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Method Development and Validation for the Quantification of Molnupiravir by RP HPLC in Capsule Dosage form

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ABSTRACT

The aim of this study was to devise an RP-HPLC method that is straightforward, accurate, and economical for determining Molnupiravir levels in pharmaceutical formulations. The chromatographic analysis was conducted using Kromasil C18 column (150 mm x 4.6 mm, 3µ) with a mobile phase consisting of a Buffer solution (Phosphate buffer pH 4) and methanol in a 55:45 % v/v ratio. The flow rate was maintained at 1.0 ml/min, with an injection volume of 10 µL and a total run time of 10 minutes. Detection was performed at a wavelength of 272 nm. The detector exhibited a linear response within the concentration range of 40 – 300 µg/ml, with a correlation coefficient of 1.000. Validation of this analytical method was carried out following ICH guidelines. The developed method has been successfully employed for the estimation of Molnupiravir in pharmaceutical formulations.

Keywords- Molnupiravir, RP-HPLC method development, Validation

INTRODUCTION

In pandemic, known as Coronavirus Disease 2019 (COVID-19), stems from the SARS-CoV-2 virus, presenting a persistent global threat to public health. Its impact is profound, with soaring rates of illness and death worldwide. By April 2023, confirmed cases had exceeded 762 million, with nearly 6.9 million resulting in fatalities. While vaccination efforts have curbed transmission, some communities remain unprotected. Moreover, the emergence of new viral variants and waning vaccine efficacy have fueled recovery in cases. Oral antiviral treatments offer promise in halting disease progression and containing viral spread.

Molnupiravir demonstrates efficacy against Corona viruses, including all known variants of SARS-CoV-2, and boasts a high resistance barrier. Its excellent oral bioavailability enables outpatient administration during the early stages of COVID-19. Upon oral intake, Molnupiravir undergoes rapid metabolism, yielding an active metabolite in the bloodstream, thus enhancing its onset and effectiveness. FDA approval for treating mild to moderate COVID-19 in high-risk patients aged 18 and above underscores its significance. With promising safety profiles and proven efficacy against new Coronavirus strains, Molnupiravir was the great choice for the treatment.

Molnupiravir offers versatility as it can serve as a standalone treatment or be combined with other medications. When used in combination, its antiviral effectiveness against SARS-CoV-2 appears to amplify. For instance, research investigating the concurrent use of Molnupiravir with favipiravir revealed an augmented antiviral response, enabling the use of Molnupiravir in reduced dosages. Furthermore, combining Molnupiravir with sotrovimab demonstrated a decrease in severe COVID-19 outcomes. Studies have also explored the synergistic effects of Molnupiravir with Remdasvir and various other antiviral drugs.

MATERIAL METHODS

2.1 Instrumentation and chromatographic conditions

The investigation employed a Shimadzu LC30 system coupled with a UV detector for high-performance liquid chromatography. This setup comprised a quaternary solvent delivery pump, automatic sample injector, and column thermostat. Data acquisition and analysis were facilitated by Lab solution software. Chromatographic separation utilized a Kromasil C18 column (4.6 mm X 150 mm, 3.0 µm), operating at a

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flow rate of 1.0 mL/min and a temperature of 25°C. The mobile phase consisted of 10 mM potassium dihydrogen phosphate (pH 4.0): Methanol (55:45% v/v), achieving satisfactory retention time. Optimization was performed at 272 nm. The entire process, from data acquisition to processing, was managed through Lab solution software. Each run lasted 10 minutes at ambient temperature.

2.2. Materials

Molnupiravir, the active pharmaceutical ingredient, was kindly provided as gift samples by BDR Pharmaceuticals, located in Baroda, Gujarat. The pharmaceutical dosage form (Molulife-200 – BDR Pharmaceuticals, Baroda, Gujarat) was obtained from a nearby pharmacy. To ensure accuracy in our study, we sourced high-quality solvents meeting HPLC (reverse phase high-performance liquid chromatography) standards from Merck Specialties Private Limited in Mumbai.

2.3 Preparation of Mobile phase.

Mix 10mM Potassium dihydrogen phosphate buffer (pH-4): Methanol (55:45%v/v)

2.4 Buffer Preparation

Preparation of 10mM potassium dihydrogen phosphate buffer pH 4.0: Weigh 1.36 gm of potassium dihydrogen phosphate and dissolve into 1000 mL of water, adjust pH to 4.0 with Orthophophoric acid. Filter the buffer and sonicate it before use.

2.5 Preparation of Diluent:

Water: Methanol (50:50%v/v) was used as a diluent for preparation of various solutions.

2.6 Preparation of standard solution. (200 µg/mL)

A precisely measured quantity of Molnupiravir standard (10 mg) was transferred into clean, dry 10 mL volumetric flasks, dissolved through sonication, and filled up to the final volume with the same diluent (1000 µg/mL). Then, 2 mL of the above stock solution was pipette out and transferred into 10 mL volumetric flasks, followed by dilution with diluent to achieve a solution concentration of 200 µg/mL.

2.7 Preparation of sample solution. (200 µg/mL)

Transfer powder equivalent to 50 mg of Molnupiravir to 50 mL of volumetric flask. Add 35 mL diluent and sonicate to dissolve the content with intermittent shaking for 30 minutes. After sonication dilute to volume with diluent, mix well (1000 µg/mL). Pipette out 2 mL of above sample solution and dilute up to 10 mL with diluent, mix well. (200 µg/mL)

METHOD VALIDATION

The method was validated according to ICH guidelines in terms of Specificity, Linearity, Accuracy, Precision, and Robustness.

3.1 Specificity

Specificity refers to the capacity to conclusively evaluate the analyte even in the presence of potentially interfering components such as impurities, degradation products, matrix, and others.

3.2 System suitability Parameters

System suitability parameters were assessed by creating standard solutions of Molnupiravir and injecting these solutions five times. Parameters including peak tailing, retention time, peak area, and USP plate count were subsequently determined.

3.2. Linearity

The method's linearity was evaluated by preparing a series of solutions within the range of 20 to 400 µg/mL The resulting peak areas were plotted against the corresponding concentrations.

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3.2.1. Preparation of linearity solutions

Preparation of Standard Stock Solutions: 20 mg of Molnupiravir working standards were accurately weighed and transferred into a clean, dry 50 mL volumetric flask. 15 mL of diluent was added, and sonication was performed to dissolve. The flask was labeled as Standard Stock Solution. (400 µg/mL) From this stock solution, solutions were prepared to obtain concentrations of 20%, 50%, 80%, 100%, 150%, and 200% of the standard solutions. Details for preparing the Linearity Solution are provided in Table-1.

3.3. Precision

3.3.1. Method precision (repeatability)

Method precision or repeatability can be assessed by injecting six working standard solutions and conducting six sample injections. The areas of all injections were recorded, and the % Relative Standard Deviation as well as the % assay was calculated.

3.3.2. Intermediate precision

Intermediate precision can be evaluated by injecting six working standard solutions and conducting six sample injections on different days, performed by different operators or using different instruments. The study of intermediate precision was conducted at 100% concentration in triplicate on different days. The areas of all injections were recorded, and % Relative Standard Deviation as well as % assay was calculated.

3.4. Accuracy

Accuracy is tested by the standard addition method at three different levels 80, 100 and 120%. The percentage recoveries of Molnupiravir present in the pharmaceutical dosage form were calculated.

3.5. Method robustness

The robustness of the developed method was evaluated by intentionally making minor adjustments, including variations in flow rate (± 0.1 mL/min), changes in mobile phase pH (±0. 2%), column oven temperature (± 5°C), and alterations in detection wavelength (± 2 nm), while adhering to the optimized method.

RESULTS AND DISCUSSION.

4.1. Development and optimization of HPLC

The primary objective of this study was to develop a stability-indicating RP-HPLC (reverse phase high performance liquid chromatography) method for the simultaneous determination of Molnupiravir in pharmaceutical formulations. Various solvents, including methanol, water, and acetonitrile, were evaluated for the solubility of the active pharmaceutical ingredient. Ultimately, a mixture of water and methanol (50:50%v/v) was selected as the diluent due to its optimal solubility. Several mobile phase compositions, such as acetonitrile with Ammonium acetate buffer, Methanol with OPA buffer, and acetonitrile with OPA buffer, were initially tested with a flow rate of 1 mL/min. However, the desired theoretical plate count, retention time, and tailing factor were not achieved. Consequently, a mobile phase consisting of Potassium dihydrogen phosphate buffer pH-4.0 and Methanol at a flow rate of 1.0 mL/min was chosen. Various columns at different temperatures (30, 35, 40, 45°C) were initially investigated, but satisfactory retention time, run time, and peak shape were not achieved until an Inertsil Kromasil C18 column (4.6 mm X 150mm, 3.0 µm) was utilized at ambient temperature with a run time of 10 minutes. The method was further optimized by adjusting the mobile phase composition and ratio. The optimized wavelength for detecting Molnupiravir was found to be 272 nm. Details of the optimized chromatographic conditions are provided in Table-2.

4.2. System suitability parameters

System suitability tests were conducted prior to validation, and the parameters met acceptable criteria: retention time for Molnupiravir was 3.38 min, plate count exceeded 2000, peak tailing was below 2, and the %RSD of peak areas from six injections was ≤ 2%. Consequently, the proposed method was effectively employed for routine analysis without any complications. Results are mentioned in Table-3.

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4.3. Linearity range

The linearity range was in the interval of Favipiravir (40–300 µg/ml). These were represented by a linear regression equation as follows:

y = 11,879 x+ 2506.6. (r2 = 1.000), Regression line was established by least squares method and correlation coefficient (r2) for favipiravir was found to be greater than 0.999. Hence the curves established were linear. Results are mentioned in Table-4 and graph is provided in figure-3.

4.4. Precision

4.4.1 Method Precision: Six replicate injections at a consistent concentration were analyzed on both the same day and two separate days to assess precision variation. The %RSD for Molnupiravir was found to be within the acceptable limit of ≤ 2%. Thus, the method demonstrates reproducibility across same days, different days indicating its precision. Results of precision are mentioned in Table 5(a).

4.4.2 Intermediate Precision:

Intermediate precision was performed on same days and different days on standard solution. The results are mentioned in Table-5 (b). Results show that the developed method was precise.

4.5. Accuracy

The percentage recoveries for Molnupiravir at levels of 80%, 100%, and 120% were determined to be 99.8%, 100.2%, and 100.9%, respectively. These recovery study results unmistakably indicate the accuracy of the proposed method. Moreover, the % RSD of three replicates was found to be less than 2%, confirming the method's accuracy across the considered range. Results are mentioned in Table-6.

4.6 Robustness

The robustness of the method was assessed under four distinct conditions: alterations in flow rate, pH, column oven temperature, and wavelength. The study demonstrated that minor deliberate adjustments in these conditions did not notably impact the method's performance, as indicated by the values obtained in the measurement of peak area of the analytes. Results of robustness are mentioned in Table-7.

CONCLUSION

This paper introduces a novel, straightforward, efficient, rapid, and precise stability-indicating Reverse Phase High Performance Liquid Chromatographic method for the simultaneous estimation of Molnupiravir in pharmaceutical formulations. The method was validated following ICH guidelines, covering aspects such as Linearity, Specificity, Accuracy, Precision, and Robustness. Our findings suggest that the developed method is specific to Molnupiravir in pharmaceutical drug products. Therefore, this method holds promise for effective application in drug testing laboratories and pharmaceutical industries for the estimation of Molnupiravir. This method can be helped for estimation of Molnupiravir in biological matrixes as well.

List of tables

Table-1: Preparation of linearity solution.

Linearity Stock preparations: 20 mg of Molnupiravir → upto 50 mL With Diluent (400 µg/mL)			
Linearity Dilutions			
Level	Linearity stock taken (mL)	Diluted upto(mL)	Target concentration (µg/mL)
Level-1 (20%)	2.5	25	40

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Level-2 (50%)	2.5	10	100
Level-3 (80%)	10	25	160
Level-4 (100%)	**5**	**10**	**200**
Level-5 (120%)	6	10	240
Level-6 (150%)	7.5	10	300

Table-2: Optimized chromatographic conditions

Parameters	**Description**
Column	Kromasil C18 (4.6 mm X 150mm, 3.0 µm)
Flow rate	1.0 mL/min
Column Temp (°C)	Ambient
Injection volume	10 µL
Detection wavelength	272 nm
Diluent	Water : Methanol (50:50% v/v)
Run time	10 minutes (Retention time- 3.38 minute)
Mobile phase	10 mM potassium dihydrogen phosphate (pH 4.0): Methanol (55:45% v/v)

Table-3: Result of system suitability parameter.

Injection	**Retention time**	**Area**	**Tailing factor**	**Theoretical plates**
Injection-1	3.386	2388864	1.1	3715
Injection-2	3.381	2398701	1.1	3726
Injection-3	3.376	2382770	1.1	3705
Injection-4	3.376	2392791	1.1	3704
Injection-5	3.386	2397766	1.1	3716
Mean		2392178	1.1	3713
%RSD		0.3	NA	NA

Table-4: Results of Linearity parameter.

Level	**Concentration (µg/mL)**	**Area**
Level-1 (20%)	40.00	479084

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Level-2 (50%)	100.00	1181364
Level-3 (80%)	160.00	1899509
Level-4 (100%)	200.00	2390085
Level-5 (120%)	240.00	2802849
Level-6 (150%)	300.00	3586248
Correlation co-efficient (r)		**1.000**
Y-intercept		**2506.614**
Slope		**11879**
Plot (Visual)		**Linear**

Table-5(a) Results of Method Precision

Sample ID	% Assay	% Mean	% RSD
Set-1	102.0	100.2	1.2
Set-2	100.2		
Set-3	98.5		
Set-4	99.3		
Set-5	101.1		
Set-6	100.0		

Table-5(b) Results of intermediate precision.

Sample ID	Interday precision			Intraday precision		
	% Assay	% Mean	% RSD	% Assay	% Mean	% RSD
Set-1	102.0	100.3	1.7	100.3	100.2	0.3
Set-2	100.4			99.9		
Set-3	98.5			100.5		

Table-6: Results of accuracy

Level	Sample ID	Amount Added (µg)	Amount Recovered (µg)	% Recovery	Mean	% RSD
80%	Set-1	158.025	159.067	100.7	99.8	1.3
	Set-2	161.713	158.992	98.3		
	Set-3	158.523	158.992	100.3		
100%	Set-1	198.403	199.032	100.3	100.2	0.3
	Set-2	199.400	199.129	99.9		

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	Set-3	198.204	199.274	100.5		
120%	Set-1	231.304	231.520	100.1	100.9	0.8
	Set-2	229.310	233.365	101.8		
	Set-3	230.307	232.025	100.7		

Table-7: Results of robustness parameters.

Sr. No.	Parameters	Altered Condition	% Assay	% RSD of 5 injections of standard	Theoretical Plates	Tailing Factor
1	Flow Rate	0.9 mL/min	99.3	0.8	2987	1.1
		1.0 mL/min (As such)	100.2	0.3	3713	1.1
		1.1 mL/min	99.9	1.1	3411	1.0
2	pH of Mobile phase Buffer	2.8	98.9	1.4	2768	1.1
		3.0 (As such)	100.2	0.3	3713	1.1
		3.2	99.1	0.6	2710	1.0
3	Change in wavelength	270 nm	100.0	1.2	3786	1.0
		272 nm (As such)	100.2	0.3	3713	1.1
		274 nm	99.8	1.0	3462	1.1
4	Change in column oven temperature	35°C	99.7	0.8	3346	0.8
		40°C (As such)	100.2	0.3	3713	1.1
		45°C	99.6	1.0	4353	1.9

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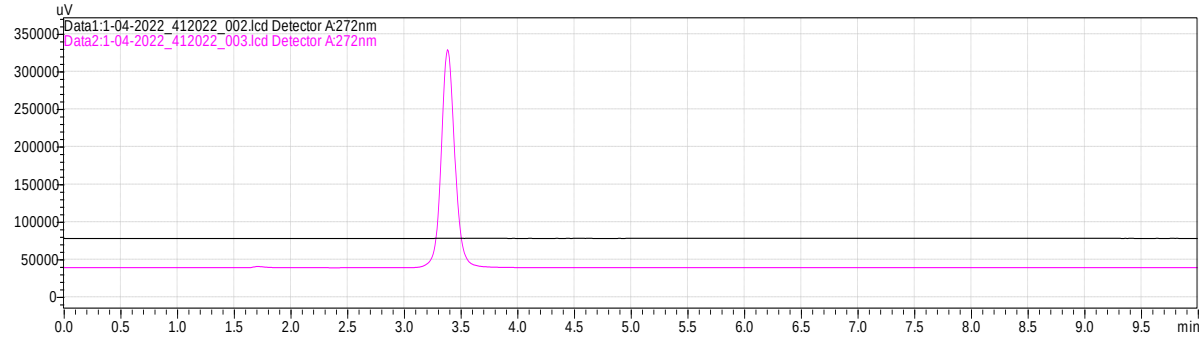

Fig-1: Overlay chromatogram of blank and Molnupiravir:

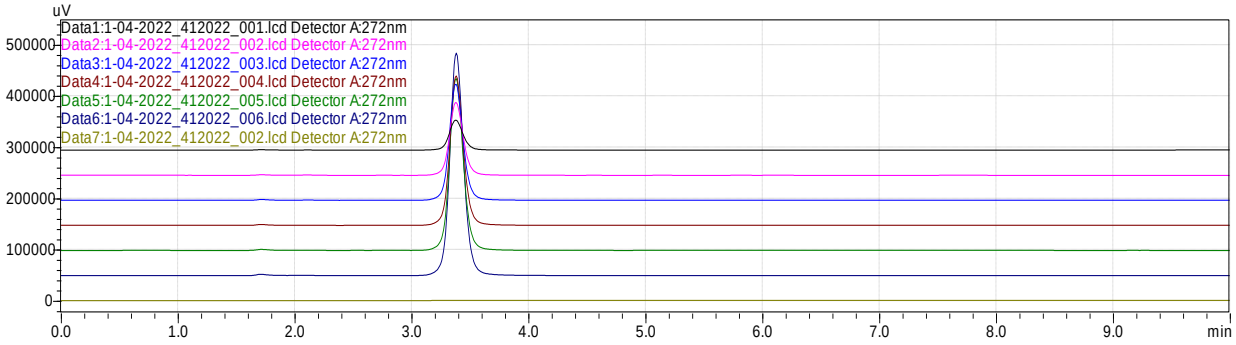

Fig-2 Linearity Overlay of Molnupiravir

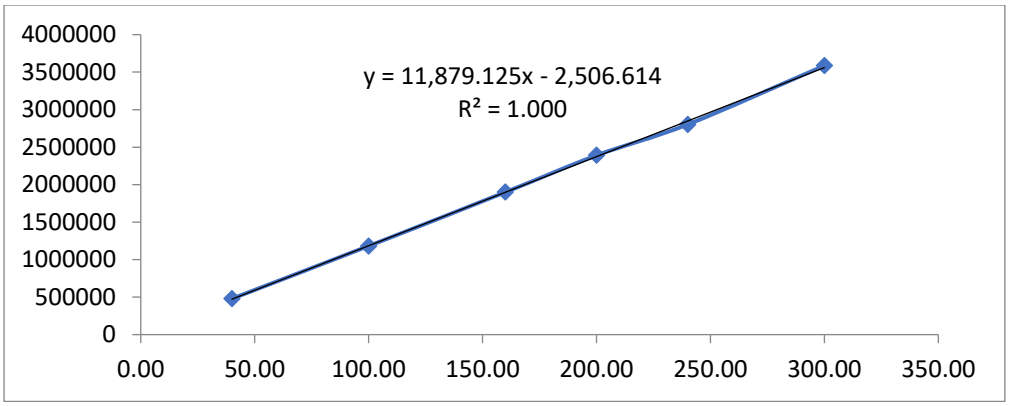

Fig-3: Linearity graph

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