RESEARCH PAPER

DEVELOPMENT AND VALIDATION OF A SIMPLE, RAPID AND ROBUST RP-HPLC METHOD FOR THE ESTIMATION OF MOLNUPIRAVIR IN BULK DRUG AND MARKETED CAPSULE FORMULATION

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Abstract
Background: Molnupiravir is an orally active antiviral nucleoside analogue prodrug used for the management of COVID-19. The development of a reliable, rapid and reproducible analytical method is essential for the quality control of Molnupiravir bulk drug and pharmaceutical formulations.
Objective: The present study aimed to develop and validate a reverse-phase high-performance liquid chromatographic (RP-HPLC) method for the quantitative determination of Molnupiravir in bulk drug and marketed capsule formulation.
Methods: Chromatographic separation of Molnupiravir was performed using a reversed-phase C18 analytical column. The developed method was evaluated for system suitability, linearity, accuracy, precision, robustness, ruggedness, limit of detection (LOD), limit of quantification (LOQ), and assay of the marketed formulation in accordance with analytical method-validation principles. Linearity was evaluated over 10–50 µg/mL. Accuracy was investigated at 80%, 100%, and 120% concentration levels. Precision was evaluated through repeatability and intra-day/inter-day studies.
Results: The developed method exhibited satisfactory chromatographic performance with a retention time of approximately 3.6 min. The calibration response was evaluated over 10–50 µg/mL. The LOD and LOQ were found to be 0.4548 and 1.3782 µg/mL, respectively. Recovery studies were within 98–102%. Repeatability demonstrated 101.34% mean assay with %RSD of 1.44. Analysis of Molnatris® 200 mg capsules showed 99.51% label claim with %RSD of 0.064. Ruggedness studies using different analysts showed acceptable reproducibility.
Conclusion: The developed RP-HPLC method demonstrated satisfactory linearity, accuracy, precision, sensitivity, robustness and ruggedness and can be considered suitable for routine quantitative analysis of Molnupiravir in bulk drug and marketed capsule formulation.
Keywords: Molnupiravir, RP-HPLC, method development, method validation, pharmaceutical analysis, assay, antiviral drug.
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INTRODUCTION

Molnupiravir is an orally administered antiviral agent belonging to the nucleoside analogue class. It acts as a prodrug of N4-hydroxycytidine and interferes with viral RNA replication. The thesis describes Molnupiravir as a nucleoside analogue prodrug with activity against RNA viruses.

Analytical method development plays an important role in pharmaceutical quality control because reliable analytical procedures are required for identification, quantification and quality assessment of drug substances and pharmaceutical formulations. A properly validated chromatographic method provides confidence in the accuracy and reproducibility of analytical measurements.

Several analytical procedures have been reported for Molnupiravir using different stationary phases, mobile phases, flow rates and detection wavelengths. For example, published methods have used C18 columns with different aqueous-organic mobile-phase compositions and detection wavelengths ranging from approximately 230 to 240 nm.

Previous literature also reports RP-HPLC methods with linear ranges such as 10–150 µg/mL and 10–50 µg/mL, with satisfactory recovery and precision.

Therefore, the present investigation was undertaken to develop and validate a simple, reliable and reproducible RP-HPLC method for estimation of Molnupiravir in bulk drug and marketed capsule formulation.

OBJECTIVES

1. To develop an RP-HPLC method for estimation of Molnupiravir.
2. To select suitable chromatographic conditions.
3. To establish suitable system suitability parameters.
4. To determine the linearity and range of the proposed method.
5. To evaluate accuracy by recovery studies.
6. To determine repeatability and precision.
7. To investigate robustness of the method.
8. To determine LOD and LOQ.
9. To evaluate ruggedness.
10. To apply the developed method for assay of

Molnupiravir in marketed capsules.

1. MATERIALS AND METHODS

1.1 Selection and Procurement of Drug

Molnupiravir was selected as the model drug and procured from **Swapnroop Drug and Pharmaceutical**. The marketed Molnupiravir formulation, **Molnatris Capsules (200 mg)**, was obtained from the local market for analysis.

1.2 Reagents and Chemicals

HPLC-grade **acetonitrile, methanol, water, 0.1% OPA, and 0.1% formic acid** were used for the analytical study. The chemicals were obtained from Merck Ltd., India.

1.3 Selection of Analytical Technique

Reverse-phase HPLC was selected for the estimation of Molnupiravir. Analysis was performed using an **Agilent HPLC system with an autosampler and DAD detector**, equipped with an Agilent C18 column (250 × 4.6 mm, 5 µm) and ChemStation software.

Column	Agilent C18, 250 × 4.6 mm, 5 µm
UV-Spectrophotometer	Analytical Technologies Ltd.
pH Meter	VSI 1-B
Balance	WENSAR™ High Resolution Balance
Sonicator	Ultrasonic electronic instrument

2. EXPERIMENTAL WORK

2.1 HPLC

1) Selection of Analytical Technique

HPLC was selected for the estimation of Molnupiravir. The analysis was performed using an **Agilent HPLC system with autosampler and DAD/UV detector**, equipped with an Agilent C18 column (4.6 × 250 mm, 5 µm) and ChemStation 10.1 software.

a) Selection of Stationary Phase

An **Agilent C18 reverse-phase column** (4.6 × 250 mm, 5 µm) was selected because of its reproducibility, stability and suitability for pharmaceutical HPLC analysis.

b) Solubility Study

Solubility studies were performed using different solvents. Molnupiravir was found to be **freely soluble in methanol** and poorly soluble in water containing 0.1% formic acid/buffer at pH 3.2.

c) Chromatographic Conditions

Table No. 7: Chromatographic Conditions Used for Method Development

Sr. No.	Parameter	Condition
1	HPLC	Agilent Tech. Gradient System with Auto Injector, UV Detector
2	Software	ChemStation 10.1
3	Column	Agilent C18, 4.6 × 250 mm
4	Particle size	5 µm
5	Stationary phase	C18

1.4 Standard and Sample Preparation

A standard stock solution of Molnupiravir was prepared by dissolving **10 mg in 10 mL methanol** to obtain a concentration of **1000 µg/mL**. The formulation stock solution was prepared by dissolving **11.15 mg sample in 10 mL methanol**.

For accuracy studies, solutions corresponding to **80%, 100%, and 120% levels** were prepared using the marketed formulation and standard Molnupiravir solution and diluted with mobile phase.

Tab no Instruments and Equipment

Instrument	Details
HPLC	Agilent, DAD detector, ChemStation

6	Mobile phase	Methanol : 0.1% Formic acid water (85:15)
7	Detection wavelength	236 nm
8	Flow rate	1 mL/min
9	Temperature	Ambient
10	Injection volume	20 µL
11	pH	3.2
12	Run time	15 min
13	Filter	0.45 µm

2.2 UV–Visible Spectrophotometer

UV–Visible spectrophotometry was selected for preliminary estimation and spectral investigation of Molnupiravir in the wavelength range of **200–400 nm**.

Instrument

A double-beam Analytical Technologies® UV–Visible spectrophotometer equipped with a deuterium lamp and computer-based data-processing system was used. Quartz cuvettes with a **1 cm path length** were employed.

2.3 Selection of UV Spectrum of Molnupiravir

About **10 mg of Molnupiravir** was accurately weighed and transferred into a 10 mL volumetric flask. Methanol was added and the solution was sonicated for approximately 15 min. The volume was made up with methanol to obtain a **1000 µg/mL stock solution**. An appropriate aliquot was further diluted with methanol and subjected to UV spectral scanning in the **200–400 nm** range.

2.4 Study of Chromatographic Conditions

A 10 mg quantity of Molnupiravir working standard was dissolved in methanol and diluted to obtain a **1000 µg/mL stock solution**. After sonication, an aliquot was diluted with the mobile phase to obtain a **10 µg/mL working solution**.

The solution was chromatographed using an Agilent C18 column, 20 µL injection volume, 1 mL/min flow rate, 236 nm detection wavelength and 15 min run time.

HPLC Conditions

- **Column:** Agilent C18, 250 × 4.6 mm, 5 µm
- **Injection volume:** 20 µL
- **Flow rate:** 1 mL/min
- **Detection:** 236 nm
- **Run time:** 15 min

1) Method Development of HPLC

Different mobile-phase compositions were evaluated during method development.

Table No. 8: Selection of Mobile Phase

Sr. No.	Mobile Phase	pH	Flow Rate	Detection
1	0.1% Formic acid water : Methanol (95:5)	3.0	1.0 mL/min	236 nm
2	0.1% Formic acid water : Methanol (90:10)	3.0	1.0 mL/min	236 nm
3	0.1% Formic acid water : Methanol (80:20)	3.0	0.7 mL/min	236 nm
4	Methanol : 0.1% Formic acid water (80:20)	3.0	0.7 mL/min	236 nm
5	Methanol : 0.1% Formic acid water (80:20)	3.0	0.7 mL/min	236 nm
6	Methanol : 0.1% Formic acid water (60:40)	3.0	1.0 mL/min	236 nm

Preparation of Stock Standard Solution

Accurately weigh **10 mg of Molnupiravir** and transfer it into a 10 mL volumetric flask. Add methanol, sonicate for 15 min and make up the volume with the same solvent to obtain a **1000 µg/mL stock solution**. Further, 0.1 mL of the stock solution was diluted to 10 mL with mobile phase to obtain the required working solution.

2.5 Analytical Method Validation

The developed HPLC method was validated according to **ICH Q2(R1) guidelines** for linearity, accuracy, precision, robustness, detection limit, and quantitation limit.

1) Linearity

Linearity was evaluated using Molnupiravir concentrations of **10–50 µg/mL**. Peak area was plotted against concentration and the calibration curve and regression parameters were determined.

Table No. 9: Linearity Concentrations

Sr. No.	Molnupiravir Concentration (µg/mL)
1	10
2	20
3	30
4	40
5	50

2) Accuracy

Accuracy was determined by the **standard addition/recovery method** at 80%, 100%, and 120% levels. The prepared solutions were analyzed by HPLC in triplicate, and percentage recovery was calculated.

Table: Accuracy Levels

Level	Standard Added
80%	8 µg/mL
100%	10 µg/mL
120%	12 µg/mL

3) Repeatability

Repeatability was evaluated using a **40 µg/mL Molnupiravir solution**. The sample was injected repeatedly and peak areas were recorded. The **%RSD** was calculated to assess repeatability.

4) Precision

Precision was assessed as intra-day and inter-day precision and expressed as **standard deviation and %RSD**.

a) Intra-day Precision

Molnupiravir solutions of **20, 30 and 40 µg/mL** were analyzed three times on the same day and %RSD was calculated.

b) Inter-day Precision

Molnupiravir solutions of **20, 30 and 40 µg/mL** were analyzed on different days and
%RSD was calculated.

5) Robustness

Robustness was evaluated using a **40 µg/mL Molnupiravir solution** by deliberately varying chromatographic conditions, including mobile-phase composition and detection wavelength. The effect of these changes was evaluated in triplicate.

6) Detection Limit

The detection limit (DL) was calculated using:

DL = 3.3σ/S

Where:

σ = Standard deviation of the y-intercepts S = Slope of the ca

7) Quantitation Limit

The quantitation limit (QL) was calculated using:

QL = 10σ/S

Where:

σ = Standard deviation of the y-intercepts of regression lines S = Slope of the ca

8) Analysis of Marketed Formulation

A quantity equivalent to **11.15 mg of marketed Molnupiravir capsule powder** was dissolved in 10 mL methanol and sonicated for 15 min. About 0.3 mL of the supernatant was diluted to 10 mL with mobile phase and injected into the HPLC system. The drug content was calculated using the regression equation obtained from the calibration curve.

9) Specificity

Specificity was evaluated by preparing and injecting Molnupiravir sample solutions into the HPLC system. The obtained chromatograms were examined for the presence of the Molnupiravir peak and possible interference from other components.

3. RESULTS AND DISCUSSION

3.1 Preliminary Studies on Molnupiravir

1) Melting Point

The procured Molnupiravir reference standard showed a melting point in the range of **156–159°C**, indicating satisfactory agreement with the reported characteristics of the drug.

2) Solubility

Molnupiravir was found to be:

- **Soluble:** Methanol, DMSO and DMF
- **Insoluble:** n-Heptane and Hexane

3) UV Spectroscopy

The UV spectrum of Molnupiravir was recorded in methanol over the range of **200–400 nm**. The drug showed maximum absorption (**λmax**) at **236 nm**. Therefore, **236 nm** was selected as the detection wavelength for further HPLC analysis.

Fig. No. 6: UV spectrum of Molnupiravir.

4) Chromatographic Method Development

Different mobile-phase compositions were evaluated using an Agilent C18 column to obtain suitable peak shape, retention and chromatographic efficiency.

Table No. 10: Optimization of Chromatographic Conditions

Trial	Column	Mobile Phase	Flow Rate	λmax	Observation	Decision
1	C18, 250 × 4.6 mm, 5 µm	0.1% Formic acid water:Methanol (5:95)	1 mL/min	236 nm	Broad peak	Rejected
2	C18, 250 × 4.6 mm, 5 µm	0.1% Formic acid water:Methanol (10:90)	1 mL/min	236 nm	Broad peak	Rejected
3	C18, 250 × 4.6 mm, 5 µm	0.1% Formic acid water:Methanol (20:80)	1 mL/min	236 nm	Broad peak	Rejected

4	C18, 250 × 4.6 mm, 5 µm	0.1% For mic acid water:Methanol (20:80)	1 mL/min	236 nm	Ghost peak	Rejected
5	C18, 250 × 4.6 mm, 5 µm	Methanol:0.1% Formic acid water (60:40)	1 mL/min	236 nm	Unsatisfactory peak	Rejected
6	**C18, × 4.6 mm, 5 µm**	**Methanol:0.1% Formic acid water (85:15)**	**1 mL/min**	**236 nm**	**Sharp peak**	**Selected**

Chromatogram of Final Trial 6:

Fig No 12: Chromatogram of final Trial 6

Table No .16: Result for Chromatogram of Final Trial 6

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	3.605	2801.60059	10873	0.70	-

Observation: Sharp peak were obtained, so method was selected.

The final chromatographic conditions selected were as follow:

- Analytical column : Agilent C18 Column (250mm x 4.6 mm, 5µm particle size).
- Injection volume : 20µl
- Flow rate : 1 ml/min
- Mobile phase : Methanol : water (0.1% Formic acid) (85: 15 % V/V)
- Detection : 236 nm
- Run Time : 15 min

Blank chromatogram:

Chromatogram of blank Standard chromatogram of Molnupiravir

Fig No.13: Chromatogram of standard Molnupiravir

\Table No.17: Result for standard Chromatogram of Molnupiravir

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	3.576	736.02448	10717	0.98	-

3.2 Analytical of Method Validation: ‘

1 **Linearity:**

From Molnupiravir standard stock solution, different working standard solution (1050 µg/ml) for HPLC were prepared in mobile phase 20µl of sample solution was injected into the chromatographic system using volume loop injector. Chromatograms were recorded.

Table No 29. Linearity of Molnupiravir (HPLC)

Sr. No.	entration µg/ml	Area Molnupiravir
1	10	735.25
2	20	1339.7
3	30	1902.7
4	40	2531.06
5	50	3112.81

Overlay of Linearity (10-50 µg/ml)

Fig.No.25 Calibration curve of Molnupiravir (HPLC)

2 **Accuracy :-**

Table No 33: Result for Chromatogram of Accuracy 100%

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	3.595	2529.02783	10572	0.93	-

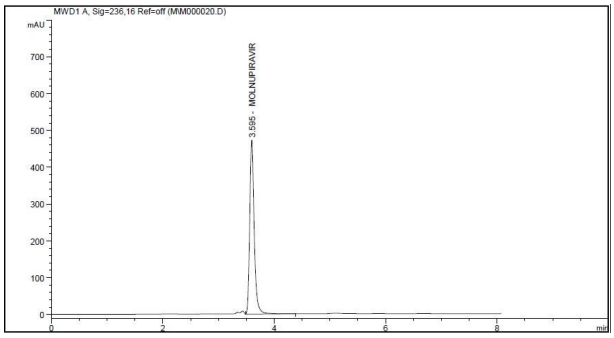


Fig. No .28 Chromatogram of Accuracy 100%

3 **System suitability parameters:**

To ascertain the resolution and reproducibility of the proposed chromatographic system for estimation of Molnupiravir system suitability parameters were studied

Table No 39: Result for Chromatogram of System suitability

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	3.584	2576.60986	10974	0.92	-

Fig No.32 Chromatogram of System suitability

4 **Precision:-**

The method was established by analyzing various replicates standards of Molnupiravir. All the solution was analyzed thrice in order to record any intra-day & inter-day variation in the result that concluded.

Table No 43: Result for Chromatogram of Precision (20 mcg)

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	3.399	1358.37708	11547	0.94	-

Chromatogram of Intra-day Precision:

5 **Robustness:**

The Robustness of a method is its ability to remain unaffected by small deliberate changes in parameters. To evaluate the robustness of the proposed method, small but deliberate variations in the optimized method parameters were done. The effect of changes in mobile phase composition and flow rate, wavelength on retention time and tailing factor of drug peak was studied.

The mobile phase composition was changed in (±1 ml/min^{-1}**)** proportion and the flow rate was varied by of optimized chromatographic condition. The results of robustness studies are shown in **(Table No.64**).Robustness parameters were also found satisfactory; hence the analytical method would be concluded.

1) Mobile phase composition Change : 84 ml Meoh + (0.1%Formic acid) 16 ml Water

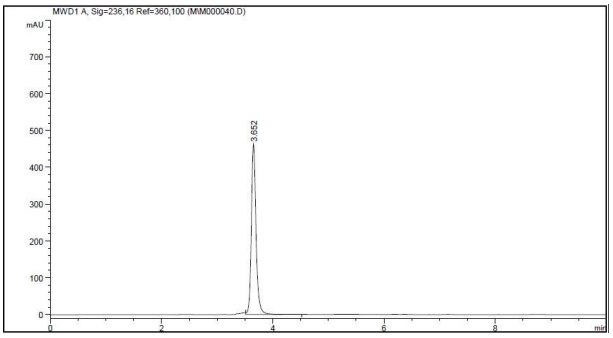


Fig No .47 Chromatogram of Mobile phase composition change 84 ml Meoh +0.1 %(Formic acid) 16 ml Water

Table No 56: Result for Chromatogram of Mobile phase composition change

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	3.652	2706.46289	10102	0.92	-

2) Mobile phase composition Change: 86 ml Meoh+ 0.1%(Formic acid) 14 ml Water

Fig No.49 Chromatogram of Mobile phase composition change 86 ml Meoh +0.1% (Formic acid) 14 ml Water

Table No 58: Result for Chromatogram of Mobile phase composition change

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	3.668	2710.5456	10753	0.97	-

3) Wavelength Change 235 nm

Fig. No 51: Chromatogram of wavelength change 235 nm

***Table No 60: Result for Chromatogram of wavelength change 235* nm**

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	3.636	2580.77588	10759	0.96	-

4) Wavelength Change 259 nm

Fig. No 53. Chromatogram of wavelength change 237 nm

Table No 62: Result for Chromatogram of wavelength change 237 nm

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	3.636	2835.5556	10754	0.96	-

5) Limit Detection

The LOD is the lowest limit that can be detected. Based on the S.D. deviation of the response and the slope The limit of detection (LOD) may be expressed as:

LOD = 3.3 (SD)/S
= 3.3 X 8.20/ 59.46
= 0.4548

Where, SD = Standard deviation of Y intercept S = Slope

- The LOD of Molnupiravir was found to be 0.4548 (µg/mL) analytical methods that concluded.

6) Limit Quantification

The LOQ is the lowest concentration that can be quantitatively measured. Based on the S.D. deviation of the response and the slope,

The quantitation limit (LOQ) may be expressed as:

LOQ = 10 (SD)/ S
=10 X 8.20/ 59.46
= 1.3782

Where, SD = Standard deviation Y intercept
S = Slope

The LOQ of Molnupiravir was found to be **1.3782** (µg/mL) analytical methods that concluded.

3.3 Analysis of formulation Procedure

Equivalent to 11.15 mg Molnupiravir into 10 ml volumetric flask. Add about 10 ml of diluents and Sonicate to dissolve it completely and make volume up to the mark with diluent. Mix well and filter through 0.45 µm filter. Further pipette 0.3 ml of the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluents. (30 µg/ml). The simple chromatogram of test Molnupiravir Shown in the amounts of Molnupiravir per capsules were calculated by extrapolating the value of area from the calibration curve.

Analysis procedure was repeated three times with formulation. Assay for %Lable claim for
%RSD Calculated,

Brand Name : Molnatris (200 mg)

Total weight of 20 Powder wt. = 2.25 gm
Avg. Powder Weight = 0.223g/cap
Eq.Wt for 10 mg= 10 x 223 / 200 = 11.15 mg
Take 11.15 mgs in 10 ml Methanol i.e. = 1000 µg/ml tab solution ***-TAB STOCK –II***

- Take 0.3 mlz in 10 ml Mobile Phase i.e. = 30µg/ml tab solution

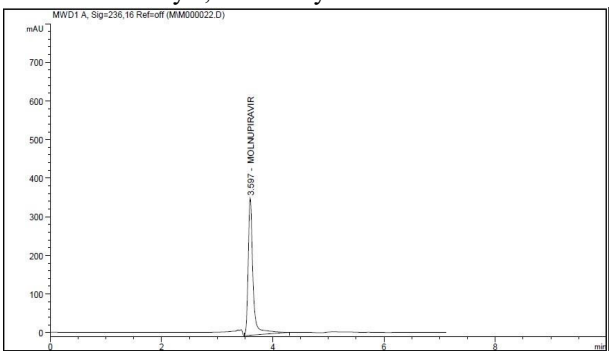
Fig No.55: Chromatogram for Marketed Formulation

Table No 65: Result for Chromatogram of Marketed Formulation

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	3.597	1914.52026	11156	0.92	-

3.4 Ruggedness

The degree of reproducibility of test result obtains by the nalysis of same sample under variety of Condition. Such as different analyst, laboratory Different instrument.



Conclusion ***Fig No.57: Chromatogram for Analyst-1 Ruggedness (30 mcg) Table .No 69. Chromatogram of Ruggedness***

No.	RT[min]	Area[mV*s]	TP	TF	Resolution
1	3.597	1914.52026	11156	0.92	-

The developed **RP-HPLC method provided selective quantification of Molnupiravir**. The method demonstrated satisfactory **accuracy, precision, robustness, and specificity** for the estimation of Molnupiravir. The optimized method offered a **short retention time, isocratic operation, simple and economical mobile phase, readily available C18 column, UV detection, and satisfactory peak resolution**. Therefore, the developed RP-HPLC method can be considered suitable for the **routine analysis and quality control of Molnupiravir in pharmaceutical formulations**.

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