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Analytical Method Development and Validation for the Estimation of Molnupiravir in Bulk and Pharmaceutical Tablet Dosage Form by RP-HPLC

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ABSTRACT

A novel, specific, accurate, rugged, precise reversed-phase high-performance liquid chromatography (RP-HPLC) method has been developed for the quantitative determination of Molnupiravir in active pharmaceutical ingredients and its Pharmaceutical dosage form by using Phenomenex LunaC18 (4.6mm x 150mm, 5µm) column with a mobile phase containing a mixture of Acetonitrile and Potassium dihydrogen phosphate buffer adjusted to pH-2.8 with Ortho phosphoric acid in the ratio of 25:75%v/v. The flow rate was 1.0 ml/min and effluent was monitored at 220 nm and a peak eluted at 3.174 min and column oven temperature was maintained ambient. The calibration curve was plotted with a range from 10- 30 µg/ml. The LOD and LOQ values of Molnupiravir were found to be 1.3µg/ml and 3.9µg/ml respectively. The percentage recovery of the Molnupiravir was found to be within the limits. The developed RP-HPLC method was validated according to the current International Conference on Harmonization (ICH) guidelines for specificity, LOD, LOQ, linearity, accuracy, precision, intermediate precision, and robustness. The results of the study showed that the proposed RP-HPLC method is simple, rapid, precise, and accurate, which is useful for the routine determination of Molnupiravir in bulk drugs and its pharmaceutical dosage form. The proposed method was applied for the analysis of tablet formulations, to improve QC and assure therapeutic efficacy.

INTRODUCTION

Coronavirus disease (COVID-19): CO' stands for corona, 'VI' for the virus, and 'D' for disease. Formerly, this disease was referred to as the '2019 novel coronavirus or '2019-nCoV.' The COVID-19 virus is a new virus linked to the same family of viruses as Severe Acute Respiratory Syndrome (SARS) and some types of the common cold. A highly contagious respiratory disease caused by the SARS-CoV-2 virus. SARS-CoV-2 is thought to spread from person to person through droplets released when an infected person coughs, sneezes, or talks. It may also be spread by touching a surface with the virus on it and then touching one's mouth, nose, or eyes, but this is less common. The most common signs and symptoms of COVID-19 are fever, cough, and trouble breathing. Fatigue, muscle pain, chills, headache, sore throat, runny nose, nausea or vomiting, diarrhea, and a loss of taste or smell may also occur. The signs and symptoms may be mild or severe and usually appear 2 to 14 days after exposure to the SARS-CoV-2 virus. Some people may not have any symptoms but are still able to spread the virus. Most people with COVID-19 recover without needing special treatment. But other people are at higher risk of serious illness. Those at higher risk include older adults and people with serious medical problems, such as heart, lung, or kidney disease, diabetes, cancer, or a weak immune system. Serious illnesses may include life-threatening pneumonia and organ failure. Research is being done to treat COVID-19.

Molnupiravir inhibits viral reproduction by promoting widespread mutations in the replication of viral RNA by RNA-directed RNA polymerase. It is metabolized into a ribonucleoside analog that resembles cytidine, β-D-N4-Hydroxycytidine 5′-triphosphate (also called EIDD-1931 5′-triphosphate or NHC-TP). During replication, the virus's enzyme incorporates NHC-TP into newly made RNA instead of using real cytidine. Oral molnupiravir was found to be effective for the treatment of Covid-19, without evident safety concerns when initiated within 5 days after the onset of signs or symptoms in this population of no hospitalized, unvaccinated adults who were at risk for progression to severe disease.Molnupiravir is indicated for the treatment of mild-to-moderate coronavirus disease (COVID-19) in adults with positive results of direct SARS-CoV-2 viral testing, and who are at high risk for progression to severe COVID-19.

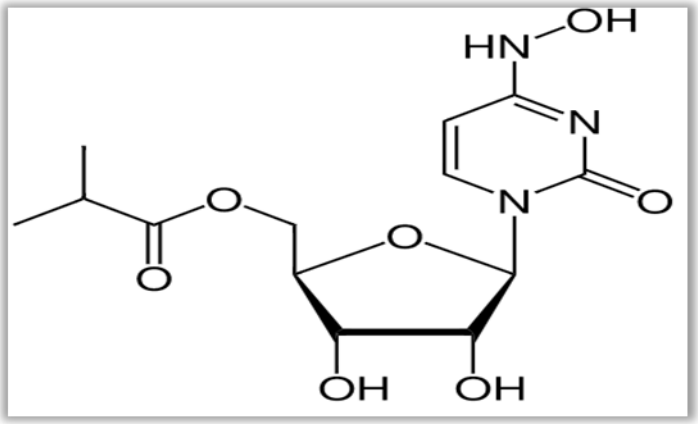

Figure no 1: MOLNUPIRAVIR

Molecular Formula: C13H19N3O7

Molecular weight: 329.309 g·mol−1

Melting Point: 169–172 °C

Solubility: DMF: 30 mg/ml, DMSO: 30 mg/ml, PBS: 1 mg/ml

PKa (Strongest acidic): 8.21

Table No. 1: Marketed Drug Formulation

S.NO.	Drug Name	Brand Name	Label Claim	Company Name
1	Molnupiravir	Molcovir	200mg	Optimus
2	Molnupiravir	Molflu	200mg	Dr.Reddy's

MATERIALS AND METHODS

Instruments used:

The liquid chromatographic system used was WATERS, software: Empower 2, Alliance 2695 separation module 996 PDA detectors, Phenomenex LunaC18 (4.6mm x 150 mm, 5 µ) column, P^H meter Lab India, Ultrasonicator-Denver.

Chemicals used:

A gift sample of molnupiravir was procured from Sure Labs, Hyderabad, and HPLC grade water, methanol, acetonitrile, and Potassium dihydrogen phosphate were purchased from MERCK laboratories, Mumbai.

Method Development

Preparation of Standard Solution:

10 mg of Molnupiravir working standard was weighed and transferred into 10ml of clean dry volumetric flasks added about 7ml of Methanol and sonicated to dissolve and remove air completely and make volume up to the mark with the same Methanol.

Further pipetted 2ml of the above Molnupiravir stock solutions into a 10ml volumetric flask and diluted up to the mark with Methanol.

Preparation of Sample Solution:

Took the average weight of the Powder and weighed 10 mg equivalent weight of the Molnupiravir sample into a 10mL clean dry volumetric flask and added about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Further pipetted 2ml of the above Molnupiravir stock solutions into a 10ml volumetric flask and diluted up to the mark with Methanol.

Mobile Phase Optimization:

Initially, the mobile phase tried was methanol: Water and ACN: Water with varying proportions. Finally, the mobile phase was optimized to Acetonitrile: Phosphate Buffer (25:75% v/v) respectively.

Optimization of Column:

The method was performed with various C18 columns like Symmetry, Zodiac, and Xterra. Phenomenex Luna C18 (4.6mm x 150mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

Method development was initiated using liquid chromatographic system WATERS, software: Empower 2, Alliance 2695 separation module. 996 PDA detectors, Phenomenex LunaC18 (4.6 x 150 mm, 5 µ) column. Initially varied concentrations of methanol: water were used and were finally optimized by methanol and acetate buffer in the ratio of 25:75v/v, Phenomenex LunaC18 (4.6mm x 150 mm, 5 µ) column, flow rate l ml/min, detection wavelength at 220 nm. The retention time of molnupiravir in the optimized chromatogram was found to be 3.174 min. The Chromatogram is shown in **figure-2.** The developed method was validated for specificity, accuracy, precision, linearity, LOD & LOQ as per the ICH guidelines.

Method Validation:

System suitability: A Standard solution was prepared by using Curcumin and Piperine working standards as per the test method and was injected in replicates five times into the HPLC system. The system suitability parameters like theoretical plates, tailing factor, and resolution was evaluated from standard chromatograms.

The standard and sample solutions were injected five times and peak areas of injections were measured in HPLC. The % RSD for the area of five replicate injections was found to be within the specified limits. The results were given in Table 2.

Three replicate injections of standard and sample solutions were injected and the assay was calculated by using the formula:

% ASSAY =

$$\frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of the tablet}}{\text{Label claim}} \times 100$$

Linearity: A Series of solutions were prepared using the molnupiravir working standard at concentration levels from 60 ppm to 140 ppm of the target concentration. Each sample solution was injected into the HPLC system in replicates and the peak areas were measured. A graph was plotted with peak areas vs concentrations and the r2 value was calculated. The results were shown in table – 3.

Accuracy: The accuracy of the newly developed method was evaluated by recovery studies at three different levels equivalent to 50,100&150%. At each level, the target concentration

was spiked in triplicates and the amount recovered was calculated the percentage recovery at each level was calculated and reported in table-5.

Precision:

Repeatability:

The standard solution was injected five times and the peak area for all five injections was measured. The % RSD for the area of five replicate injections was found to be within the specified limits. The results were given in table -5.

Intermediate precision:

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days by maintaining the same conditions. The standard solution was injected six times and measured the area for all six injections in HPLC was. The %RSD for the area of six replicate injections was found to be within the specified limits. The results were given in table –6.

Limit of Detection and Limit of Quantification:

From the linearity data, the limit of detection and quantification were calculated using the following formulae.

$$LOD = \frac{3.3\ \sigma}{S}$$

σ = standard deviation of the response

S = slope of the calibration curve of the analyte

$$LOQ = \frac{10\ \sigma}{S}$$

The values were given in table -

Robustness: A study was conducted to determine the effect of variation in flow rate, change in mobile phase composition, and detection of wavelength. A standard solution prepared as per the test method was injected into the HPLC system using flow rates, of 1.0 ml/min and

1.2 ml/min. The same studies were also performed by varying mobile phase composition and detection wavelength. The system suitability parameters were evaluated and reported in table 7.

RESULTS AND DISCUSSION

Method development:

Optimized Chromatogram (Standard)

Column	:	Phenomenex Luna C18 (4.6mm x 150mm, 5µm)
Column temperature	:	Ambient
Wavelength	:	220 nm
Mobile phase ratio	:	Acetonitrile: Phosphate Buffer (Ph-2.8) (25:75% v/v)
Flow rate	:	1.0mL/min
Injection volume	:	10 µl
Run time	:	8 minutes

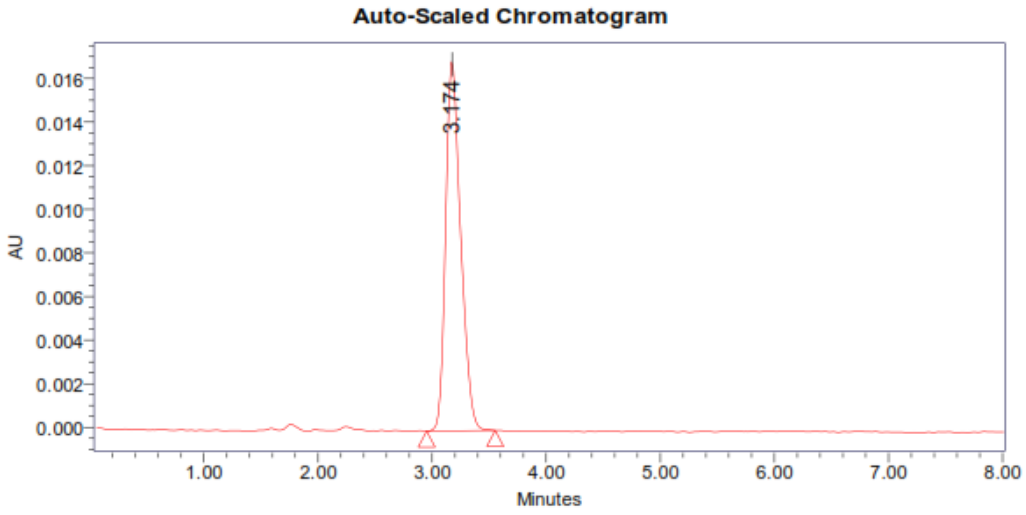

Figure no 2: Optimized Chromatogram (Standard)

Table-2: Peak results for Optimized Chromatogram (Standard)

S.No.	Peak name	R_t	Area	Height	USP Tailing	USP plate count
1	Molnupiravir	3.174	856985	69854	1.25	8547

Method Validation:

System suitability: The theoretical plates are more than 2000 and the tailing factor is less than 2 in each injection for both analytes. The values were within the acceptance criteria.

Table 2: Results of system suitability for Molnupiravir

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Molnupiravir	3.146	856985	69854	8569	1.26
2	Molnupiravir	3.123	856857	68954	8547	1.25
3	Molnupiravir	3.192	857894	68975	8596	1.25
4	Molnupiravir	3.164	857468	69854	8541	1.26
5	Molnupiravir	3.181	854785	69856	8616	1.25
Mean			856797.8			
Std.Dev.			1197.992			
%RSD			0.139822			

Linearity: A graph was plotted with peak areas vs concentration and the correlation coefficient was calculated. The r^2 values were found to be 0.999 which was within the limits confirming the linearity of the method.

Table no 3: Data for Linearity of Molnupiravir

Concentration µg/ml	Average Peak Area
10	442986
15	652547
20	856985
25	1063654
30	1268475

Accuracy: Three target concentrations 50%, 100%, and 150% were prepared concerning target assay and injected into the HPLC system in triplicates. At each spike level, the mean recovery values are between 98 to 102 % which were in agreement with the acceptance criteria. The recovery values indicate the method is accurate.

Table no 4: The accuracy results for Molnupiravir

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	429549.7	10	9.916	99.16%	99.68%
100%	856189.3	20	20.036	100.18%	
150%	1272534	30	29.912	99.706%	

Precision: Repeatability was performed in five replicate injections and the % RSD of the peak areas was calculated. The % RSD for the peak areas of five standard injections was found to be0.183536which was within the limits.

Intermediate precision was also performed on two different days the % RSD for the peak areas of six standard injections was found to be 0.119311 which was in agreement with the acceptance criteria.

Table no 5: Results of method precision for Molnupiravir:

S. No.	Peak name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Molnupiravir	3.165	856985	69856	8569	1.26
2	Molnupiravir	3.163	856898	69845	8597	1.25
3	Molnupiravir	3.158	856789	69865	8589	1.26
4	Molnupiravir	3.167	859854	69874	8569	1.25
5	Molnupiravir	3.171	854789	69798	8564	1.26
6	Molnupiravir	3.167	856978	69859	8599	1.25
Mean			857048.8			
Std. dev			1617.106			
%RSD			0.188683			

Table no 6: Results of Intermediate precision for Molnupiravir

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Molnupiravir	3.173	878548	70254	8758	1.26
2	Molnupiravir	3.134	874598	70265	8798	1.27
3	Molnupiravir	3.161	874589	69989	8742	1.26
4	Molnupiravir	3.174	875984	70145	8759	1.26
5	Molnupiravir	3.199	875981	70158	8746	1.27
6	Molnupiravir	3.199	875984	69998	8796	1.27
Mean			875947.3			
Std.Dev.			1444.511			
%RSD			0.164908			

Specificity: There is no interference observed in the blank. The chromatograms of the Standard and Sample were identical with the same retention time.

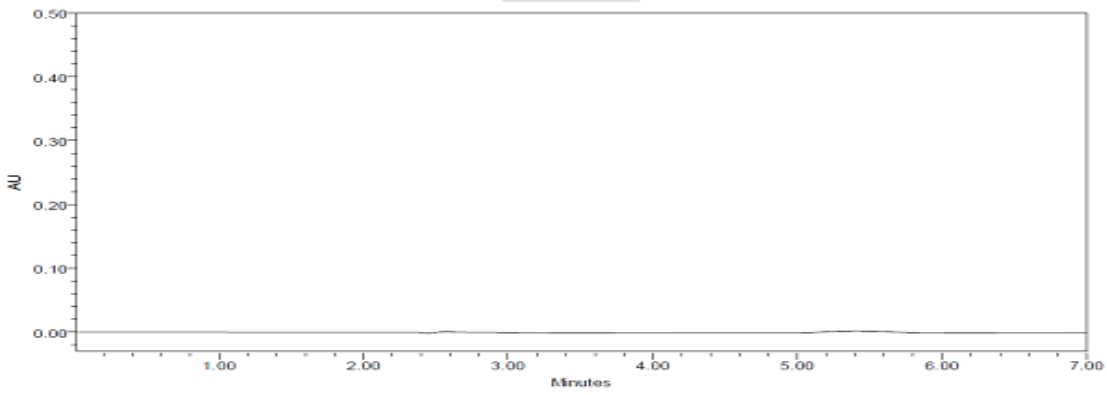

Figure No. 3: Chromatogram of blank

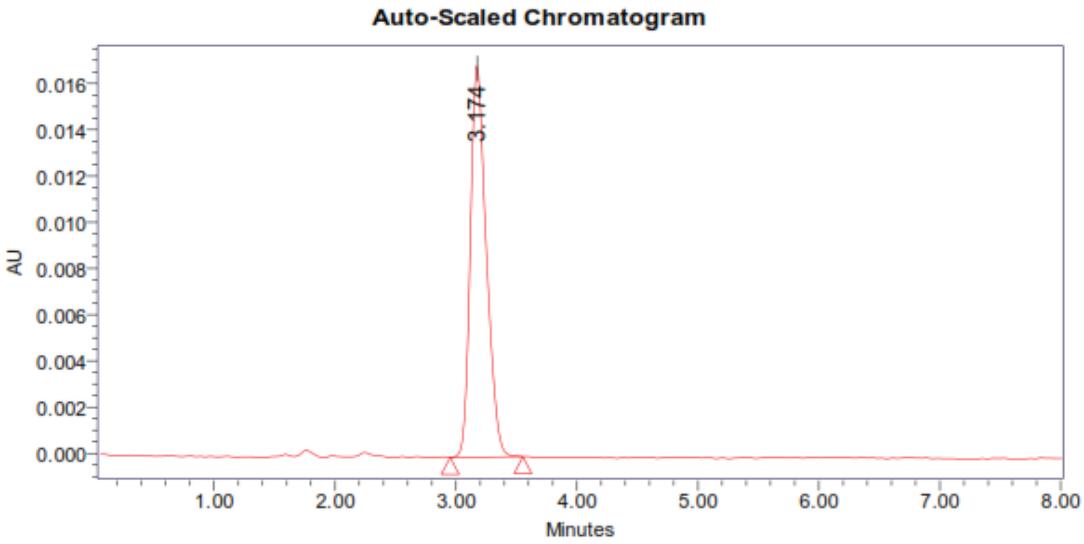

Figure 4: Chromatogram of Standard

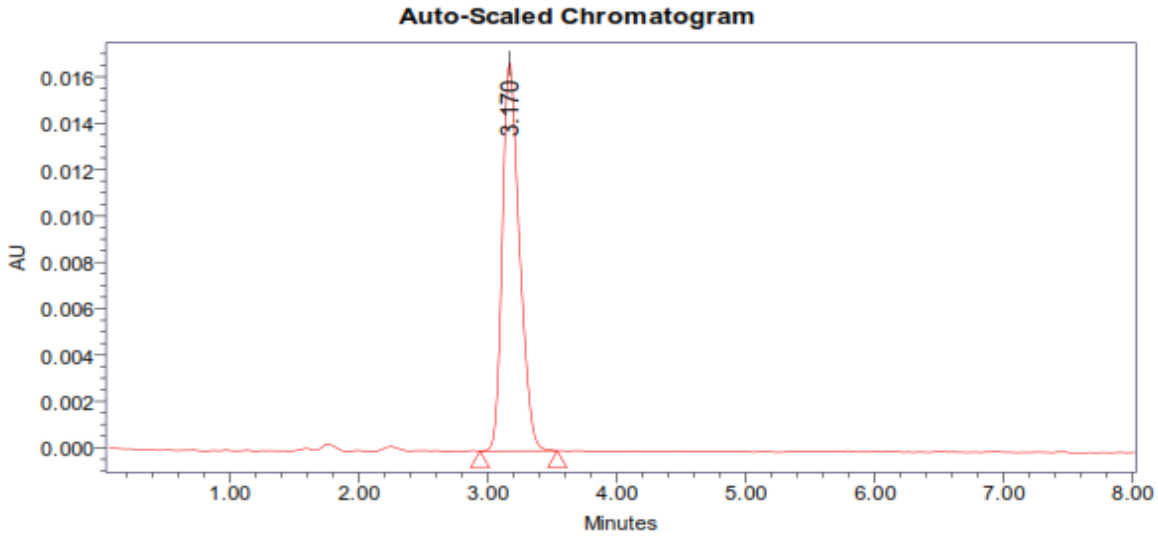

Figure-5: Chromatogram of Sample

Limit of Detection and Quantitation (LOD and LOQ): The LOD and LOQ of favipiravir were found to be 1.54µg/ml and 4.56µg/ml respectively. The results indicate that the method was sensitive.

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 the more organic phase to the less organic phase ratio for Favipiravir. The method is robust only in fewer flow conditions and the method is robust even by a change in the Mobile phase ±5%. The standard and samples of Favipiravir 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.

Table no 7: Robustness data of molnupiravir

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
The actual Flow rate of 1.0 mL/min	856985	3.174	8547	1.25
Less Flow rate of 0.9 mL/min	841542	3.488	8256	1.23
More Flow rate of 1.1 mL/min	812546	2.877	8146	1.20
Less organic phase	802654	4.705	8365	1.16
More organic phase	826549	2.090	8154	1.14

CONCLUSION

A Rapid and Precise RP-HPLC method was developed and validated for the quantification of molnupiravir in bulk as well as in tablet dosage form. Chromatography was carried out by using Phenomenex LunaC (150 x 4.6mm, 5µm) column. The method was optimized using a mixture of Acetonitrile and Potassium dihydrogen phosphate buffer adjusted ph-2.8 with Ortho phosphoric acid in a ratio of 25:75% v/v, as the mobile phase at a flow rate of 1.0ml/min, the detection was carried out at 220nm. The retention time of molnupiravir was found to be 3.174. The method was linear in the concentration range of 10-30µg/ml. The method was precise since the % RSD values of peak areas were found to be below “2". The % recovery values for both the analytes were found to be “99.68” indicating the method was accurate. The specificity of the method was assessed by injections of standard, sample, and blank solutions separately and the chromatograms were recovered. The LOD& LOQ values were 1.3 and 3.9µg/ml respectively. There are very few methods reported on the estimation of molnupiravir. Therefore it was contemplated to develop a simple RP-HPLC method for the routine analysis of molnupiravir. The results of validation were in agreement with the acceptance criteria. This indicates that the method is suitable and can be adopted for regular quality control analysis.

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