AN ANALYTICAL NEW RP-HPLC METHOD FOR THE QUANTITATIVE DETERMINATION OF MOLNUPIRAVIR IN BULK AND TABLET DOSAGE FORM

Dr. Ch. V. Suresh, Tallamalla Mamatha, Santhosh Illendula, Dr. K. N. V. Rao*
Department of Pharmaceutical Analysis, Nalanda College of Pharmacy, Charlapally, Nalgonda, Telangana

ABSTRACT: ***A rapid and precise reverse phase high performance liquid chromatographic method has been developed for the validated of Molnupiravir, in its pure form as well as in pharmaceutical dosage form. Chromatography was carried out on a Phenomenex Luna C18, 150 mm x 4.6 mm and 5µm column using a mixture of Methanol and Phosphate Buffer was in the ratio of 25:75%v/v (pH-3.4) as the mobile phase at a flow rate of 1.0ml/min, the detection was carried out at 270 nm. The retention time of the Molnupiravir was found to be 2.784 ±0.02min respectively. The method produce linear responses in the concentration range of 5-15µg/ml of Molnupiravir. The method precision for the determination of assay was below 2.0 %RSD. The method is useful in the quality control of bulk and pharmaceutical formulations.***

Keywords: Molnupiravir, RP-HPLC, Method Development, Validation, Accuracy, Precision.

I. INTRODUCTION

Molnupiravir is an orally bioavailable prodrug of EIDD-1931, the synthetic ribonucleoside derivative N4-hydroxycytidine and ribonucleoside analog, with potential antiviral activity against a variety of RNA viruses. Upon oral administration, Molnupiravir[1], being a prodrug, is metabolized into its active form EIDD-1931 and converted into its triphosphate (TP) form. The TP form of EIDD-1931 is incorporated into RNA and inhibits the action of viral RNA-dependent RNA polymerase. This results in the termination of RNA transcription and decreases viral RNA production, and viral RNA replication. 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. A Remdesivir resistant mutant mouse hepatitis virus has also been shown to have increased sensitivity to N4-hydroxycytidine. Molnupiravir[2] is indicated for treatment of mild to moderate coronavirus disease (COVID-19) in adults with a positive SARS-COV-2 diagnostic test and who have at least one risk factor for developing severe illness. The IUPAC Name of Molnupiravir[3] is [(2R, 3S, 4R, 5R)-3, 4-dihydroxy-5-[4-(hydroxy amino)-2-oxopyrimidin-1-yl] oxolan-2-yl] methyl 2-methyl propanoate. The Chemical Structure of Molnupiravir is as following

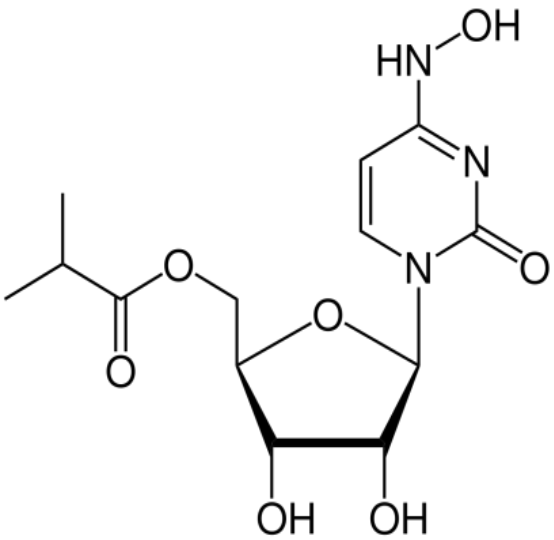

Fig.1. Chemical Structure of Molnupiravir

II. MATERIALS AND METHODS

Table-1: List of Equipments

S.No.	Instruments/Equipments/Apparatus
1.	HPLC WATERS with Empower2 Software with Isocratic with UV-Visible Detector.
2.	T60-LABINDIA UV – Vis spectrophotometer
3.	High Precision Electronic Balance
4.	Ultra Sonicator (Wensar wuc-2L)
5.	Thermal Oven
6.	Symmetry C_{18} Column, 250 mm x 4.6 mm and 5µm particle size
7.	P^H Analyzer (ELICO)
8.	Vaccum Filtration Kit (Labindia)

Table-2: List of Chemicals used

S.No.	Name	Grade	Manufacturer/Supplier
1.	HPLC grade water	HPLC	Sd fine-Chem ltd; Mumbai
2.	Methanol	HPLC	Loba Chem; Mumbai.
3.	Ethanol	A.R.	Sd fine-Chem ltd; Mumbai
4.	Acetonitrile	HPLC	Loba Chem; Mumbai.
5.	DMSO	A.R.	Sd fine-Chem ltd; Mumbai
6.	DMF	A.R.	Sd fine-Chem ltd; Mumbai

HPLC Instrumentation & Conditions:

The HPLC system employed was **HPLC WATERS** with Empower2 Software with Isocratic with UV-Visible Detector[4].

Standard preparation for UV-spectrophotometer analysis:

The standard stock solutions – 10 mg of Molnupiravir standard was transferred into 10 ml volumetric flask, dissolved & make up to volume with Methanol. Further dilutions were done by transferring 1 ml of the above solution into a 10ml volumetric flask and make up to volume with methanol to get 10ppm concentration.

It scanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Molnupiravir, so that the same wave number can be utilized in HPLC UV detector for estimating the Molnupiravir.

Different Trials for Chromatographic Conditions:

Table-3: Different Chromatographic Conditions

Column Used	Mobile Phase	Flow Rate	Wave length	Observation	Result

Inertsil C_{18}, 250 mm x 4.6 mm and 5µm Column	Acetonitrile : Water (60:40)	0.8 ml/min	270nm	Broad Peak	Method rejected
Symmetry C_{18}, 150 mm x 4.6 mm and 5µm Column	Methanol : Water (70:30)	0.9 ml/min	270nm	Negligible Peaks	Method rejected
Hypersil C_{18}, 250 mm x 4.6 mm and 5µm Column	Methanol : Acetonitrile (80:20)	0.9 ml/min	270nm	Multiple Peaks	Method rejected
Develosil C_{18}, 150 mm x 4.6 mm and 5µm Column	Methanol : Acetonitrile (70:30)	1.0 ml/min	270nm	Base line noise is high	Method rejected
Zorbax C_{18}, 250 mm x 4.6 mm and 5µm Column	Methanol : Acetonitrile (40:60)	1.0 ml/min	270nm	Stabilization not done properly	Method rejected
Phenomenex Luna C_{18}, 150 mm x 4.6 mm and 5µm Column	Methanol : Phosphate Buffer (45:55) (pH-4.2)	1.0 ml/min	270nm	Extra peaks	Method rejected
Phenomenex Luna C_{18}, 150 mm x 4.6 mm and 5µm Column	Methanol : Phosphate Buffer (35:65) (pH-3.8)	1.0 ml/min	270nm	Extra peaks & stabilization was not done properly	Method rejected
Phenomenex Luna C_{18}, 150 mm x 4.6 mm and 5µm Column	Methanol : Phosphate Buffer (25:75) (pH-3.4)	1.0 ml/min	270nm	Good sharp peak	Method accepted

Preparation of Phosphate buffer pH-3.4:

Accurately weighed 6.8 grams of KH2PO4 was taken in a 1000ml volumetric flask, dissolved and diluted to 1000ml with HPLC water and the pH was adjusted to 3.4 with orthophosphoric acid solution.

Preparation of Mobile Phase:

The mobile phase used in this analysis containing of a mixture of Methanol and Phosphate Buffer in the ratio of 25:75% v/v was prepared in the volume of 1000ml in which 250ml of Methanol was mixed with 750ml of Water.

Preparation of Standard Solution:

Working concentration should be about 10µg/ml. Correctly weigh around 10mg of Molnupiravir working standard, poured into a clean and dry 10 ml volumetric flask. Then dissolved and diluted to volume with the mobile phase to obtain a solution having a known concentration of about 1000 mcg/ml or 1000ppm. Further dilutions have been made to get the final concentration of 10µg/ml.

III. RESULTS AND DISCUSSION

Method Development

Selection of Wavelength:

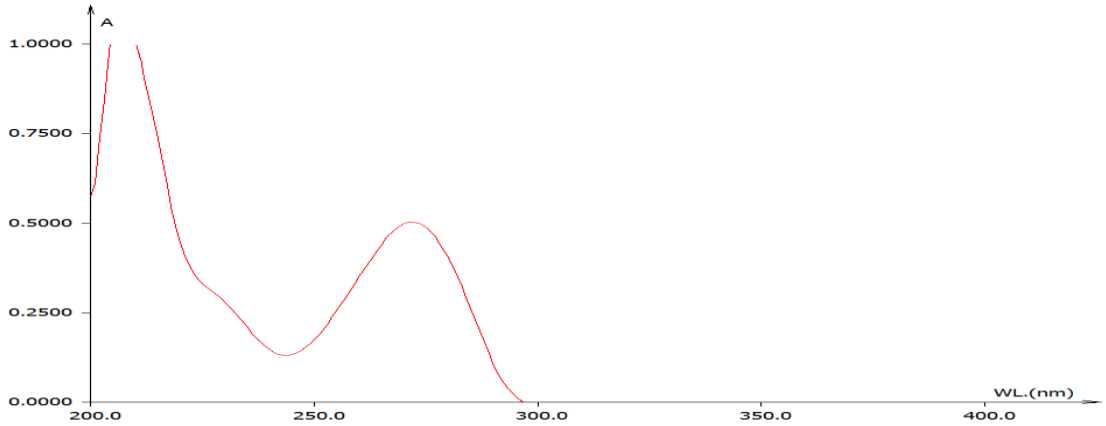

Fig.2. UV-Spectrum for Molnupiravir

Observation: While scanning the Molnupiravir solution we observed the maxima at 270nm.

Optimized Chromatographic Conditions:

Table-4: Optimized Chromatographic Conditions

Column	Phenomenex Luna C_{18}, 150 mm x 4.6 mm and 5µm
Mobile Phase	Methanol : Phosphate Buffer (25:75) (pH-3.4)
Flow Rate	1.0ml/minute
Wave length	270 nm
Injection volume	10 µl
Run time	7 minutes
Column Temperature	Ambient

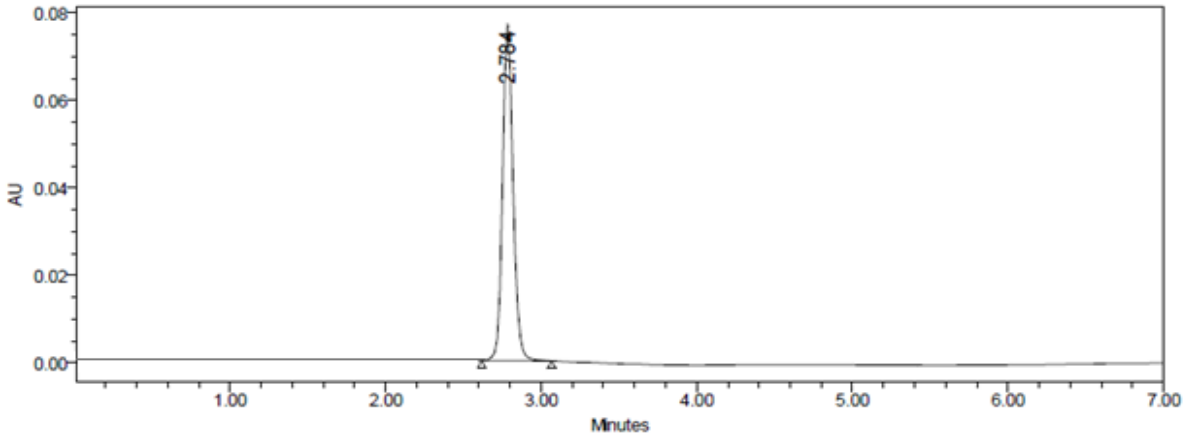

Fig.3. Optimized Chromatogram for Molnupiravir

Method Validation

System Suitability Test

System suitability testing[5-7] is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analysed constitute an integral system that can be evaluated as such. Following system suitability test parameters were established. The data are shown in Table-5.

Table-5: Data of System Suitability Test

S.No	Injection no	RT	Area	Height	USP Plate Count	USP Tailing
1	Injection 1	2.768	2435648	7542	8745	1.38

2	Injection 2	2.784	2452417	7659	8758	1.39
3	Injection 3	2.786	2453654	7582	8975	1.35
4	Injection 4	2.794	2454576	7635	8965	1.38
5	Injection 5	2.780	2455645	7659	8859	1.34
6	Injection 6	2.789	2453524	7698	8869	1.37
Mean			**2450911**			
S.D			**7555.422**			
%RSD			**0.30827**			

Accuracy:

Recovery study:

To determine the accuracy[8] of the proposed method, recovery studies were carried out by adding different amounts (50%, 100%, and 150%) of pure drug of Molnupiravir were taken and 3 replications of each has been injected to HPLC system. From that percentage recovery values[9] were calculated from the linearity equation y = 242891x + 28359. The results were shown in table-6.

Table-6: Accuracy Readings

Sample ID	Concentration (µg/ml)		Peak Area	% Recovery of Pure drug	Statistical Analysis	
	Amount Injected	Amount Recovered				
S_1 : 50 %	5	4.998	1242548	99.960%	Mean = 100.22% S.D. = 0.004854 % R.S.D.= 0.484321	% Mean Recovery = 100.16%
S_2 : 50 %	5	4.996	1241879	99.920%		
S_3 : 50 %	5	5.039	1252436	100.780%		
S_4 : 100 %	10	9.998	2456854	99.980%	Mean = 100.05% S.D. = 0.002621 % R.S.D. = 0.261976	
S_5 : 100 %	10	9.983	2453216	99.830%		
S_6 : 100 %	10	10.034	2465648	100.340%		
S_7 : 150 %	15	15.016	3675847	100.106%	Mean = 100.20% S.D. = 0.000911 % R.S.D. = 0.090875	
S_8 : 150 %	15	15.043	3682345	100.286%		
S_9 : 150 %	15	15.033	3679868	100.220%		

Observation: From the Accuracy Method, we observed that the mean %Recovery of the drug are 100.22%, 100.05% and 100.20% which is within the range of 98-102% and %RSD is within the range <2, i.e. 0.484321%, 0.261976% and 0.090875% respectively.

Precision:

Repeatability

The precision[10] of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount of drug Molnupiravir (API). The percent relative standard deviation[11] was calculated for Molnupiravir.

Table-7: Repeatability readings

HPLC Injection Replicates of Molnupiravir	Retention Time	Peak Area	Theoretical Plates	Tailing Factor
Replicate – 1	2.777	2453658	8652	1.38
Replicate – 2	2.799	2469854	8694	1.34
Replicate – 3	2.789	2458677	8672	1.39
Replicate – 4	2.797	2459689	8692	1.37
Replicate – 5	2.797	2477898	8679	1.35
Replicate – 6	2.799	2469853	8638	1.36
Average		**2464938**		
Standard Deviation		**9058.437**		
% RSD		**0.367491**		

Observation: From the Precision method, we observed that the %RSD of the Peak Area is 0.367491 which are within the acceptable range as per ICH guidelines[12-15].

Intermediate precision:

The Intermediate Precision[16] consists of two methods:-

Intra Day: In Intra Day process, the 50%, 100% and 150% concentration are injected at different intervals of time in same day.

Inter Day: In Inter Day process, the 50%, 100% and 150% concentration are injected at same intervals of time in different days.

Intra-Day (Day 1):

Table-8: Results of Intermediate precision Day 1 for Molnupiravir

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Molnupiravir	2.768	2489851	7584	8658	1.38
2	Molnupiravir	2.789	2478658	7598	8699	1.37
3	Molnupiravir	2.784	2478785	7596	8693	1.39
Mean			**2482431**			
Std. Dev.			**6425.934**			
% RSD			**0.258856**			

Inter Day (Day 2):

Table-9: Results of Intermediate precision Day 2 for Molnupiravir

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Molnupiravir	2.777	2536542	7869	8987	1.57
2	Molnupiravir	2.780	2547154	7889	8967	1.56
3	Molnupiravir	2.797	2569853	7896	8969	1.59
Mean						
Std. Dev.						
% RSD						

Observations: The intra & inter day variation[17] of the method was carried out for standard deviation & % RSD (% RSD <2%) within a day & day to day variations for Molnupiravir revealed that the proposed method is precise.

Linearity & Range:

To evaluate the linearity, serial dilution of analyte were prepared from the stock solution was diluted with mobile phase[18] to get a series of concentration ranging from 5-15µg/ml. The prepared solutions were sonicated. From these solutions, 10µl injections of each concentration were injected into the HPLC system[19] and chromatographed under the optimized conditions. Calibration curve[20] was constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis).

Table-10: Concentration of Molnupiravir

Concentration in ppm	Peak Area
0	0
5	1275475
7.5	1857648
10	2456587
12.5	3082546
15	3642447

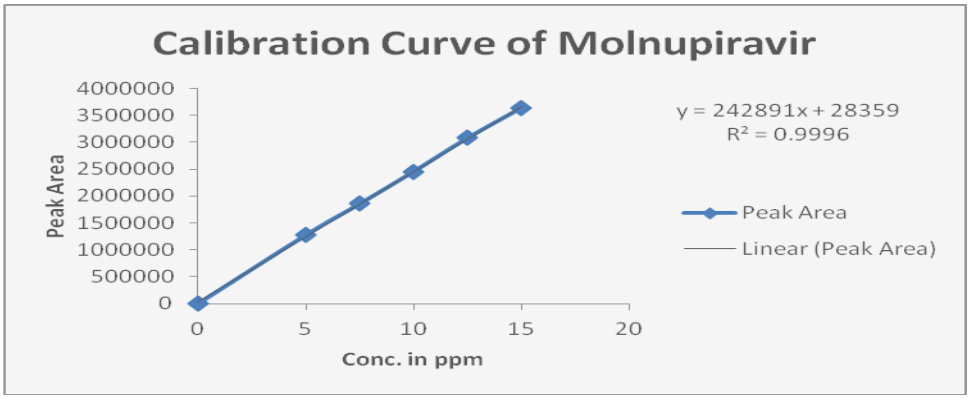

Fig.4. Calibration Curve of Molnupiravir (API)

Observation: We observed that the calibration curve showed good linearity in the range of 5-15 µg/ml, for Molnupiravir (API) with correlation coefficient (R^2) of 0.9996. A typical calibration curve has the regression equation[21] of y = 242891x + 28359 for Molnupiravir.

Method Robustness:

Influence of small changes in chromatographic conditions such as change in flow rate 1ml (± 0.1ml/min), Wavelength of detection 270nm (±2nm) & organic phase content in mobile phase (±5%) studied to determine the robustness[22] of the method are also in favour of (Table-11, % RSD < 2%) the developed RP-HPLC method for the analysis of Molnupiravir (API).

Table-11: Results of Method Robustness Test

Change in Parameter	Theoretical Plates	Tailing Factors	% RSD
Flow (1.0 ml/min)	8645	1.38	0.265
Flow (0.9 ml/min)	8754	1.37	0.487
More Organic (75+5)	8952	1.32	0.698
Less Organic (75-5)	8214	1.34	0.458
Wavelength of Detection (272 nm)	8368	1.39	0.597
Wavelength of detection (268nm)	8952	1.34	0.856

Limit of Detection (LOD) & Limit of Quantitation (LOQ):

The detection limit[23] (LOD) and quantization limit[24] (LOQ) may be expressed as:

L.O.D. = 3.3(SD/S).

L.O.Q. = 10(SD/S)

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.6902µg/ml & 2.091µg/ml respectively.

Estimation of Molnupiravir in TABLET Dosage Form

Twenty Capsules were taken and the I.P. method was followed to determine the average weight. Above weighed Capsules were finally powdered and triturated well. A quantity of powder equivalent to 10 mg of drug were transferred to 10 ml volumetric flask, and 8 ml of mobile phase was added and solution was sonicated for 15 minutes, there after volume was made up to 10 ml with same solvent. Then 1ml of the above solution was diluted to 10 ml with HPLC grade methanol. The solution was filtered through a membrane filter (0.45 µm) and sonicated to degas. From this stock solution (1.0 ml) was transferred to five different 10 ml volumetric flasks and volume was made up to 10 ml with same solvent system.

The solution prepared was injected in five replicates into the HPLC system and the observations were recorded.

A duplicate injection of the standard solution was also injected into the HPLC system and the peak areas were recorded. The data are shown in Table-12.

ASSAY

$$\% \text{ Assay} = AT/AS \times WS/DS \times DT/WT \times P/100 \times AW/LC \times 100$$

Where:

AT = Peak Area of Molnupiravir obtained with test preparation

AS = Peak Area of Molnupiravir obtained with standard preparation

WS = Weight of working standard taken in mg

WT = Weight of sample taken in mg

DS = Dilution of Standard solution

DT = Dilution of sample solution

P = Percentage purity of working standard

Results obtained are tabulated below:

Table-12: Assay of Molnupiravir Capsule

Brand Name of Capsules	Labelled amount of Drug (mg)	Mean (±SD) amount (mg) found by the proposed method (n=5)	Assay + % RSD
Molflu Capsules (Dr. Reddy's)	200	199.867 (± 0.324)	99.867% (± 0.457)

Result & Discussion: The %Purity[25] of Molflu Capsule containing Molnupiravir was found to be 99.867% (± 0.457).

Stability Studies:

Results of Degradation Studies: The results of the stress studies[26-30] indicated the specificity of the method that has been developed. Molnupiravir was stable in thermal, Oxidation and Photolytic stress conditions. The results of forced degradation studies are given in the following table-13.

Table-13: Results of Forced Degradation Studies of Molnupiravir

Stress Condition	Time	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1N HCl)	24Hrs.	76.302	23.698	100%
Basic Hydrolysis (0.1N NaOH)	24Hrs.	84.426	15.574	100%
Thermal Degradation (60^0C)	24Hrs.	91.105	8.895	100%
UV (254nm)	24Hrs.	85.121	14.879	100%
3% Hydrogen peroxide	24Hrs.	86.315	13.685	100%

IV. SUMMARY AND CONCLUSION

The analytical method was developed by studying different parameters. First of all, maximum absorbance was found to be at 270 nm and the peak purity was excellent. Injection volume was selected to be 10µl which gave a good peak area. The column used for study was Phenomenex Luna C18, 150 mm x 4.6 mm and 5µm because it was giving good peak. Ambient temperature was found to be suitable for the nature of drug solution. The flow rate was fixed at 1.0ml/min because of good peak area and satisfactory retention time.

Mobile phase is Methanol and Phosphate Buffer was taken in the ratio of 25:75% v/v at pH-3.4 was fixed due to good symmetrical peak. So this mobile phase was used for the proposed study. Run time was selected to be 7 min because analyze gave peak around 2.784 ±0.02min respectively and also to reduce the total run time. The percent recovery was found to be 98.0-102 was linear and precise over the same range. Both system and method precision was found to be accurate and well within range.

The analytical method was found linearity over the range 5-15µg/ml of Molnupiravir of the target concentration. The analytical passed both robustness and ruggedness tests. On both cases, relative standard deviation was well satisfactory.

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