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(RESEARCH ARTICLE)

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A validated stability indicating RP-HPLC method for the determination of molnupiravir in pharmaceutical dosage form

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Abstract

A simple, Precise, Accurate method was developed for the estimation of Molnupiravirby RP-HPLC technique. Chromatographic conditions used are stationary phase Agilent C18 (250 mm*4.6mm3.6µ), Mobile phase Orthophopoaricacid: Acetonitrile in the ratio of 60:40 and flow rate was maintained at 1.0 mL/min, detection wave length was 254 nm, column temperature was set to 30o C and diluent was mobile phase Conditions were finalized as optimized method. System suitability parameters were studied by injecting the standard six times and results were well under the acceptance criteria. The retention time for Molnupiravir was 2.46 min. The method was validated for linearity, accuracy, precision, specificity, limit of detection, limit of quantification and robustness. Limit of detection and limit of quantification were found 0.06 µg/ml and 0.21 µg/ml respectively and recovery of Molnupiravir from tablet formulation was found 99.73%. The proposed method was successfully applied for the quantitative determination of Molnupiravir in tablet formulation.

Keywords: HPLC; Molnupiravir; Method development; Method validation; Degradation studies ICH Guidelines

1. Introduction

Molnupiravir:Chemically[1-7][(2R,3S,4R,5R)-3,4-dihydroxy-5-[(4Z)-4-(hydroxyimino)-2-oxo-1,2,3,4-tetrahydropyrimidin-1-yl]oxolan-2-yl]methyl 2-methylpropanoate is used as anti- viral drug. Molecular Formula: $C_{13}H_{19}N_3O_7$, Molecular weight: 329.309, Solubility: 5.77 mg/mL

Molnupiravir is hydrolyzed in vivo to N4-hydroxycytidine, which is phosphorylated in tissue to the active 5'-triphosphate form, and incorporated into the genome of new virions, resulting in the accumulation of inactivating mutations, known as viral error catastrophe.1,3 A remdesivir resistant mutant mouse hepatitis virus has also been shown to have increased sensitivity to N4-hydroxycytidine.

A RP-HPLC [8-14] method was developed for the determination of Molnupiravir and applied for in vitro permeability studies of self-emulsifying drug delivery system formulations using Caco-2 cell line. Discovery® HS C_{18} Column (250 ×4.6 mm, 5 µm) was used at 30 °C. Isocratic elution was performed with OPA: Acetonitrile 60:40 v/v) mixture. The flow rate was 0.5 ml/min and UV detection was at 253 nm. Molnupiravir eluted within 5 min.

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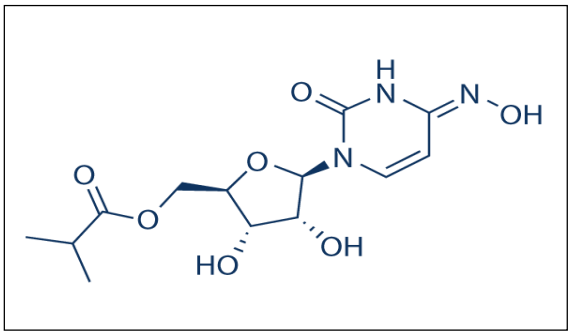

Figure 1 Chemical Structure of Molnupiravir

2. Material and methods [15]

2.1. Instrumentation

HPLC instrument used was of WATERS HPLC 2965 SYSTEM with Auto Injector and PDA Detector. Software used is Empower 2. UV-VIS spectrophotometer PG Instruments T60 with special bandwidth of 2mm and 10mm and matched quartz was be used for measuring absorbance for Molnupiravir solutions.

2.2. Chemicals

Molnupiravir reference standard was obtained from the tablets of brand Lagevrio (Merck) of 200mg was obtained from MSN Pharma LabsHyderbad. Methanol was used as the solvent for the experiment.

2.3. Chromatographic conditions

Glass waresusedineachprocedureweresoakedovernightinamixtureofchromicacidandsulphuricacidrinsedthoroughlywithdoubledistilledwateranddried inhotairoven. HS C18 Column (250 ×4.6 mm, 5 µm) was used at 30 °C. Isocratic elution was performed with ACN: water (60:40 v/v) mixture. The flow rate was 1.0 mL/min and UV detection was at 240 nm. Molnupiravir eluted within 5 min.

2.4. Preparation of Standard stock solutions:

Accurately weighed 20mg of Molnupiravir transferred 50ml and volumetric flasks, 3/4 Th of diluents was added and sonicated for 10 minutes. Flasks were made up with diluents and labelled as Standard stock solution (400µg/ml of Molnupiravir).

2.5. Method Validation

The Proposed method was validated as per the ICH Q2 (R1) guidelines for linearity, range, accuracy, precision, ruggedness.

2.5.1. Robustness

Small deliberatechanges in method like Flow rate, mobile phase ratio, and temperature are made but there were no recognized change in the result and are within range as per ICH Guide lines.

2.5.2. Accuracy

To assess the accuracy of the proposed method, recovery studies were carried out three different levels i.e., 50%, 100% and 150%. To the pre- analyzed sample solution, a known amount standard drug solution was added at three different levels, absorbance was recorded. The % recovery was then calculated by using formula

$$\% \text{ Recovery} = A - B / C$$

Where A = Total amount of drug estimated'; B = Amount of drug found on preanalysed basis
C = Amount of Pure drug added

3. Results

3.1. Screening and optimization

3.1.1. Selection of the detection wavelength

The UV spectra of Molnupiravir in 60:40 v/v mixtures of OPA: Acetonitrilein the region between 200 and 400 nm are shown in Fig 1. It shows that at 253 nm Molnupiravir has maximum absorbance. Hence max of molnupiravir in mobile phase was selected as an optimum detection wavelength for the quantification of Molnupiravir.

3.1.2. Calibration of standards

The standard calibration curve was constructed for Molnupiravir. Different volumes of stock solutions of eachwere accurately transferred in to 10mL volumetric flasksand diluted to mark to yield a concentration of 40µg/mlsolutions.Thecalibration line was obtained by plotting the peak areaagainst concentration of drug.

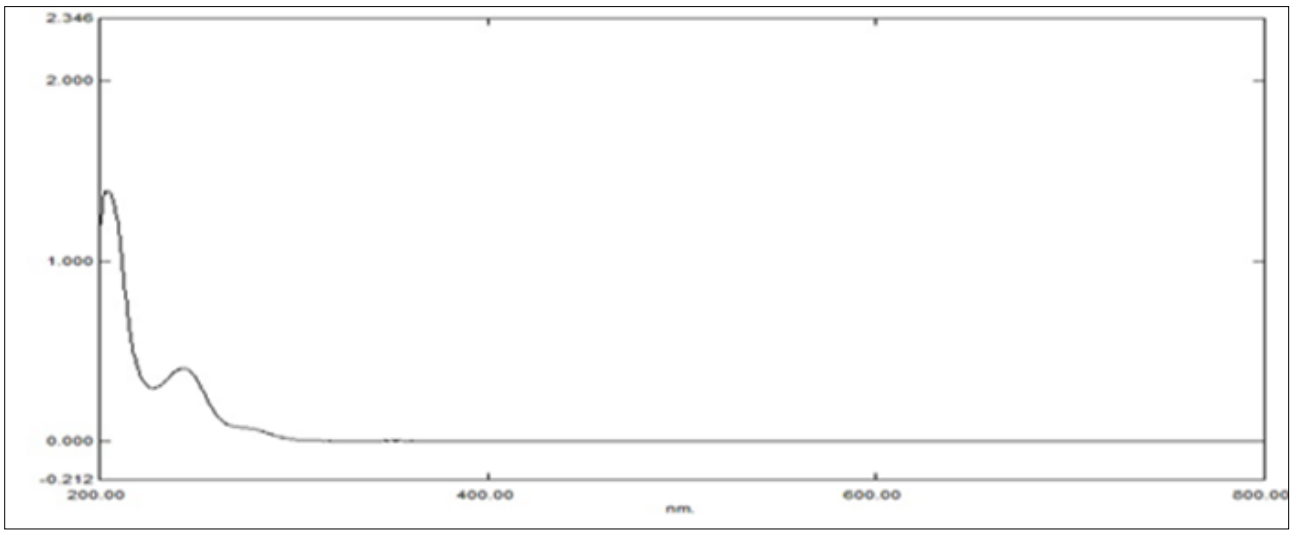

Figure 2 Absorbance spectrum of Molnupiravir

3.2. Method Development

3.2.1. Optimized Chromatographic Conditions

- Column:Agilent C_{18} 250mm x 4.6 mm, 5µ).
- Mobile phase: OPA: Aetonitrile (60:40)
- Flow rate: 1.0 ml/min
- Detector: PDA 253nm
- Temperature: 30^0C
- Injection Volume: 10µL

3.3. Robustness, limit of detection and limit of quantification

Precisionwascalculatedbypercentagerelativestandard deviation. The accuracy was expressed in terms of percent recovery of the know amount of the standard drugs added to the known amount of the pharmaceuticaldosageforms.Variousvalidationparameters areperformed.

3.3.1. System Suitability

A Standard solution of Molnupiravir working standard was prepared as per procedure and was injected five times into the HPLC system. The system suitability parameters were evaluated from standard Chromatograms obtained by calculating the % RSD of retention time, tailing factor, theoretical plates and peak areas from five replicate injections are within range and Results were shown in table 1.

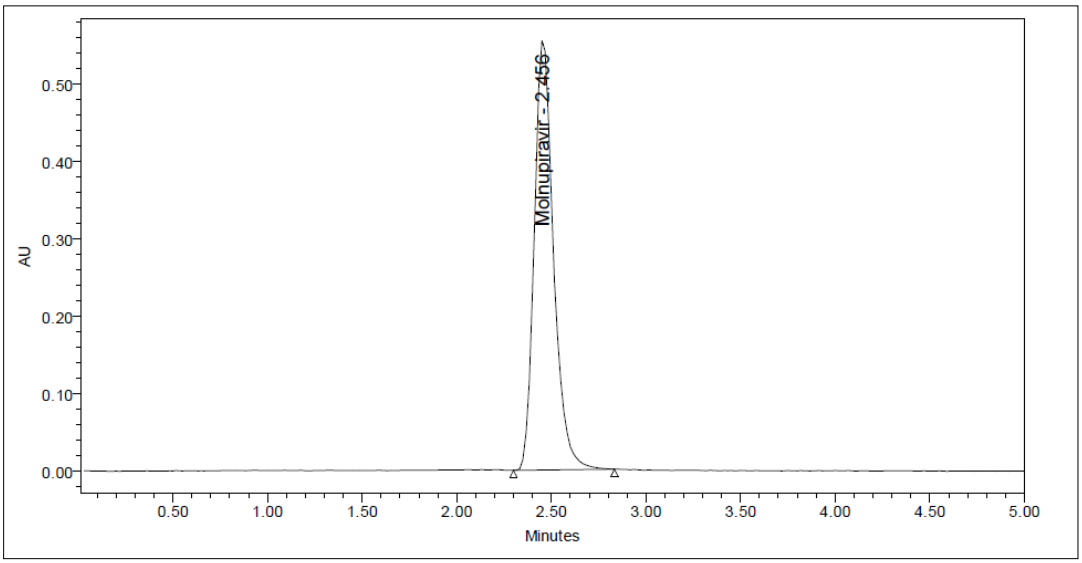

Figure 3 Optimized chromatographic condition

Table 1 System suitability data

Sr.No.	Peak name	Rt	Peak Area	USP plate count	USP tailing
1.	Molnupiravir	2.4	3800986	3107	1.20
2.	Molnupiravir	2.4	3740420	2767	1.20
3.	Molnupiravir	2.4	3095207	2971	1.33
4.	Molnupiravir	2.4	3555454	3136	1.21
5.	Molnupiravir	2.4	3822067	3202	1.21

3.3.2. Linearity

It is the ability of the method to elicit test result that is directly proportional to analytic concentration within a given range. It is generally reported as variance of slope or regression line. Appropriate volume of standard stock solution was transferred to volumetric flask of 10 ml capacity. The volume was adjusted to the mark with mobile phase to give a solution scontaining 10-60 ppm. The slope, Y intercept and correlation coefficient were calculated. The regression line relating standard concentrations of drug using regression analysis was calculated. The calibration curves were linear in the studied range and equations of the regression analysis were obtained.

Table 2 Linearity data of Molnupiravir

Concentration (µg/mL)	Peak Area
10	1066416
20	1925671
30	3028643
40	3970103
50	4975021
60	6013506

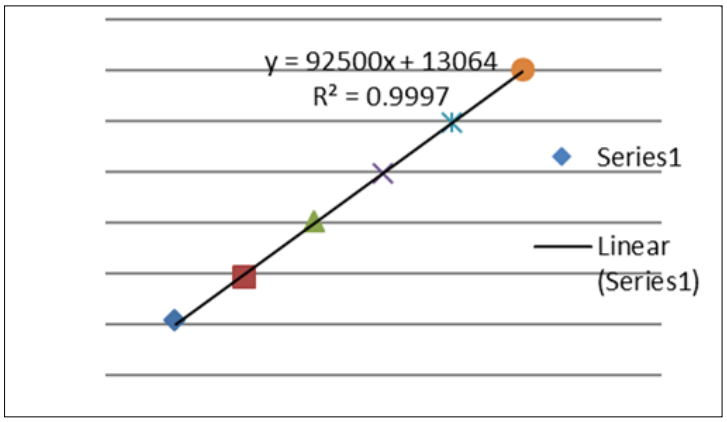

Figure 4 Calibration curve of Molnupiravir

3.3.3. Precision

Six working sample solutions of 40ppm are injected and the % Amount found was calculated and %RSD was found to be 0.9.

Table 3 Precision Data

Sr.No	Peak Area
1	3950268
2	3908540
3	3899032
4	3895431
5	3984869
6	3918533
AVG	3926112
Std. deviaton	34861.9
%RSD	0.9

Intermediate precision: Five working sample solutions of 40ppm are injected on the next day of the preparation of samples and the % Amount found was calculated and %RSD was found to be within the specified limitsAcceptance Criteria: The % RSD for all the five standard injections results should not be more than 2%

3.3.4. Accuracy

Three Concentrations of 50%, 100%, 150% are Injected in a triplicate manner and %Recovery was calculated as 100.45.

3.3.5. Robustness

Small Deliberate change in the method is made like Flow minus, flow plus, Mobile phase minus, Mobile phase plus, Temperature minus, Temperature Plus. %RSD of the above conditions are calculated.

Table 4 Accuracy data of Molnupiravir

%Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
50%	20	20.21	100.01	100.31%
	20	20.21	101.06	
	20	20.01	100.03	
100%	40	40.75	101.86	
	40	40.70	101.74	
	40	40.29	100.72	
150%	60	59.24	98.71	
	60	59.94	99.91	
	60	59.36	98.94	

3.4. LOD and LOQ

LOD and LOQ were calculated from the formula 3.3 x(σ/S) and 10 x (σ/S), respectively where, σ is standard deviation of intercept and S is the mean of slope. The LOD and LOQ can also be determined by S/N.

The value for LOD should be 3-5 whilst for LOQ 10-15.

3.5. Assay of marketed formulation

Standard solution and sample solution were injected separately into the system and chromatograms were recorded and drug present in sample was calculated using before mentioned formula.

Table 5 Assay of marketed formulation

Sample No	%Assay
1	100.92
2	99.86
3.	99.61
4.	99.52
5.	101.81
6.	100.11
AVG	100.31
STDEV	0.89
%RSD	0.9

3.6. Forced degradation studies

The stability of Molnupiravir was studied under stress conditions. For hydrolytic degradation, different strengths of acid (0.1 N, 1 N, 2 N, 5 N HCl), base (0.1 N, 1 N NaOH) and hydrogen peroxide (H_2O_2) (3%, 6%) were employed. Accurately weighed 50 mg of MOLNUPIRAVIR was dissolved in 1 ml of respective acid, base or H_2O_2 and kept for specified period of time at room temperature. For degradation with 0.1 N HCl, 0.1 N NaOH and 3% H_2O_2, solutions were refluxed at 80° for 30 min. The volume was made up to 50 ml with OPA: Acetonitrile (60:40 v/v) and 5 ml of this solution was again diluted to get a final concentration of 20 µg/mL. The stressed samples from acid or base hydrolysis were neutralized (with NaOH or HCl of appropriate strength) prior to injection. Thermal degradation was studied by exposing

10 mg dry powder of Molnupiravir at 80° in a temperature controlled oven for 4 h. Photolytic degradation was studied by exposing 10 mg dry powder of Molnupiravir to 253 nm UV light for 4 h in UV chamber. Using OPA: Acetonitrile (60:40 v/v) as solvent, 20 µg/mL solutions of these stressed samples were prepared, filtered and injected. Analysis was performed by the developed method.

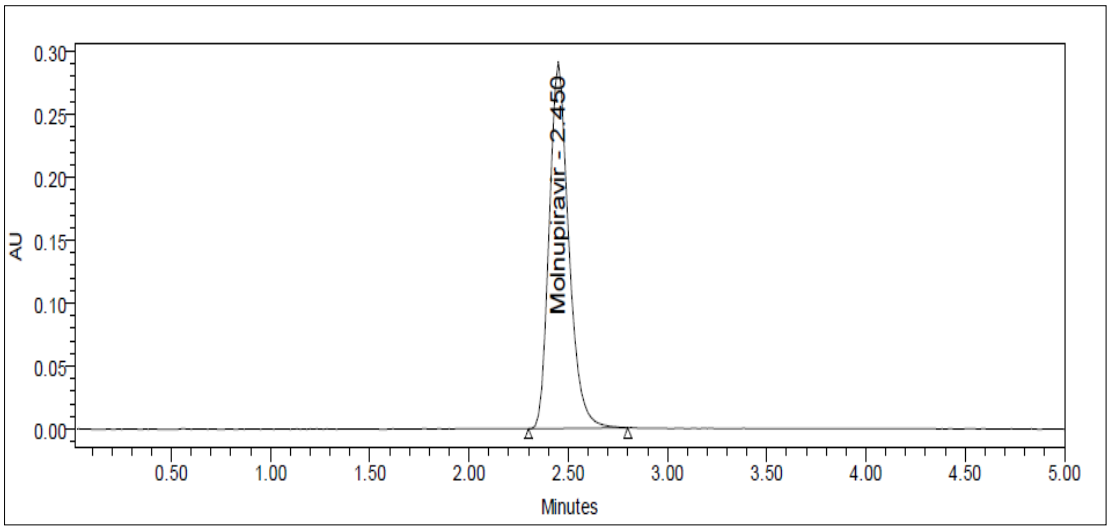

Figure 5 Assay Chromatogram

3.7. Degradation Studies

Degradation studies were performed with the formulation and the degraded samples were injected. Assay of the injected samples was calculated and all the samples passed the limits of degradation

3.7.1. Degradation procedure

Oxidation

To 1 ml of stock solution of Molnupiravir 1 ml of 20% hydrogen peroxide (H_2O_2) was added separately. The solutions were kept for 30 min at 60^{0}c. For HPLC study, there sultan solution was diluted to obtain (40ppm) solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Acid Degradation Studies

To 1 ml of stock solution Molnupiravir1 ml of 2N Hydrochloric acid was added and refluxed for 30mins at 1c.The resultant solution was diluted to obtain (40ppm) solutionand10µl solutions were injected in to the system and the chromatograms were recorded to assess the stability of sample.

Alkali Degradation Studies

To 1 ml of stock solution Molnupiravir 1 ml of 2 N sodium hydroxide was added and refluxed for 30mins at 60^{0}c. There sultan solution was diluted to obtain (40ppm) solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Dry Heat Degradation studies

The standard drug solution was placed inovenat 105^{0}c for6h to study dry heat degradation. For HPLC study, the resultant solution was diluted to (40ppm) solutionand10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

Photo stability studies

The photochemical stability of the drug was also studied by exposing the (400ppm) solution to UV Light by keeping the beaker in UV Chamber for 7days or 200 Watt hours/m^2 in photo stability chamber. For HPLC study, the resultant

solution was diluted to obtain (40ppm)solutions and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Neutral degradation studies

Stress testing under neutral conditions was studied by refluxingthedruginwaterfor6h r s at Temperature of 60ºc. For HPLC study, the resultant solution was diluted to (40ppm) solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

Table 6 Degradation Data of Molnupiravir

S.NO	Degradation Condition	% Drug Undegraded	% Drug Degraded
1	Acid	94.03	5.97
2	Alkali	95.07	4.93
3	Oxidation	95.36	4.64
4	Thermal	97.25	2.75
5	UV	98.61	1.39
6	Water	99.88	0.12

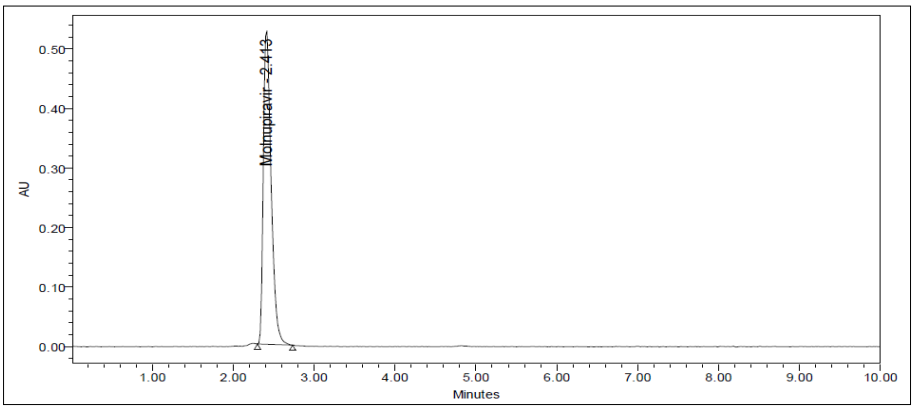

Figure 6 Acid degradation Chromatogram

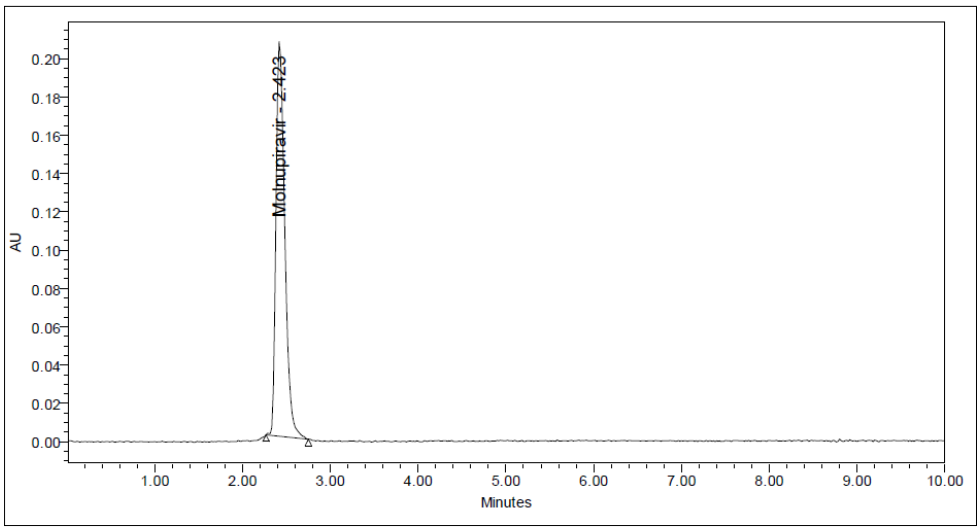

Figure 7 Base degradation chromatogram

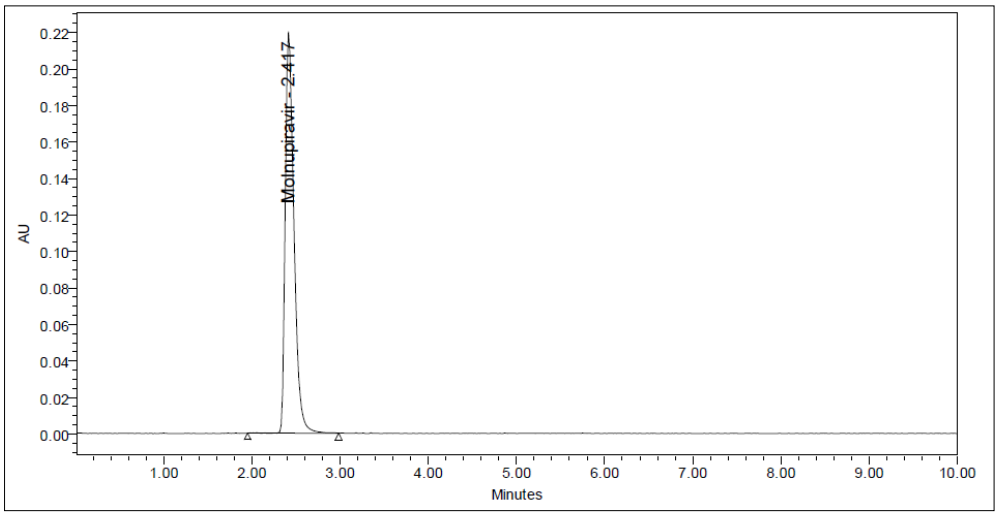

Figure 8 Peroxide degradation chromatogram

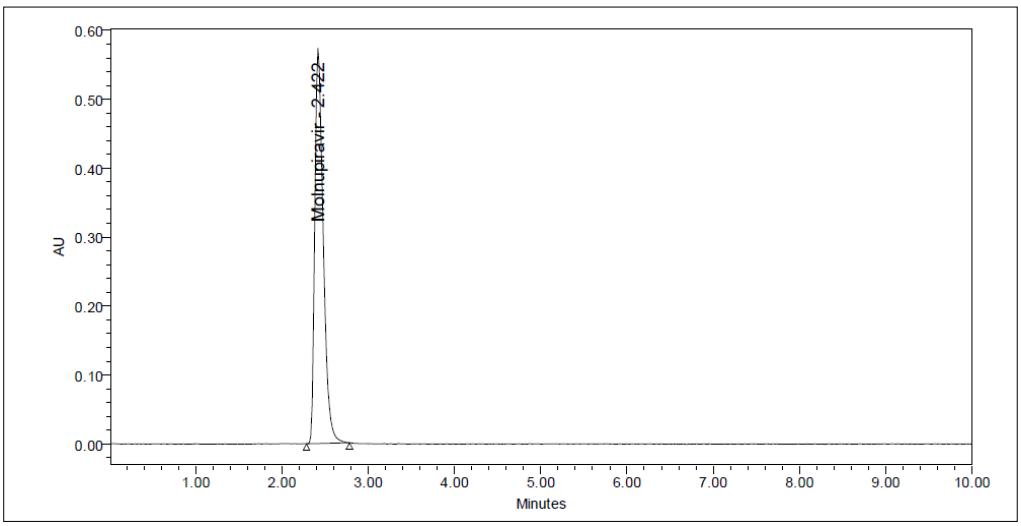

Figure 9 Thermal degradation chromatogram

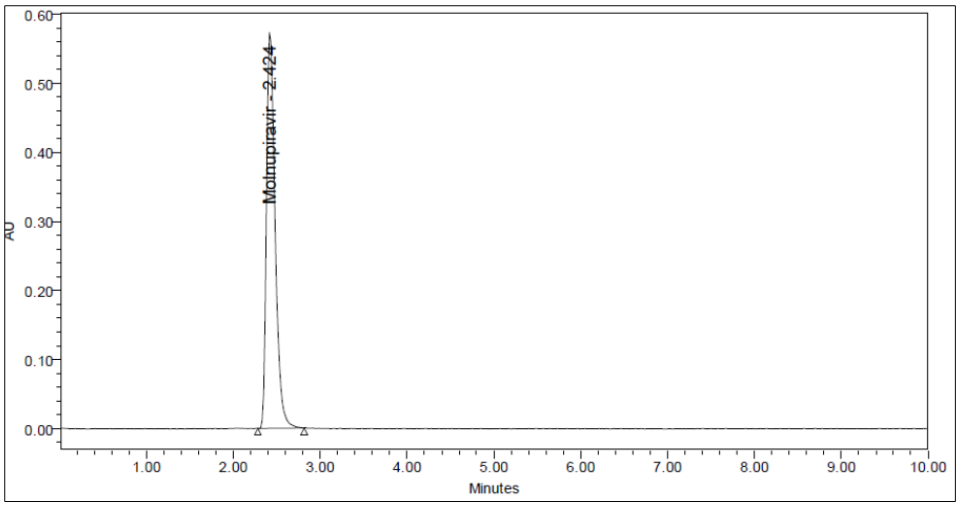

Figure 10 UV degradation chromatogram

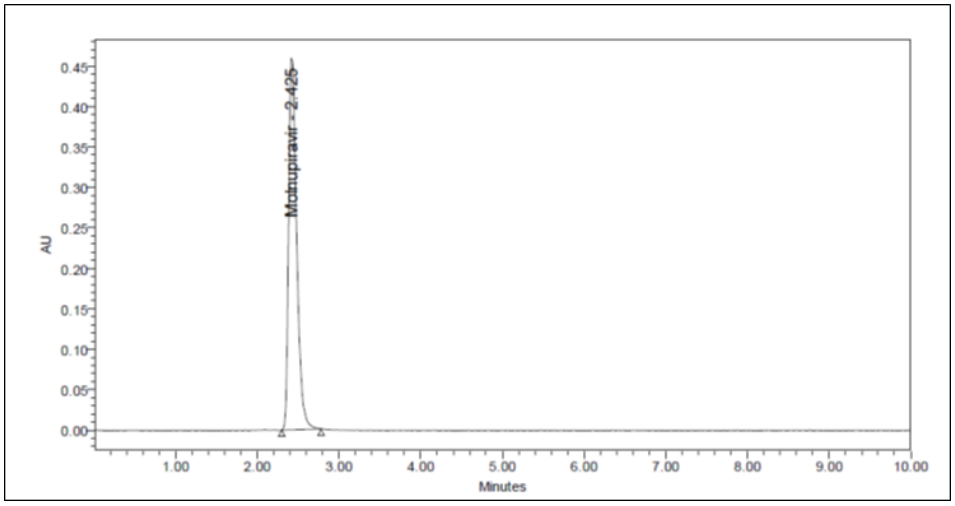

Figure 11 Water degradation chromatogram

4. Discussion

Regarding the pHadjustment in mobile phase for the acid and base degradation studies have movement in retention time of drugs. But due to neutralized acid sample with 2N Base solution and base sample with 2N Acid solution there will be no change in retention time. the summarized data shown in table 7.

Table 7 Summarized data

Parameters		**Molnupiravir**	**LIMIT**
Linearity :Range(µg/ml)		20-60 µg/ml	R< 1
Regression coefficient		0.999	
Slope(m)		99651	
Intercept(c)		7534	
Regression equation (Y=mx+c)		y = 99651x + 7534.	
Assay(% mean assay)		100.31%	90-110%
Specificity		Specific	No interference of any peak
System precision %RSD		0.8	NMT 2.0%
Method precision %RSD		0.9	NMT 2.0%
Accuracy %recovery		100.45%	98-102%
LOD		0.09	NMT 3
LOQ		0.27	NMT 10
Robustness	FM	0.6	%RSDNMT2.0
	FP	0.5	
	MM	1.1	
	MP	1.1	
	TM	1.2	
	TP	0.5	

5. Conclusion

Chromatographic conditions used are stationary phase Zodiasil C18 (150mm*4.6mm5µ), Mobile phase Water: Methanol in the ratio of 60:40 and flow rate were maintained at 0.8ml/min, detection wave length was 25nm, column temperature was set to 30°C and diluent was mobile phase Conditions were finalized as optimized method. System suitability parameters were studied by injecting the standard six times and results were well under the acceptance criteria. Linearity study was carried out between25% to150 % levels, R^2 value was found to be as 0. 999.Precision was found to be 0.9 for repeatability and 0.8 for intermediate precision.LOD and LOQ are 0.23µg/ml and 0.69µg/ml respectively. By using above method assay of marketed formulation was carried out 100.31% was present. Degradation studies of Molnupiravir were done, in all condition's purity threshold was more than purity angle and within the acceptable range. Full length method was not performed; if it is done this method can be used for routine analysis of Molnupiravir.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors agree no conflict of interest.

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