RESEARCH ARTICLE

New Stability Indicating RP-HPLC Method for Estimation of the Drug Molnupiravir

Mahesh Deshpande[1*], Farhat Shaikh[1], Vijay Sable[2], Khushabu Patil[3], Machchhindra R. Holam[4], Harshal Tare[5]

[1]*Department of Pharmaceutical Quality Assurance, Amrutvahini College of Pharmacy, Sangamner, Dist. Ahmednagar, Affiliated to Savitribai Phule Pune University, Pune, Maharashtra, India.*
[2]*Department of Pharmaceutics, Lokmangal College of Pharmacy, Solapur, Affiliated to Dr. Babasaheb Ambedkar Technological University, Lonere, Maharashtra, India.*
[3]*Department of Quality Assurance, Arunamai College of Pharmacy, Mamurabad, Affiliated to Kavayitri Bahinabai Chaudhari North Maharashtra University, Jalgaon, Maharashtra, India.*
[4]*Department of Pharmaceutical Chemistry, Sant Gajanan Maharaj College of Pharmacy, Mahagaon, Affiliated to Shivaji University, Kolhapur, Maharashtra, India.*
[5]*Department of Pharmacognosy, Sharadchandra Pawar College of Pharmacy, Otur, Affiliated to Savitribai Phule Pune University, Pune, Maharashtra, India.*

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ABSTRACT

Background: Molnupiravir was granted approval by the UKS medicines and health product regulatory agency on 04 November 2021 and on 23 December 2021, granted emergency use of authorization by FDA.

Objective: Provide a technique for measuring Molnupiravir in active pharmaceutical ingredients and formulations.

Method: The wavelength maximum was found to be 236 nm. ICH guidelines were followed. The forced degradation study in the form of acidic, alkali, thermal, photolytic, hydrolytic, and oxidative stress conditions was carried out for Molnupiravir.

Results: The method was linear, as measured by a coefficient of correlation (R2) of 0.9991 in the 10 to 50 µg/mL range. The %RSD for precision, accuracy, limit of detection (LoD), limit of quantitation (LoQ), ruggedness, and robustness was within acceptable limits per ICH Q2 (R1).

Conclusion: HPLC equipped with a UV detector is used to create and verify the proposed method. An acetonitrile mobile phase component of 20% was used, demonstrating the more cost-effective technique. The extensive data of mobile phase optimization gives a complete idea of final chromatographic conditions, which can be further implemented for future analysis. Molnupiravir shows less than 4% degradation under different stress conditions. The forced degradation data helps show stability, indicating the behavior of Molnupiravir.

Keywords: Molnupiravir, COVID-19, RP-HPLC, Forced degradation.

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INTRODUCTION

The Molnupiravir (C2R, 3S, 4R, 5R)-3, 4-dihydroxy-5-((4Z)-4-(hydroxyimino)-2-oxo-3, 4-dihydropyrimidine-1(2H)-yl oxolan-2-yl) methyl 2-methyl propanoate having antiviral action[1]. Molnupiravir was granted approval by the UKS medicines and health product regulatory agency on 04 November 2021 and, on 23 December 2021, granted emergency use authorization by FDA[2]. As Molnupiravir was recently approved for COVID-19, three methods are available, including HPLC, UV, and LC-HRMS as a single or combined with another drug. One bioanalytical method for its metabolite is available. There is no economical method available as in all the methods, and acetonitrile is one of the components of the mobile phase. Also, mostly hyphenated techniques were implemented for analysis.[3-6]

Analysis of Physical Characteristics

The physical characteristics like practical solubility, melting point, and IR interpretation of the Molnupiravir were performed before starting method development (Tables 1-3 and Figure 1).[7-10]

**Author for Correspondence: maheshdeshpande83@gmail.com*

Table 1: Practical solubility of drug

Solvent	*Solubility Description*
Water	Soluble
Methanol	Freely soluble
Acetonitrile	Slightly soluble
DMSO	Freely soluble

Table 2: Determination of melting point

Drug	*Observed melting point*	*Reference melting point*
Molnupiravir	171°C	169-172°C

Table 3: IR interpretation

Functional group	*Observed value (cm^{-1})*	*Standard value (cm^{-1})*
N-H	3368	3300 – 3500
O-H	3563.52	3400 – 3650
C=O	1686.02	1685 – 1650
C-O	1121.20	1050 -1150
C-N	1074.86	1030 – 1230
$-CH_3$	1448.79	1470 – 1430
Pyrimidine ring	1381	1380.9–1142.5
imino group	2138.66	2129 – 2166

MATERIALS AND METHOD

An Agilent HPLC Instrument (1260 Infinity II Agilent HPLC) equipped with UV/PDA detector and Open Lab Software was used for method development. The chromatographic analysis used a C-18 column (250 mm x 4.6 mm, 0.5 m particle size), with an injection volume of 20 µL. HPLC Prominence-I, LC2030C Plus, Open Lab Solution Software validated the analytical method. Analytical balance (Shimadzu), digital pH meter (systolic), Digital Balance (Shimadzu), SonicatorPCi (3.5L), and Borosil Calibrated glassware were employed during the analysis.[11-16]

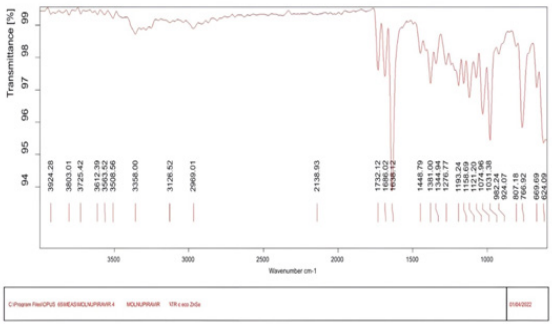

Figure 1: IR spectrum of molnupiravir.

Reagents and Materials

The working standard of molnupiravir (Potency = 98%) was obtained from Swapnaroop Research Pvt. Ltd, Aurangabad. The HPLC-grade solvents were used for analysis. All the solvents and solutions were degassed before the actual analysis. The Molflu Capsules containing 200 mg Molnupiravir were procured from the local market.[17]

Mobile Phase Preparation

The pH 3.1 is achieved by adding orthophosphoric acid to a mixture of acetonitrile, methanol, and water (20:50:30% v/v/v) to create the mobile phase.[18-20]

Preparation of Standard and Working Stock

The medicine's active pharmaceutical ingredient (API), weighing 10 mg, was transferred to a volumetric flask for further analysis. A 15 minute sonication process utilizing the mobile phase yielded the stock solution. The concentration of the combination was 1000 µg/mL, and the volume was 100 mL.

Table 4: Optimization of mobile Phase and Chromatographic conditions

Sr no.	*Chromatographic condition*				*Observation*	*Remark*	*Retention time (min)*
	Mobile phase	*Solvent ratio*	*λmax*	*Flow rate (mL/min)*			
1	Methanol: Water	50:50.	236	1	Tailing observed	Method Rejected	3.7
2	Methanol: Water	60:40	236	1	The theoretical plate was not observed	Method Rejected	3.04
3	Methanol: Water	65:35	236	1	The theoretical plate was not observed	Method Rejected	2.913
4	Methanol: Water	70:30	236	1	The theoretical plate was not observed	Method Rejected	2.767
5	Methanol: Water	80:20	236	1	The peak shape was not good	Method Rejected	2.63
6	Methanol:Water: ACN	60:20:20	236	1	Tailing observed	Method Rejected	2.493
7	Methanol:Water: ACN	50:20:30	236	1	The peak shape was not good	Method Rejected	2.540
8	Methanol:ACN: Water (pH 3.1)	50:20:30	236	1	The peak shape was good, and the Theoretical plate was observed	Method Accepted	2.427

Table 5: Chromatographic condition for Trial–1

Parameter	*Condition*
Column	Agilent (250 x 4.6 mm, 0.5 µm)
Mobile phase	Methanol:Water (50:50 v/v)
Flow rate	1-mL/min
Run time	10 mins
Column temperature	Ambient
Injection volume	20 µL
Detection wavelength	236 nm
Diluent	Mobile phase

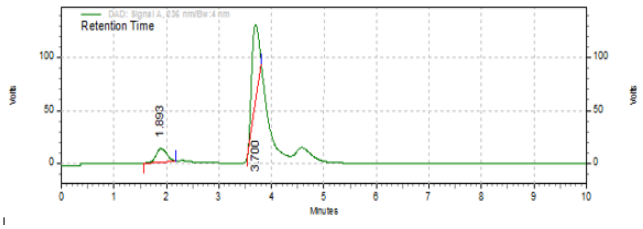

Figure 2: Chromatogram of Trial–1

Table 6: Chromatographic condition for Trial–2

Parameter	*Condition*
Column	Agilent (250 x 4.6 mm, 0.5 µm)
Mobile phase	Methanol:Water (60:40 v/v)
Flow rate	1-mL/min
Run time	10 mins
Column temperature	Ambient
Injection volume	20 µL
Detection wavelength	236 nm
Diluent	Mobile phase

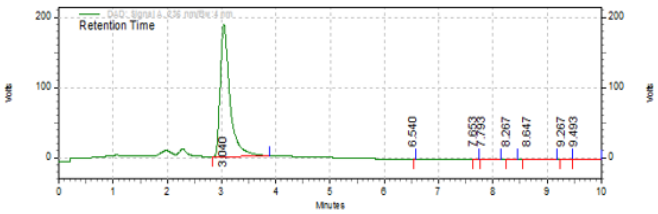

Figure 3: Chromatogram of Trial–2

Table 7: Chromatographic condition for Trial–3

Parameter	*Condition*
Column	Agilent (250 x 4.6 mm, 0.5 µm)
Mobile phase	Methanol: Water (65:35 v/v)
Flow rate	1-mL/min
Run time	10 mins
Column temperature	Ambient
Injection volume	20 µL
Detection wavelength	236 nm
Diluent	Mobile phase

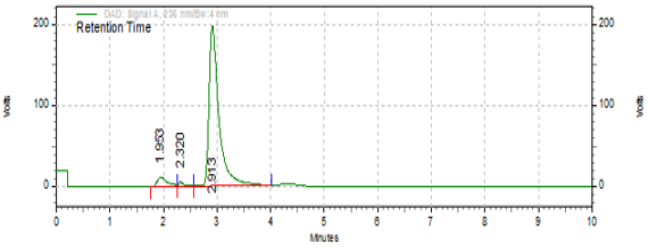

Figure 4: Chromatogram of Trial–3.

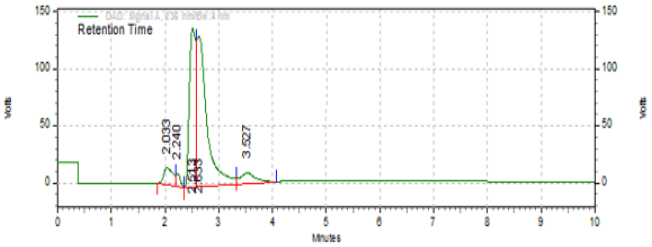

Figure 6: Chromatogram of Trial–5

To produce various concentrations, a mobile phase was used to further dilute the standard stock solution.[21]

Table 8: Chromatographic condition for Trial–4

Parameter	Condition
Column	Agilent (250 x 4.6 mm, 0.5 µm)
Mobile phase	Methanol: Water (70:30 v/v)
Flow rate	1-mL/min
Run time	10 mins
Column temperature	Ambient
Injection volume	20 µL
Detection wavelength	236 nm
Diluent	Mobile phase

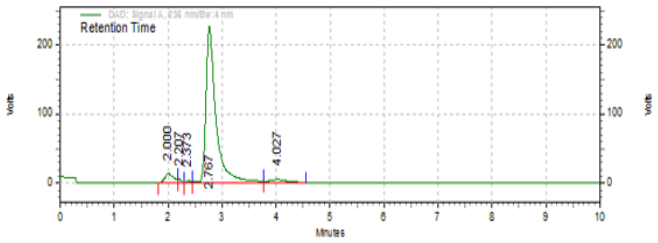

Figure 5: Chromatogram of Trial–4

Table 9: Chromatographic condition for Trial–5

Parameter	*Condition*
Column	Agilent (250 x 4.6mm, 0.5µm)
Mobile phase	Methanol: Water (80:20 v/v)
Flow rate	1-mL/min
Run time	10 mins
Column temperature	Ambient
Injection volume	20 µL
Detection wavelength	236 nm
Diluent	Mobile phase

Table 10: Chromatographic condition for Trial – 6

Parameter	Condition
Column	Agilent (250 x 4.6 mm, 0.5 µm)
Mobile phase	Methanol: Water: ACN (60:20:20 v/v/v)
Flow rate	1-mL/min
Run time	10 min
Column temperature	Ambient
Injection volume	20 µL
Detection wavelength	236 nm
Diluent	Mobile phase

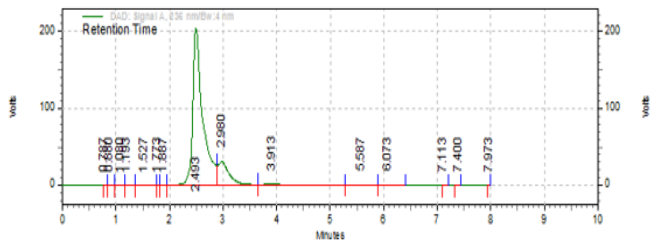

Figure 7: Chromatogram of Trial – 6.

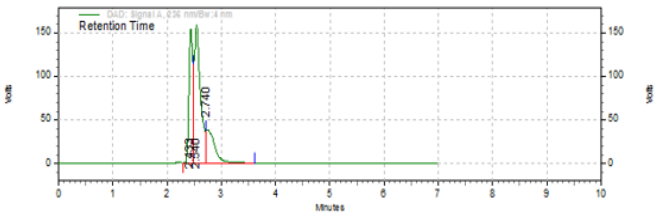

Figure 8: Chromatogram of Trial – 7

Table 11: Chromatographic condition for Trial–7

Parameter	*Condition*
Column	Agilent (250 x 4.6mm, 0.5µm)
Mobile phase	Methanol:Water: ACN (50:20:30 v/v/v)
Flow rate	1mL/min
Run time	10 min
Column temperature	Ambient
Injection volume	20 µl
Detection wavelength	236 nm
Diluent	Mobile Phase

Sample Solution's Preparation

Twenty capsules (average) was established. The capsules were broken up, and a fine powder measuring 14.1 mg was taken. The volume was then raised to the appropriate level to obtain 1000 µg/mL. For 30 minutes, the solution was sonicated. In order to obtain various sample concentrations, this sample solution was further diluted.

Chromatographic Conditions and Mobile Phase Optimization

During the method optimization stage, various combinations of methanol, water, and acetonitrile were tried to get the optimized method with accepted levels of system suitability.[22-23]

Table 12: Chromatographic condition for Trial – 8

Parameter	Condition
Column	Agilent (250 x 4.6 mm, 0.5 µm)
Mobile phase	Methanol:ACN: Water(50:20:30v/v/v)
Flow rate	1-mL/min
Run time	10 min
Column temperature	Ambient
Injection volume	20 µL
Detection wavelength	236 nm
Diluent	Mobile phase

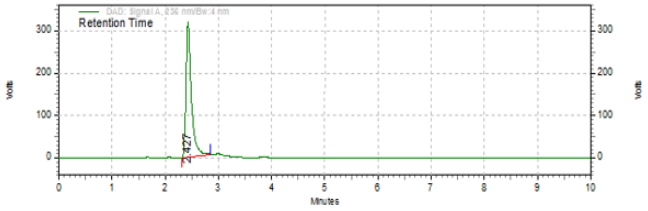

Figure 9: Chromatogram of Trial – 8

Table 13: Final Accepted Chromatogram

Parameter	Condition
Mobile phase column	C-18, 250×4.6mm, 0.5µm
Flow rate	1 mL /min
Wavelength	236 nm
Injection volume	20 µL
Column temperature	Ambient
Pump mode	Isocratic
Run time	10 min
Retention time	2.427 min
Mobile phase	Methanol, Acetonitrile, and Water (pH 3.1 with Ortho Phosphoric acid) in the ratio of 50:20:30 % v/v/v

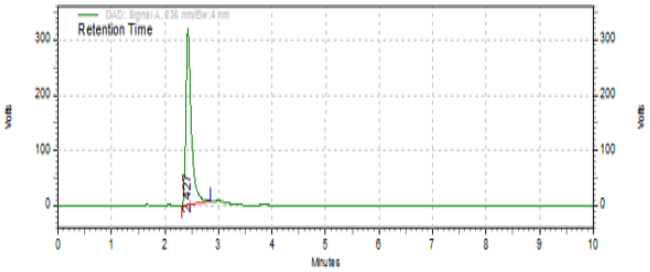

Figure 10: Chromatogram of Molnupiravir of 50µg/mL

The details of the optimization of the mobile phase final accepted chromatographic conditions are given in Tables 4 to 14 and Figures 2 to 10.

Validation of HPLC Method

Linearity

The technique responded linearly to concentrations between 10 to 50 µg/mL. It was discovered that the correlation coefficient was 0.9991. (Table 15 and Figure 11)

Table 14: System suitability parameter

Conc. µg/mL	*Retention Time*	*Theoretical plates*	*Asymmetry*
50	2.427	3251	1.82028
50	2.420	3215	1.81497
50	2.420	3218	1.81781
50	2.420	3225	1.82817
50	2.420	3291	1.87253
50	2.420	3239	1.86734
Mean	2.4211	3239.8	1.8368
SD	0.00285	28.4845	0.02605
%RSD	0.1180%	0.8791%	1.4182%

Table 15: Linearity of molnupiravir

Conc.(µg/mL)	*Area*
10	195565
20	283761
30	387494
40	489241
50	593470

Table 16: Found concentration of intra-day precision (morning)

Conc. in (*µg/mL)*	*Peak area*	*Found conc.*	*%Found conc.*	*Mean*
	184465	9.532	95.539	
10	184478	9.661	96.632	96.31
	184455	9.678	96.788	
	288762	19.802	99.666	
20	284781	19.712	98.120	98.54
	285494	19.572	97.86	
	389431	29.921	99.66	
30	387611	29.726	99.00	99.26
	384781	29.745	99.291	
SD 1.538062 %RSD 1.5689			Mean	98.03

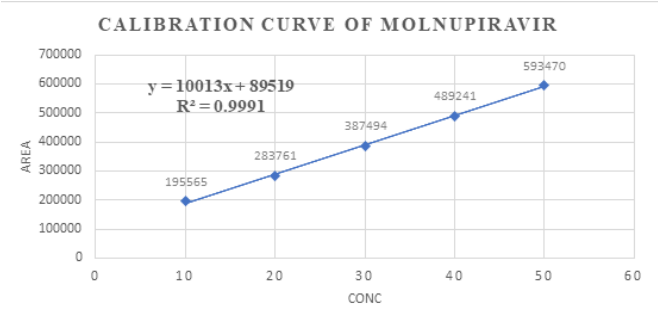

Figure 11: Calibration curve of molnupiravir

Table 17: Found concentration of intraday precision (evening)

Conc. in (µg/mL)	Peak Area	Found conc.	%Found conc.	Mean
	194365	9.608	96.237	
10	184578	9.678	96.021	96.789
	185568	9.512	95.532	
	284761	19.628	96.011	
20	285782	19.732	97.238	97.555
	284410	19.780	97.580	
	389432	29.724	97.671	
30	387821	29.752	97.765	97.992
	386538	29.831	98.921	
SD 0.608952 %RSD 0.624916			Mean	97.44533

Table 18: Found concentration of inter-day precision (Day-1)

Conc. in (µg/mL)	*Peak Area*	*Found conc.*	*%Found conc.*	*Mean*
	184465	9.532	95.539	
10	184478	9.661	96.632	96.31
	184455	9.678	96.788	
	288762	19.802	99.666	
20	284781	19.712	98.120	98.54
	285494	19.572	97.86	
	389431	29.921	99.66	
30	387611	29.726	99.00	99.26
	384781	29.745	99.291	
SD 1.538062 %RSD 1.5689			Mean	98.03

Precision

Precision was assessed by taking three replicates of 10, 20, 30 µg/mL solutions. The %RSD found is given in Table 16 to 19.

Accuracy

It was obtained at 3 different levels of concentration (80, 100, and 120%). The recovery percent was obtained by obtaining the pre-analysis of formulation and then utilizing the pre-analysis values in accuracy determination. (Table 20 to 23).

Robustness

The robustness was determined under the conditions which include deliberate change in wavelength (±2 nm) flow rate (± 0.1 mL/min). Results were summarized in Tables 24 and 25.

Detection and Quantitation Limits

LoD: LoD can be calculated from the following formula:

Table 19: Found concentration of inter-day precision (Day-2)

Conc. in (µg/mL)	*Peak area*	*Found conc.*	*%Found conc.*	*Mean*
	195321	9.528	95.123	
10	184781	9.698	96.438	96.280
	185778	9.699	96.289	
	282345	19.681	96.839	
20	283368	19.672	96.790	96.221
	284781	19.786	97.655	
	388998	29.923	97.781	
30	388368	29.698	96.685	97.982
	385321	29.833	98.321	
SD 1.000117 %RSD 1.032884			Mean	96.82767

$$\text{LoD} = 3.3 \times \frac{\text{standard deviation}}{\text{slope}}$$

$$\text{LoD} = \frac{3.3 \times 4058.9}{10013}$$

$$\text{LoD} = 1.3376\ \mu\text{g/mL}$$

Table 20: Pre-analysis for accuracy

Level (µg/mL)	*Replicate*	*Area*	*Found conc.*
	R1	184444	9.480
10	R2	186789	9.714
	R3	186651	9.700
	R1	283761	19.398
20	R2	287813	19.803
	R3	286180	19.640
	R1	385321	29.541
30	R2	382458	29.255
	R3	385484	29.558

Table 21: %Recovery of 80% level

Level	*Conc. (µg/mL)*	*Replicate*	*Area*	*API added (mL)*	*Formulation added (mL)*	*Found conc.*	*Recovered conc.*	*Percent recovery*	*Mean*
	18	R1	263761	8	10	17.4231	7.94	99.25	
	18	R2	267322	8	10	17.7511	8.03	100.37	98.95
	18	R3	264547	8	10	17.4801	7.78	97.25	
	36	R1	449321	16	20	35.9332	16.58	103.31	
80%	36	R2	446257	16	20	35.6210	15.81	98.81	100.04
	36	R3	443217	16	20	35.3238	15.68	98.00	
	54	R1	624750	24	30	53.4541	23.91	99.63	
	54	R2	629829	24	30	53.9608	24.70	102.93	101.33
	54	R3	629258	24	30	53.9038	24.34	101.43	
							Mean		100.10
							SD		1.6829
							%RSD		1.6812

Table 22: Percent recovery of 100% level

Level	*Conc. (µg/mL)*	*Replicate*	*Area*	*API added (mL)*	*Formulation added (mL)*	*Found conc.*	*Recovered conc.*	*Percent recovery*	*Mean*
	20	R1	283667	10	10	19.3895	9.90	99.00	
	20	R2	288892	10	10	19.9114	9.8	98.00	99.00
	20	R3	287592	10	10	19.7841	10.0	100	
	40	R1	487321	20	20	39.7285	19.3	96.5	
100%	40	R2	488975	20	20	39.8989	19.8	99.00	98.01
	40	R3	487378	20	20	39.4346	19.7	98.5	
	60	R1	687323	30	30	59.7032	29.4	98.00	
	60	R2	688456	30	30	59.8159	29.6	98.66	98.33
	60	R3	689341	30	30	59.9043	29.5	98.33	
							Mean		98.44
							SD		0.50520
							%RSD		0.51321

Table 23: %Recovery of 120% level

Level	*Conc. (µg/mL)*	*Replicate*	*Area*	*API added (mL)*	*Formulation added (mL)*	*Found conc.*	*Recovered conc.*	*Percent recovery*	*Mean*
	22	R1	307231	12	10	21.7429	11.9	99.10	
	22	R2	309478	12	10	21.9673	11.8	98.33	98.86
	22	R3	306211	12	10	21.6410	11.9	99.16	
	44	R1	528731	24	20	43.8641	23.5	97.91	
120%	44	R2	524378	24	20	43.4294	23.6	98.33	98.60
	44	R3	526961	24	20	43.7874	23.9	99.58	
	66	R1	746781	36	30	65.6408	35.9	99.72	
	66	R2	745331	36	30	65.4960	35.8	99.44	99.44
	66	R3	742922	36	30	65.2554	35.7	99.16	
						Mean			98.96
						SD			0.430
						%RSD			0.4345

Table 24: Found concentration of robustness (Change in wavelength)

Conc.(µg/mL)	Area			Found concentration			% Found concentration			Mean
	R1	R2	R3	R1	R2	R3	R1	R2	R3	
30 (λ = 236)	388178	388611	387891	29.28	29.81	29.82	93.21	98.2	98.71	96.01
30 (λ = 237)	387861	385421	383218	29.72	29.91	29.28	97.11	97.7	98.8	98.23
30 (λ = 238)	387890	384301	389988	29.82	29.78	29.66	98.3	97.2	96.10	97.78
								Mean		97.34
								SD		0.253
								%RSD		0.289

Table 25: Found concentration of robustness (Change in flow rate (± 0.1 mL/min))

Flow rate (mL/min)	*Conc. (30µg/mL)*	*AUC*	*Found conc. (µg/mL)*	*% Found conc.*	*Mean*
	R1	387896	29.79	99.3	
0.9mL/min	R2	379989	29.00	96.66	96.30
	R3	368786	27.89	92.96	
	R1	388983	29.90	99.66	
1mL/min	R2	385782	29.58	98.6	97.95
	R3	376789	28.68	95.6	
	R1	379289	28.93	96.43	
1.1mL/min	R2	369998	28.01	93.36	94.36
	R3	369875	27.99	93.30	
				Mean	96.20
				SD	1.7969
				%RSD	1.8678

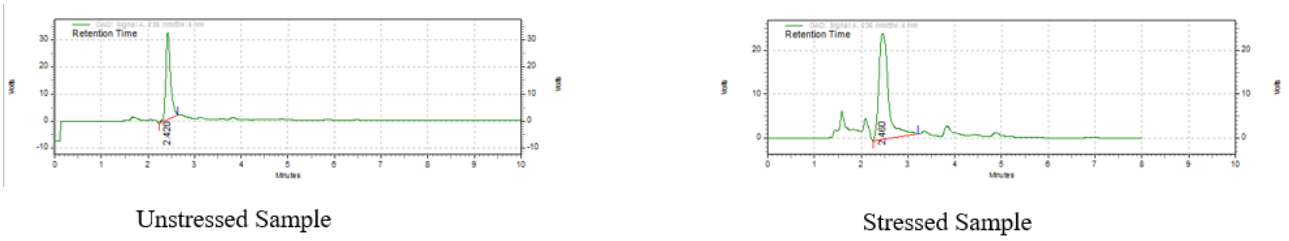

Figure 12: Chromatograms of thermal degradation sample.

$$LoD = 3.3 \times \frac{\text{standard deviation}}{\text{slope}}$$

$$LoD = \frac{3.3 \times 4058.9}{10013}$$

$$LoD = 1.3376\ \mu g/mL$$

LoQ: LoQ can be calculated from the following formula

$$LoQ = 10 \times \frac{\text{standard deviation}}{\text{slope}}$$

$$LoQ = \frac{10 \times 4058.9}{10013}$$

$$LoQ = 4.0536\ \mu g/mL$$

LoD and LoQ were found to be 1.3376 and 4.0536 µg/mL by UV as per ICH guidelines.

Force Degradation Study

The forced degradation of molnupiravir was determined by stressing the sample with different stress conditions like thermal, oxidative, acidic, alkali, and photolytic degradation. The final observations are given in Table 26.

Thermal Degradation

The standard medication stock was diluted with 1-mL of water to make 0.1 mL. The following day, this was kept at 40°C for two hours. After reaching room temperature, it was diluted to achieve a 20 µg/mL concentration before being injected into the HPLC apparatus. (Figure 12).

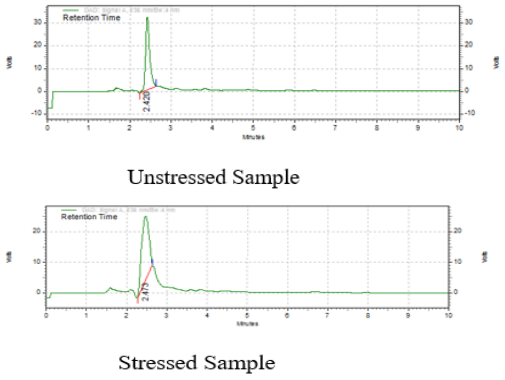

Figure 13: Chromatograms of oxidative degradation samples.

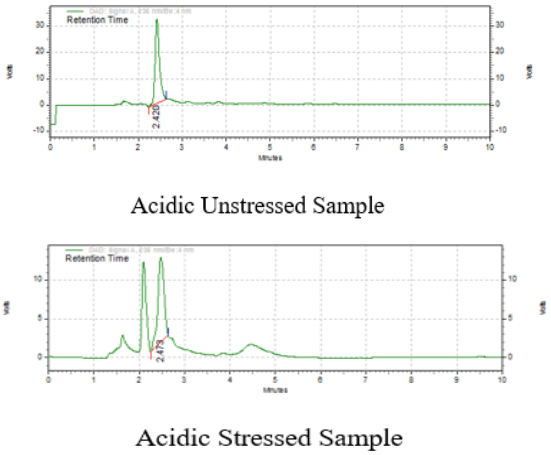

Figure 14: Chromatograms of acidic degradation sample.

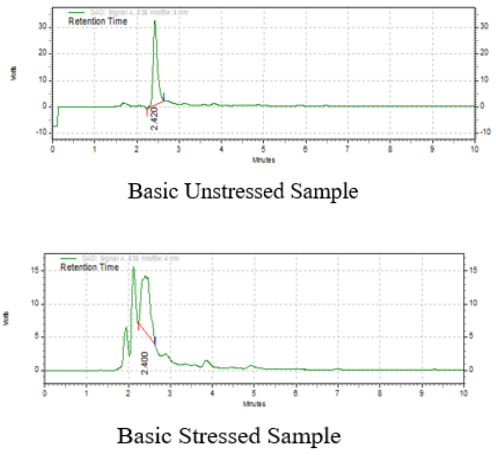

Figure 15: Chromatograms of basic degradation sample.

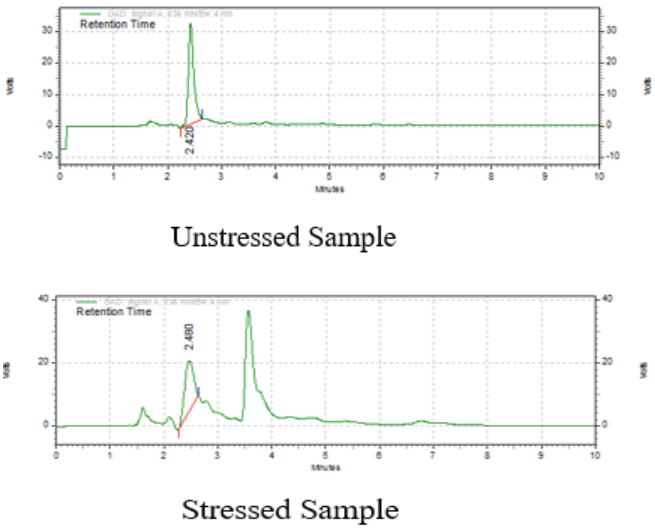

Figure 16: Spectra of photolytic degradation.

Table 26: Observation of forced degradation study

Degradation parameter	*Area of unstressed sample*	*Area of stressed sample*	*%Degradation*
Thermal degradation	283761	281001	0.972%
Oxidative degradation	284781	274998	3.4%
Acidic degradation	283661	275678	2.8%
Basic degradation	284898	281111	1.3%
	285333	283437	0.66%

Oxidative Degradation

The 1-mL of 0.1M H2O2 was added to 0.1 mL of the drug's standard stock solution. For two hours, the solution was maintained at 40°C. These solutions were neutralized with an appropriate quantity of HCI before being chilled. The volume was made up to 20 µg/mL and analyzed by HPLC. (Figure 13)

Acid and Alkali Hydrolysis

In 1-mL of 0.1M HCI and 0.1M NaOH, 0.1 mL of the drug's standard was diluted. For two hours, the solutions were maintained at 40°C. The temperature was reduced to room temperature. The solutions were neutralized by HCl and NaOH, respectively. 20 µg/mL was injected in HPLC. (Figure 14 and 15)

Photolytic Degradation

The standard stock solution of the medication was diluted with water from 0.1 to 1 mL. It was carried out by exposing the solution to UV radiation for 24 hours. Before being put into the HPLC equipment, it was diluted with mobile phase to a 20 µg/mL concentration. (Figure 15).

RESULT AND DISCUSSION

The analysis of the physical characteristics like practical solubility, melting point and FTIR of the API proves the purity of the drug under analysis. The extensive data obtained during mobile phase optimization shows behavior of the drug in different mobile phase combinations. The medication separates most effectively in a 20:50:30 (v/v/v) mixture of acetonitrile, methanol, and water. The drug's linear response between 10 and 50 µg/mL has a correlation coefficient close to 1, indicating that this range is suitable for human use per ICH standards. The method's repeatability was evaluated in low, medium, and high concentrations. As required by ICH criteria, all obtained values of relative standard deviation for precision are less than 2%. The precision of the procedure was tested at three different concentrations (80, 100, and 120%) using the usual addition method. The method's acceptability, according to ICH guidelines is demonstrated by the fact that the percent recovery figures for all levels are close to 100%. The method's sensitivity was measured by establishing its LoD and LoQ. The obtained LoD and LoQ values demonstrate the sensitivity of the approach. The forced degradation study was carried out by thermal, oxidative, acidic and basic stress degradation study. The values obtained for each degradation parameter show the drug is stable with the respective stress procedure and the results of which can used for further formulation development of the drug.

[MQC-medium quality control sample, LQC-lowest quality control sample, HQC-Highest quality control sample]

CONCLUSION

The new, simple, economical reverse phase HPLC method with forced degradation was performed. Extensive data were obtained during the method development stage, including practical solubility, melting point, and FTIR analysis which can be applied in other method development and further formulation development. All results are within acceptable limits as stated in ICH guidelines. The molnupiravir shows less than 4% degradation under different stress conditions. The forced degradation data helps show stability indicating the behavior of molnupiravir. Method validation was performed in accordance with ICH standards. The extensive data of mobile phase optimization gives a complete idea of final chromatographic conditions, which can be further implemented for future analysis. The less use of a high-cost component, acetonitrile, proves the more economical method.

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