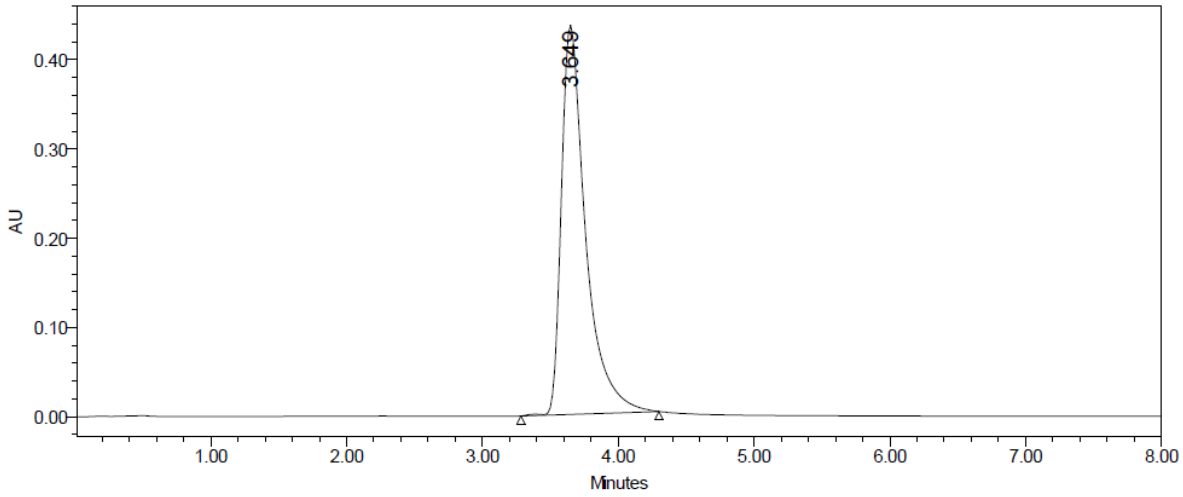

Fig-3: Chromatogram of Molnupiravir in Optimized Chromatographic Condition.

Validation of Method

1. Accuracy

Preparation of Standard Solution

Accurately weigh and transfer 10 mg of Molnupiravir working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 0.5ml of the above Molnupiravir stock solutions[9] into a 10ml volumetric flask and dilute up to the mark with Methanol.

For Preparation of 80% Standard Stock Solution

Accurately weigh and transfer 10 mg of Molnupiravir working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Take 0.4ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

For Preparation of 100% Standard Stock Solution

Accurately weigh and transfer 10 mg of Molnupiravir working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Take 0.5ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.[10]

For Preparation of 120% Standard Stock Solution

Accurately weigh and transfer 10 mg of Molnupiravir working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Take 0.6ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

Recovery Study

To determine the accuracy of the planned technique, recovery studies were distributed by adds completely different amounts (80%, 100%, and 120%) of pure drug of Molnupiravir were taken and extra to the pre-analyzed formulation of concentration 50µg/ml. From that proportion recovery values[11-13] were calculated. The results were shown in table-4.

Table-4: Accuracy Readings.

Sample ID	Concentration (µg/ml)		Peak Area	% Recovery of Pure drug	Statistical Analysis
	Amount Added	Amount Found			
S_1 : 80 %	40	40.141	502647	100.352	Mean= 100.3947%
S_2 : 80 %	40	40.191	503214	100.477	S.D. = 0.071319
S_3 : 80 %	40	40.142	502656	100.355	% R.S.D.= 0.071038
S_4 : 100 %	50	50.044	614215	100.088	Mean= 99.98533%
S_5 : 100 %	50	49.887	612451	99.774	S.D. = 0.183045
S_6 : 100 %	50	50.047	614254	100.094	% R.S.D.= 0.183071
S_7 : 120 %	60	60.192	728547	100.32	Mean= 100.311%
S_8 : 120 %	60	59.939	725698	99.898	S.D. = 0.408574
S_9 : 120 %	60	60.429	731211	100.715	% R.S.D.= 0.407308

2. Precision

2.1. Repeatability

Preparation of Molnupiravir Product Solution for Precision

Accurately weigh and transfer 10 mg of Molnupiravir working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Take 0.5ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

Procedure

The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

The exactitude[14] of every technique was determined one by one from the height areas & retention times obtained by actual determination of six replicates of a set quantity of drug. Molnupiravir (API). The % relative variance[15] was calculated for Molnupiravir square measure bestowed within the table-5.

Table-5: Repeatability Readings.

HPLC Injection Replicates of Molnupiravir	Retention Time (Minutes)	Peak Area
Replicate – 1	3.649	5674158
Replicate – 2	3.684	5654715
Replicate – 3	3.687	5665841
Replicate – 4	3.688	5654578
Replicate – 5	3.688	5652284
Replicate – 6	3.687	5641487
Average		**5657177**
Standard Deviation		**11369.72**
% RSD		**0.200979**

2.2. Intermediate Precision/Ruggedness

2.2.1. Intra-Day & Inter-Day

The intra & inter day variation[16-17] of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Molnupiravir revealed that the proposed method is precise.

Procedure

Analyst 1: The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Analyst 2: The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Analyst-1/Intra Day/Day-1

Table-6: Results of Ruggedness for Molnupiravir Analyst 1.

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Molnupiravir	3.687	584968	65982	4985	1.42
2	Molnupiravir	3.688	582479	66354	4876	1.46
3	Molnupiravir	3.688	586236	67425	4896	1.48
4	Molnupiravir	3.687	586985	65982	4986	1.47
5	Molnupiravir	3.684	582679	65932	5016	1.45
6	Molnupiravir	3.649	583989	65874	4987	1.43
Mean			**584556**			
Std. Dev.			**1846.658**			
% RSD			**0.315908**			

Analyst 2/Inter Day/Day-2.

Table-7: Results of Intermediate Precision Analyst 2 for Molnupiravir.

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Molnupiravir	3.649	598698	66985	5265	1.49
2	Molnupiravir	3.684	596847	67458	5168	1.47
3	Molnupiravir	3.687	596354	66985	5436	1.46
4	Molnupiravir	3.688	598676	67854	5369	1.45
5	Molnupiravir	3.688	596874	68521	5247	1.48
6	Molnupiravir	3.687	598989	67898	5375	1.42
Mean			**597739.7**			
Std. Dev.			**1168.098**			
% RSD			**0.195419**			

3. Linearity & Range

Preparation of Drug Solutions for Linearity

Accurately weigh and transfer 10 mg of Molnupiravir working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 0.5ml of the above Molnupiravir stock solutions into a 10ml volumetric flask and dilute up to the mark with Mobile Phase.[18]

Preparation of Level – I (30ppm of Molnupiravir)

Take 0.3ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

Preparation of Level – II (40ppm of Molnupiravir)

Take 0.4ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

Preparation of Level – III (50ppm of Molnupiravir)

Take 0.5ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

Preparation of Level – IV (60ppm of Molnupiravir)

Take 0.6ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

Preparation of Level – V (70ppm of Molnupiravir)

Take 0.7ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

Procedure

Inject each level into the chromatographic system[19] and measure the peak area.

Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

The calibration curve showed good linearity in the range of 0-70µg/ml, for Molnupiravir (API) with correlation coefficient[20] (r^2) of 0.999 (Fig-4). A typical calibration curve has the regression equation of y = 11266.x + 50416 for Molnupiravir.

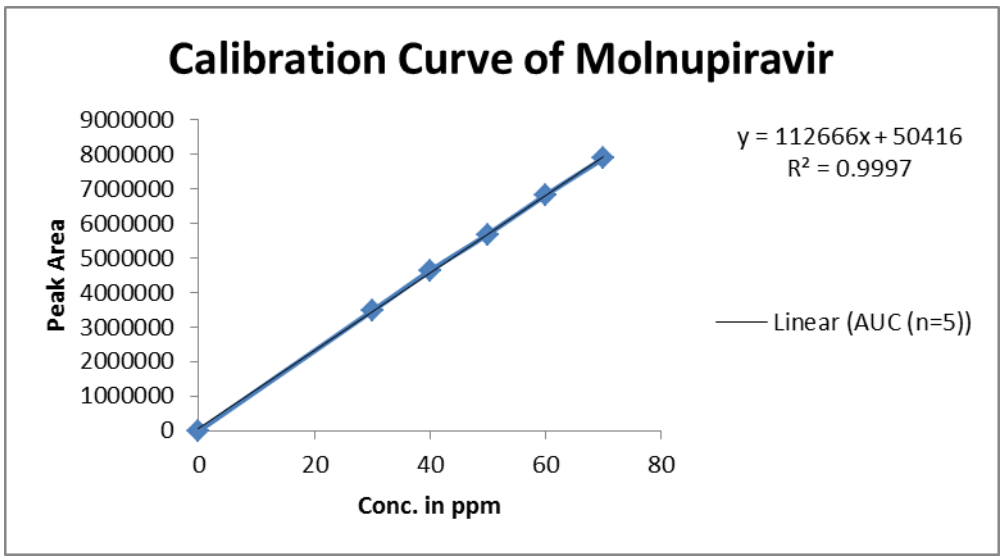

Fig-4: Calibration Curve of Molnupiravir (API).

Table-8: Linearity Results.

CONC.(µg/ml)	MEAN AUC (n=6)
0	0
30	3465974
40	4626478
50	5682284
60	6815478
70	7878721

Linearity Plot

The plot of Concentration (x) versus the Average Peak Area (y) data of Molnupiravir is a straight line.

Y = mx + c

Slope (m) = 112666

Intercept (c) = 50416

Correlation Coefficient (r) = 0.99

Validation Criteria: The response linearity[21-24] is verified if the Correlation Coefficient is 0.99 or greater.

CONCLUSION: Correlation Coefficient (r) is 0.99, and the intercept is 50416. These values meet the validation criteria.

4. Method Robustness

The robustness was performed for the flow rate variations from 0.9 ml/min to 1.1ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for Molnupiravir. The method is robust[26] only in less flow condition and the method is robust even by change in the Mobile phase ±5%. The standard and samples of Molnupiravir were injected by

changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count.

The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results.

For Preparation of Standard Solution
Accurately weigh and transfer 10 mg of Molnupiravir working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Take 0.5ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluent.

Effect of Variation of Flow Conditions
The sample was analyzed at 0.9ml/min and 1.1ml/min instead of 1ml/min, remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded.

Effect of Variation of Mobile Phase Organic Composition
The sample was analyzed by variation of mobile phase i.e. Acetonitrile: Phosphate Buffer was taken in the ratio and 40:60, 30:70 instead of 35:65, remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded.

Table 9: Results for Robustness.

Parameter Used for Sample Analysis	Peak Area	Retention Time	Theoretical Plates	Tailing Factor
Actual Flow rate of 1.0 mL/min	584624	3.649	1.42	4765
Less Flow rate of 0.9 mL/min	598676	3.687	1.49	4856
More Flow rate of 1.1 mL/min	612543	3.649	1.46	4965
Less organic phase	578642	3.688	1.49	4758
More organic phase	569896	3.684	1.47	4962

5. LOD & LOQ
LOD: The detection limit[27] of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.
LOD = 3.3 × σ / s
Where
σ = Standard deviation of the response
S = Slope of the calibration curve
LOQ: The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined.
LOQ = 10 × σ/S
Where
σ = Standard deviation of the response
S = Slope of the calibration curve

Table-10: Results of LOD & LOQ.

SE of Intercept	48846.22527
SD of Intercept	109223.4801
LOD	3.199168
LOQ	9.694449

Observation
The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 3.19 & 9.69 µg/ml respectively.

6. System Suitability Parameter
System quality testing is associate degree integral a part of several analytical procedures. The tests square measure supported the idea that the instrumentation, physics, associate degree analytical operations and samples to be analyzed represent an integral system that may be evaluated intrinsically. Following system quality check parameters [28] were established. The information square measured shown in Table-11.

Preparation of Standard Solution
Accurately weigh and transfer 10 mg of Molnupiravir working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.

Further pipette 0.5ml of the above Molnupiravir stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure
The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Table-11: Knowledge of System quality Parameter.

S.No.	Parameter	Limit	Result
1	Asymmetry	$T \leq 2$	Molnupiravir =0.98
2	Theoretical plate	$N > 2000$	Molnupiravir =4782
3	Tailing Factor	$T<2$	Molnupiravir =1.49

7. Specificity

Specificity can be determined by comparing the chromatograms obtained from the drugs with the chromatogram obtained from the blank solution. Blank solution was prepared by mixing the excipients in the mobile phase without drug. Drug solutions were prepared individually and the sample containing one drug was also prepared. Now these mixtures were filtered by passing through 0.45 µ membrane filter before the analysis. In this observation no excipient peaks were obtained near the drug in the study run time. This indicates that the proposed method was specific.

8. Estimation of Molnupiravir in Pharmaceutical Dosage Form.

Table-12: Recovery Data for estimation Molnupiravir.

Brand Name of Molnupiravir	Labelled amount of Drug (mg)	Mean (± SD) amount (mg) found by the proposed method (n=6)	Assay % (± SD)
Molflu Capsule (Dr. Reddy's)	200mg	199.589 (± 0.258)	99.698 (± 0.639)

RESULT AND DISCUSSION

The amount of drugs in Molnupiravir Capsule was found to be 199.589 (± 0.258) mg/tab for Molnupiravir & % assay was 99.698 (± 0.639).

Forced Degradation Studies

Results of Degradation Studies

The results of the stress studies[29] indicated the Specificity of the method that has been developed. Molnupiravir was stable in photolytic and peroxide stress conditions. The result of forced degradation studies are given in the following table-13.

Table-13: Results of Forced Degradation Studies of Molnupiravir API.

Stress Condition	Time	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1 M HCl)	24Hrs.	98.76	1.24	100.0
Basic Hydrolysis (0.I M NaOH)	24Hrs.	98.63	1.37	100.0
Thermal Degradation (50 ^{0}C)	24Hrs.	93.98	6.02	100.0
UV (248nm)	24Hrs.	98.84	1.16	100.0
3 % Hydrogen Peroxide	24Hrs.	94.61	5.39	100.0

SUMMARY AND CONCLUSION

The analytical method was developed by studying different parameters. First of all, maximum absorbance was found to be at 248nm and the peak purity was excellent. Injection volume was selected to be 20µl which gave a good peak area. The column used for study was Symmetry ODS (C18) RP Column, 250 mm x 4.6 mm, 5µm particle size because it was giving good peak. Ambient temperatures were found to be suitable for the nature of drug solution. The flow rate was fixed at 1.0ml/min because of good peak area and satisfactory retention time. Mobile phase is Phosphate Buffer (0.02M) and Acetonitrile were taken in the ratio of 48: 52% v/v (pH-2.80) was fixed due to good symmetrical peak. So this mobile phase was used for the proposed study.

Methanol was selected because of maximum extraction sonication time was fixed to be 10min at which all the drug particles were completely soluble and showed good recovery. Run time was selected to be 8.0 min because analyze gave peak around 3.649min and also to reduce the total run time.

The percent recovery was found to be 98.0-102 was linear and precise over the same range. Both system and method precision was found to be accurate and well within range. The analytical method was found linearity over the range of 30-70ppm of the Molnupiravir target concentration. The analytical passed both robustness and ruggedness tests. On both cases, relative standard deviation was well satisfactory.

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