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Unlocking Molnupiravir Analysis: A Validated Rp-Hplc Approach

Prashant Jaideorao Burange[1*] Akansha Eknath Borkar[2], Manohar Sakharam Kasdekar[3], Pankaj Haribhau Chaudhary[4], Aditya Rajkumar Khanzode[5], Adnya Durwasrao Bahurupi[6], Ritu Ambadas Bairagi[7], Gauri Vikrant Salode[8]

[12,3,4,5,7]Department of Pharmaceutical Quality Assurance, P. R. Pote Patil College of Pharmacy, Sant Gadge Baba Amravati University, Amravati 444604, Maharashtra, India.

[6,8]Department of Pharmaceutics, P. R. Pote Patil College of Pharmacy, Sant Gadge Baba Amravati University, Amravati 444604, Maharashtra, India.

abstract

A simple, robust, and validated reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed for the quantitative analysis of Molnupiravir in its active pharmaceutical ingredient (API) form. Chromatographic separation was achieved using an Inertsil C8 column (150 × 4.6 mm, 5 µm) with an isocratic mobile phase comprising potassium dihydrogen phosphate buffer (pH 4.1) and methanol in an 80:20 ratio (v/v). The flow rate was maintained at 1.0 mL/min, and detection was performed at 275 nm. The method showed excellent linearity across the concentration range of 50–150% with a correlation coefficient (r^2) of 0.999. Accuracy, expressed as recovery, ranged from 98.08% to 100%, and precision was confirmed by a %RSD of less than 0.2%. The method demonstrated high sensitivity with a limit of detection (LOD) of 0.478 µg/mL and a limit of quantification (LOQ) of 1.592 µg/mL. It remained stable and specific under various forced degradation conditions, including acidic, alkaline, oxidative, photolytic, and thermal stress, establishing its stability-indicating capability. Robustness and ruggedness studies confirmed the method's reliability across small deliberate variations in chromatographic conditions and different analysts and instruments. Validated in accordance with ICH Q2(R2) guidelines, this method is suitable for routine quality control and regulatory analysis of Molnupiravir in bulk drug substances. Given the critical role of Molnupiravir in COVID-19 treatment, the developed method ensures consistent drug quality and supports safe and effective pharmaceutical formulations.

Keywords: *Molnupiravir, RP-HPLC, Method validation*

1. INTRODUCTION

Analytical method development is a fundamental process in pharmaceutical research that ensures the accurate, precise, and reliable quantification of drug substances in bulk materials, formulations, and biological matrices[1]. Molnupiravir is a prodrug that is converted to a ribonucleoside analog that increases mutations in viral RNA and prevents replication. It is effective against SARS-CoV-2 and is used to treat mild-to-moderate COVID-19 in high-risk patients. Treatment should be started as soon as possible, within 5 days of symptom onset, at a dosage of 800 mg every 12 h for 5 days. Common side effects include dizziness, headaches, and gastrointestinal issues. Animal studies have suggested that Molnupiravir may cause bone and cartilage toxicity; therefore, it is not recommended for children under 18 years of age. It may also harm the fetus during pregnancy; therefore, women of childbearing potential should use effective contraception during treatment and for 4 days after. Some countries recommend that men with partners of childbearing potential also use contraception during treatment and for at least 3 months after treatment, although the risk is low. It's unclear if molnupiravir enters human milk, so breastfeeding should be avoided during treatment and for 4 days after the last dose[2].

Molnupiravir is a prodrug that converts to EIDD-1931 after oral administration. This active form is phosphorylated to β-D-N4-hydroxycytidine-triphosphate, which mimics cytidine and uridine and is incorporated into viral RNA. This leads to an accumulation of mutations during replication, inhibiting SARS-CoV-2 and other RNA viruses through a process called viral error catastrophe[3]. Molnupiravir is used to treat mild to moderate COVID-19 in adults who test positive for SARS-CoV-2, are at high risk for severe disease, and when alternative treatments are unavailable or unsuitable[4]. Approved by the MHRA on November 4, 2021, the FDA granted Emergency Use Authorization on December 23, 2021,

and the CDSCO gave restricted emergency use approval on December 28, 2021[5]. Molnupiravir undergoes hydrolysis within the body to produce N4-hydroxycytidine, which is subsequently phosphorylated in tissues to its active 5′-triphosphate form. This active metabolite is incorporated into the viral RNA during replication, causing a buildup of mutations that impair the virus, a mechanism referred to as viral error catastrophe. Furthermore, studies have demonstrated that a remdesivir-resistant strain of mouse hepatitis virus exhibits heightened susceptibility to N4-hydroxycytidine[6].

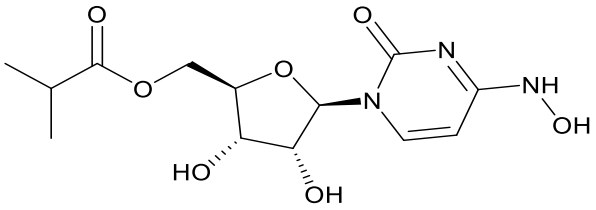

Figure 1: Structure of Molnupiravir

Chromatographic methods are key for purifying analytes and measuring their amounts. Molnupiravir, a new pharmaceutical, is crucial amid ongoing viral infection threats; however, no validated method exists for quantifying it in its raw form[7]. Accurate measurement of a drug and its impurities is vital to ensure efficacy and safety[8,9]. Identifying impurities and degradation products in raw pharmaceutical materials is vital, as they can have pharmacological and toxicological effects. Their detection and concentration are key indicators of drug quality and patient safety[10].

High-performance liquid chromatography (HPLC) is essential to identify drugs and impurities in raw materials and finished pharmaceuticals. It plays a key role in quality checks, pre-formulation, material verification, and stability assessments, making robust and validated HPLC methods crucial[11]. RP-HPLC is the preferred method for quantitatively estimating Molnupiravir due to its high resolution and compatibility with various pharmaceutical matrices[12]. Choosing a suitable stationary phase, like C_{18} silica columns, and optimizing the mobile phase's pH and polarity is crucial for effective separation and symmetrical peak shapes. Method validation parameters—accuracy, precision, linearity, specificity, limit of detection (LOD), and limit of quantitation (LOQ)—are established following ICH Q2(R2) guidelines for regulatory compliance[13]. This study presents a simple, cost-effective, and rapid HPLC method for measuring Molnupiravir content in raw materials. Validated according to the ICH guidelines, the method demonstrated specificity, accuracy, consistency, and stability, successfully accommodating minor variations. All results met the required standards, confirming their reliability for routine analysis. It ensures the quality and stability of antiviral drugs in tablets[14].

2. MATERIALS AND METHODS

2.1 Materials

The study used several materials, including Molnupiravir from Rainbow Pharma Training Lab in Hyderabad, along with HPLC-grade water, acetonitrile, methanol, sodium dihydrogen phosphate, dipotassium hydrogen phosphate, and ortho-phosphoric acid. All reagents were of analytical or HPLC grade and used as received.

2.2 Instrumentation

The analysis was conducted at the Rainbow Training Lab using a Waters HPLC system (Model e2695) with a PDA detector and the Empower 3 software. An electronic balance (Model CY64, Citizen Scale) was used for weighing, and a sonicator (Model LMUC-3, Enertech) was used for sample preparation. Chromatographic separation was performed using an Inertsil C8 column (150 × 4.6 mm, 5 µm).

2.3 Chromatographic Conditions

The chromatographic conditions consisted of a mobile phase containing an 80:20 mixture of KH_2PO_3 buffer (pH 4.1) and methanol, delivered at a flow rate of 1.0 mL/min. Detection was performed at 275 nm with an injection volume of 10 µL, and the total runtime for the analysis was set at 5 min.

3. EXPERIMENTAL WORK

3.1 Procedure:

Inject 10µL of standard was injected into the chromatographic system. The areas for the Molnupiravir peaks were measured, and the % assay was calculated using the formula.

3.2 Preparation of Standard Solution

Molnupiravir (200 mg) was accurately weighed and transferred into a 100 ml volumetric flask, 10 ml of methanol was added, sonicated for 10 min, shaken for 5 min, and filled with water. The above solution was transfer into 1 ml into a 10 ml volumetric flask and diluted to volume with water.

3.3 Preparation of Sample Solution

Accurately weighed and transfer of 200 mg Molnupiravir to the active ingredients was transferred into a 100 ml of volumetric flask and 10 ml of methanol was added, sonicated for 20 min, shaken for 10 min, and mixed with water. Transfers (> 1 ml into a 10 ml of the volumetric flask were diluted with methanol. The solution was filtered through a 0.45µm filter before injection into the HPLC system.

4. RESULTS AND DISCUSSION

4.1 Optimized Method

Table 1: Optimised chromatographic conditions

Parameter	Method
Mobile Phase	KH_2PO_4: Methanol (80:20)
Column	Inertsil C_8 ,150X4.6mm, 5µm
Flow rate	1.0ml/Min
Column Temperature	25°C
Sample Temperature	25°C
Volume	10µl
Run time	5min
Detector	275
pH	4.1
RT	2.230

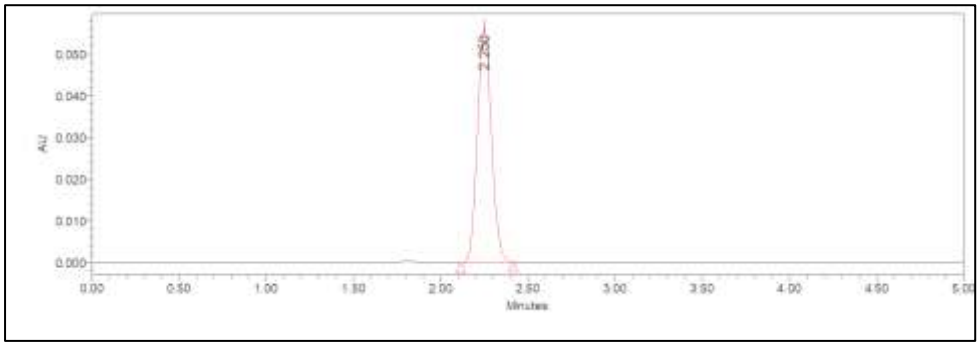

Figure 2: Chromatogram of Optimized Method

Observation: RT was good, and the peak symmetry of both drugs was good. The resolution, theoretical plate count, and tailing were within their limits and were used to validate the method.

4.2 Validation Results

1. System Suitability: A standard solution of Molnupiravir was prepared following the established protocol to ensure the readiness and performance of the testing setup, including the instrument and analyst. This solution was analysed using the HPLC system, and system suitability was assessed using parameters such as retention time, resolution, theoretical plates, tailing factor, and relative standard deviation (RSD).

Table 2: System Suitability Test

Sample Name	Peak Name	Rt (Min)	Area	Tailing	Plate Count
Blank	Molnupiravir	-	-	-	-
Std-1	Molnupiravir	2.230	312843	1.09	3633
Std-2	Molnupiravir	2.231	312786	1.09	3620
Std-2	Molnupiravir	2.233	312842	1.08	3642
Std-2	Molnupiravir	2.231	313344	1.09	3638
Std-2	Molnupiravir	2.237	313835	1.09	3770
Std-2	Molnupiravir	2.236	313625	1.08	3722
Mean Area: 313,212.5					
Standard Deviation (SD): 454.02					
% **Relative Standard Deviation (%RSD)**: 0.14%					

Observation: The method showed consistent retention times (2.230–2.237 min) with minimal variability (%RSD = 0.14%), sharp peaks (tailing factor 1.08–1.09), and sufficient efficiency (theoretical plate count ≈ 3633), indicating that the chromatographic system performed satisfactorily.

2. Specificity: To assess specificity, blank, placebo, standard, and sample solutions were injected into the HPLC system to verify that the molnupiravir peak could be clearly distinguished from any interfering substances. The chromatographic data were reviewed to ensure that no overlapping peaks from excipients, impurities, or degradation products were present, confirming the selectivity of the method for the analyte.

Table 3: Specificity Data of Molnupiravir

Sr.no.	Sample Name	RT (min)	Area (µV*sec)
1	Blank	-	-
2	Standard	2.230	312843
3	Sample	2.250	312843

Observation: No interference from the blank, placebo, or degradation products was observed at the retention time of Molnupiravir, confirming the ability of the method to specifically detect the analyte in the presence of other components.

3. Accuracy: Accuracy was evaluated for Molnupiravir at concentrations of 50, 100, and 150% of the target level using a standard analytical procedure. Each concentration was tested in triplicate, and the recovery percentages were calculated. The results showed that the method consistently yielded values close to the actual target across the tested range.

Table 4: Accuracy Data of Molnupiravir

Spiked level	Sample Weight	Sample Area	µg/ml added	µg/ml found	%Recovery	% Mean
50%	100.00	154421	99.000	98.29	99	99
50%	100.00	154101	99.000	98.08	99	
50%	100.00	154120	99.000	98.09	99	
100%	200.00	311424	198.000	198.21	100	100
100%	200.00	311451	198.000	198.23	100	
100%	200.00	311669	198.000	198.37	100	
150%	300.00	467440	297.000	297.52	100	100
150%	300.00	467402	297.000	297.49	100	
150%	300.00	467541	297.000	297.58	100	

Observation: Recovery studies at three concentration levels (50%, 100%, and 150%) showed percent recoveries between 98.08% and 100%, indicating that the method provided accurate results across the tested range.

4. Linearity: Linearity was assessed using five standard solutions of Molnupiravir at concentrations ranging from 50 ppm to 150 ppm. A calibration graph plotting the concentration on the x-axis and the peak areas on the y-axis showed strong linearity, with a correlation coefficient (r^2) of 0.999.

Table No. 5: Linearity Study of Molnupiravir

MOLNUPIRAVIR					
Sample name	**CONC%**	**Area**	**ug/ml**	**Rt.(min.)**	**Area**
Linearity-50%	50	154112	100	2.229	154112
Linearity-75%	75	232902	150.00	2.239	232902
Linearity-100%	100	311922	200.00	2.247	311922
Linearity-125%	125	390050	250	2.245	390050
Linearity-150%	150	467803	300	2.246	467803

Observation: The method exhibited excellent linearity over the tested concentration range (100–300 µg/mL), with a correlation coefficient (r^2) of 0.999, indicating a direct proportional relationship between peak area and concentration.

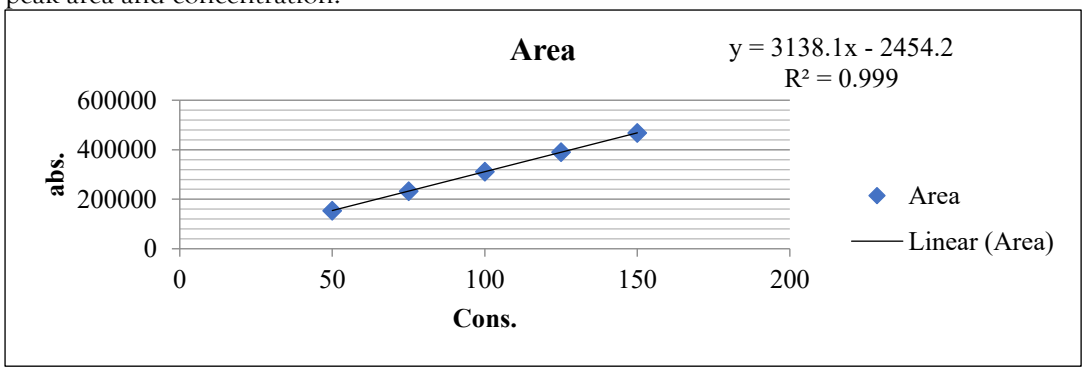

Figure 3: Linearity Curve for molnupiravir

5. Precision: Precision was assessed by analyzing six replicates of a Molnupiravir solution. The relative standard deviation (%RSD) of the peak areas indicated that the system and method demonstrate reliable precision due to low variability.

Table 6: Precision results

Sample Name	Peak Name	Rt (Min)	Area	Sample Weight	% Assay	Statistical Analysis
Precision-1	Molnupiravir	2.239	311864	200.00	99%	Average Assay = 99% Sd = 0.10 %Rds =0.10%
Precision-2	Molnupiravir	2.234	311965	200.00	99%	
Precision-3	Molnupiravir	2.232	312131	200.00	99%	
Precision-4	Molnupiravir	2.247	311273	200.00	99%	
Precision-5	Molnupiravir	2.241	312024	200.00	99%	
Precision-6	Molnupiravir	2.240	312111	200.00	99%	

Observation: Six replicate injections of the same concentration yielded %assay values consistently around 99%, with a very low %RSD of 0.10%, demonstrating the repeatability of the method.

6. Limit Of Detection (Lod) And Limit of Quantification (Loq)

Limit of Detection Determined using the standard deviation technique with the following formula:

Limit of detection = 3.3 × deviation of drug response peak area/slope of drug calibration curve

= 3.3 × 200 / 1255.9

= 0.478 µg/mL

The data gathered proved that the method has sufficient sensitivity for molnupiravir analysis.
Observation: The calculated LOD was 0.478 µg/mL, indicating that the method was capable of reliably detecting minimal amounts of Molnupiravir.
Limit of Quantification: Determined using the standard deviation technique with the following formula:
Limit of quantitation = 10 × deviation of drug response peak area/slope of drug calibration curve
= 10 × 200 / 1255.9
= 1.592 µg/mL
The data gathered proved that the method has enough sensitivity for Molnupiravir analysis.
Observation: The LOQ was determined to be 1.592 µg/mL, indicating that the method can accurately quantify low analyte levels.
Table 7: Lod & Loq results

Peak name	Sample Name	Area	Retention Time (min)
Molnupiravir	LOD	2796	2.217
Molnupiravir	LOQ	13424 3.1	2.206

Robustness: Robustness was evaluated by varying method parameters such as pH and flow rate during Molnupiravir analysis. The method remained reliable and showed no significant impact on these variations.
Table 8: Robustness Studies of Molnupiravir

Parameters	Sample Name	Rt (Min.)	Area	Tailing	Theoretical Plate Count
Wavelength (NM)	NM-1	2.230	312843	1.09	3633
	NM-2	2.231	312786	1.09	3620
Composition	Comp-1	1.829	313365	1.04	3911
	Comp-2	2.468	449601	1.09	3760
pH	pH-1	2.239	313864	1.09	3695
	pH-2	2.234	313965	1.08	3793
Flowrate	Flow-1	1.829	313365	1.04	3911
	Flow-1	2.784	521337	1.19	3587
Temperature	Temp-1	2.030	349526	1.04	3086
	Temp-1	2.468	449601	1.09	3760

Observation: The method remained unaffected by small, deliberate changes in chromatographic conditions (pH, flow rate, temperature, and wavelength). No significant changes were observed in retention time, peak shape, or system suitability metrics.
8. Ruggedness: Ruggedness was evaluated by analysing Molnupiravir samples across different analysts, instruments, and laboratories. The %RSD of the results confirms the robustness of the method for routine use.
Table 9: Ruggedness Studies of Molnupiravir

Variable	Peak area	Rt(min.)	%assay
Analyst 1	3111914.5	2.236	99.2
Analyst 2	311927	2.2395	99.4
Instrument 1	312024	2.2405	99.3
Instrument 2	312111	2.247	99

MEAN	311994.12
SD	92.01
%RSD	0.0295%

Observation: The results across different analysts and instruments showed minimal variation (%RSD = 0.0295%), confirming that the method is rugged and reproducible under various operational conditions.
9. Force degradation studies: Molnupiravir was tested under various stress conditions, including acidic, alkaline, thermal, photolytic, and oxidative stress, and the degradation products were analysed to confirm that the method was stable and that there was no interference with the main peak.

Table 10: Forced degradation studies of Molnupiravir

Degradation study	Sample wt.	Sample Area	% Assay	% Degradation
Acid	200.00	281432	89.56	10.44
Base	200.00	297905	94.81	5.19
Peroxide	200.00	291468	92.76	7.24
Heat	200.00	286013	91.02	8.98
Sunlight	200.00	299991	95.47	4.53

Observation: Molnupiravir was degraded under acidic (10.44%), heat (8.98%), peroxide (7.24%), basic (5.19%), and sunlight (4.53%) conditions. In all cases, the degradation products were well separated from the main peak, confirming that the method was stable.

5. CONCLUSION

The developed RP-HPLC method for quantifying Molnupiravir is simple, robust, and cost-effective, offering high precision, accuracy, and compliance with the ICH validation guidelines. It is well-suited for routine quality control and stability testing in pharmaceutical settings. Given the significance of Molnupiravir in COVID-19 treatment, this validated method ensures the quality, safety, and efficacy of the drug across formulations. This study emphasized the importance of method validation for regulatory compliance and public health assurance. Using analytical rigor, it supports product integrity and fosters confidence among healthcare professionals and patients in the delivery of reliable and effective therapeutic options.

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