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A new analytical method development and validation for the quantitative estimation of anti-viral agent Molnupiravir in API form and marketed pharmaceutical dosage forms by RP-HPLC

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Abstract
An Analytical, precise, accurate, robust, rugged, efficient and simple RP-HPLC method has been developed and validated for the determination of Molnupiravir in bulk and was applied on marketed pharmaceutical dosage forms. The mobile phase used for the chromatographic runs consisted of Phosphate Buffer (0.02M) and Acetonitrile in the proportion of 48:52% with maintained at pH-2.80 by diluted OPA solution. The separation was achieved on a Symmetry ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm column using isocratic mode. Drug peak were well separated and were detected by a UV detector at 248 nm. The method was linear at the concentration range 30–70 µg/ml for Molnupiravir. The method has been validated according to ICH guidelines with respect to system suitability, specificity, precision, accuracy and robustness. Molnupiravir limit of detection (LOD) and limit of quantification (LOQ) were 0.09µg/ml and 0.27µg/ml respectively.

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Introduction

Molnupiravir is a nucleoside analogue that is N (4)-hydroxycytidine in which the 5'-hydroxy group is replaced by a (2-methylpropanoyl) oxy group. It is the prodrug of the active antiviral ribonucleoside analog N (4)-hydroxycytidine (EIDD-1931), which has activity against a number of RNA viruses including SARS-CoV-2, MERS-CoV, and seasonal and pandemic influenza viruses. It is currently in phase III trials for the treatment of patients with COVID-19. It has a role as a prodrug, an anticoronaviral agent, and an antiviral drug. It is a nucleoside analog, an isopropyl ester, and a ketoxime. It is functionally related to an N (4)-hydroxycytidine. Molnupiravir [1] (EIDD-2801, MK-4482) is the isopropyl ester prodrug of [N4-hydroxycytidine]. With improved oral bioavailability in non-human primates, it is hydrolyzed in vivo, and distributes into tissues where it becomes the active 5'-triphosphate form. The active drug incorporates into the genome of RNA viruses, leading to an accumulation of mutations known as viral error catastrophe. Recent studies have shown Molnupiravir [2] inhibits replication of human and bat coronaviruses, including SARS-CoV-2, in mice and human airway epithelial cells. A [Remdesivir] resistant mutant mouse hepatitis virus has also been shown to have increased sensitivity to N4-hydroxycytidine. Molnupiravir was granted approval by the UK's Medicines and Health products Regulatory Agency (MHRA) on 4 November 2021 to prevent severe outcomes such as hospitalization and death due to COVID-19 in adults. Molnupiravir was also granted emergency use authorization by the FDA on December 23, 2021; however, it is not yet fully approved. Molnupiravir [3] is a ribonucleoside analogue and antiviral agent that is used in the therapy the severe acute respiratory syndrome (SARS) coronavirus 2 (CoV-2) infection, the cause of the novel coronavirus disease, 2019 (COVID-19). Molnupiravir therapy is given orally for 5 days early in the course of SARS-CoV-2 infection and has not been linked to serum aminotransferase elevations or to clinically apparent liver injury. The IUPAC Name of Molnupiravir is [(2R, 3S, 4R, 5R)-3, 4-dihydroxy-5-[4-(hydroxy amino)-2-oxo pyrimidin-1-yl] oxolan-2-yl] methyl 2-methyl propanoate. The Chemical Structure of Molnupiravir is as shown in follows

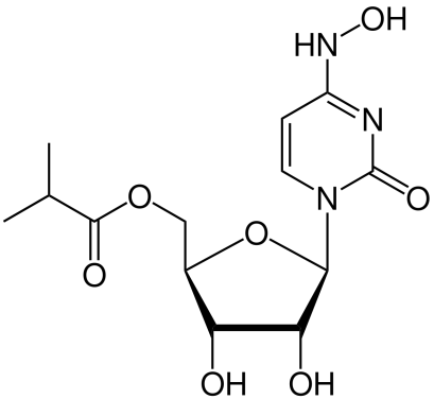

Fig 1: Chemical Structure of Molnupiravir

Literature survey [31-32] revealed that Molnupiravir was determined in bulk and pharmaceutical dosage forms by RP-HPLC as well as in biological fluids using liquid chromatography and liquid chromatography mass spectrometric methods. In the present work the authors have developed a simple, rapid, precise, accurate and robust liquid chromatographic method for the determination of Molnupiravir in pure forms and marketed pharmaceutical dosage forms as per ICH guidelines [30].

Materials and Methods

Table 1: List of Instrument Used

S. No.	Instruments/Equipments/Apparatus
1.	Waters HPLC with Empower2 Software with Isocratic with UV-Visible Detector.
2.	ELICO SL-159 UV – Vis spectrophotometer
3.	Electronic Balance (SHIMADZU ATY224)
4.	Ultra Sonicator (Wensar wuc-2L)
5.	Thermal Oven
6.	Symmetry RP C_{18}, 5µm, 250mm x 4.6mm i.d.
7.	P^H Analyzer (ELICO)
8.	Vacuum filtration kit (BOROSIL)

Chemicals / Reagents Used

Table 2: List of Chemicals Used

S. No.	Name	Specifications		Manufacturer/Supplier
		Purity	Grade	
1.	Doubled distilled water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
2.	Methanol	99.9%	HPLC	Loba Chem; Mumbai.
3.	Dipotassium hydrogen orthophosphate	96%	L.R.	Sd fine-Chem ltd; Mumbai
4.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
5.	Potassium dihydrogen orthophosphate	99.9%	L.R.	Sd fine-Chem ltd; Mumbai
6.	Sodium hydroxide	99.9%	L.R.	Sd fine-Chem ltd; Mumbai
7.	Hydrochloric acid	96%	A.R.	Sd fine-Chem ltd; Mumbai
8.	3% Hydrogen Peroxide	96%	A.R.	Sd fine-Chem ltd; Mumbai

Method Development

Selection of Wavelength

The standard & sample stock solutions were prepared separately by dissolving standard & sample in a solvent in mobile phase [4] diluting with the same solvent. (After optimization of all conditions) for UV analysis. 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. While scanning the Molnupiravir solution [5] we observed the maxima at 248 nm. The UV spectrum has been recorded on ELICO SL-159 make UV – Vis spectrophotometer model UV-2450. The scanned UV spectrum[6] is attached in the following page.

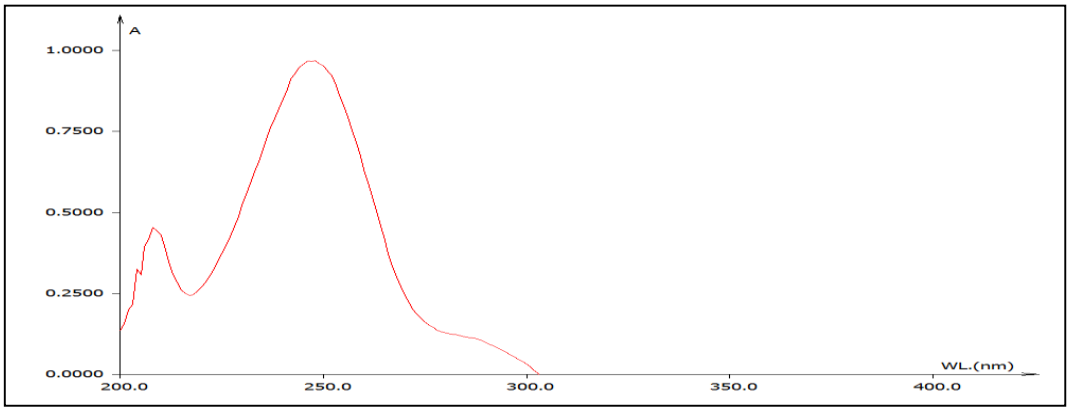

Fig 2: UV Spectrum for Molnupiravir (248nm)

Sample & Standard Preparation for the Analysis

25 mg of Molnupiravir standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with mobile phase.

Further dilution [7] was done by transferring 0.3 ml of the above solution into a 10 ml volumetric flask and make up to volume with mobile phase.

Optimization of Chromatographic Conditions:

The chromatographic conditions[8] were optimized by

different means. (Using different column, different mobile phase, different flow rate, different detection wavelength & different diluents for sample preparation [9] etc.

Table 3: Summary of Process Optimization

Column Used	Mobile Phase	Flow Rate	Wave length	Observation	Result
Develosil ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm	Methanol : Water = 40 : 60	1.0ml/min	248nm	Very Low response	Method rejected
Zorbax ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm	Acetonitrile : Water = 60 : 40	1.0ml/min	248nm	Low response	Method rejected
Inertsil ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm	Acetonitrile: Methanol = 70 : 30	1.0ml/min	248nm	Tailing peaks	Method rejected
Symmetry ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm	Phosphate Buffer : Acetonitrile = 30:70 (pH-4.0)	1.0ml/min	248nm	Resolution was not good	Method rejected
Symmetry ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm	Phosphate Buffer : Methanol = 20:80 (pH-3.8)	1.0ml/min	248nm	Tailing peak	Method rejected
Symmetry ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm	Phosphate Buffer : Acetonitrile = 48:52 (pH-2.8)	1.0ml/min	248nm	Nice peak	Method accepted

Summary of Optimized Chromatographic Conditions

The Optimum conditions[10] obtained from experiments can be summarized as below:

Table 4: Summary of Optimised Chromatographic Conditions

Mobile phase	Phosphate Buffer (0.02M): Acetonitrile = 48:52 (pH-2.80)
Column	Symmetry ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm
Column Temperature	Ambient
Detection Wavelength	248 nm
Flow rate	1.0 ml/ min.
Run time	08 min.
Temperature of Auto sampler	Ambient
Diluent	Mobile Phase
Injection Volume	20µl
Mode of Elution	Isocratic
Retention time	3.649 minutes

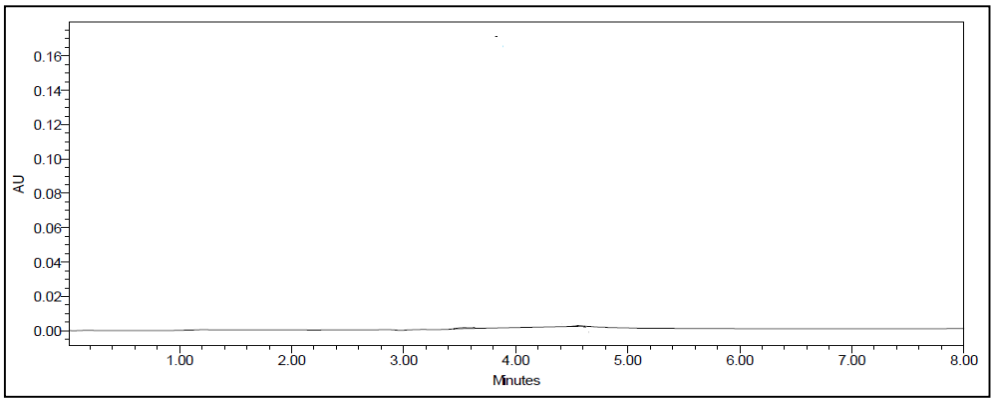

Fig 3: HPLC Spectrum of Molnupiravir (Blank Solution)

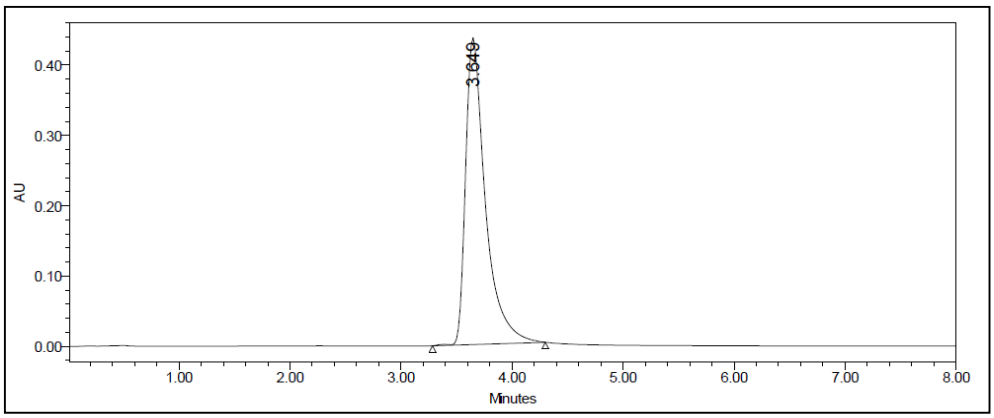

Fig 4: Chromatogram of Molnupiravir in Optimized Chromatographic Condition

Preparation of 0.02M Potassium Dihydrogen Orthophosphate Solution

About 2.72172 grams of Potassium dihydrogen orthophosphate was weighed and transferred into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC [11] Grade water. The pH was adjusted to 2.80 with diluted orthophosphoric acid Solution.

Preparation of Mobile Phase

480mL (48%) of above Phosphate buffer solution and 520mL of HPLC Grade Acetonitrile (52%) were mixed well and degassed [12] in ultrasonic water bath for 15 minutes. The resulted solution was filtered through 0.45 µm filter under vacuum filtration.

Method Validation

1. Accuracy

Recovery Study

To determine the accuracy [13] of the planned technique, recovery studies were distributed by adds completely different amounts (80%, 100%, and 120%) of pure drug of Molnupiravir were taken and extra to the pre-analyzed formulation of concentration 30µg/ml. From that proportion recovery values[14] were calculated. The results were shown in table-5.

Table 5: Accuracy Readings

Sample ID	Concentration (µg/ml)		Peak Area	% Recovery of Pure drug	Statistical Analysis
	Amount Added	Amount Found			
S_1 : 80 %	40	40.141	502647	100.352	Mean= 100.3947% S.D. = 0.071319 % R.S.D.= 0.071038
S_2 : 80 %	40	40.191	503214	100.477	
S_3 : 80 %	40	40.142	502656	100.355	
S_4 : 100 %	50	50.044	614215	100.088	Mean= 99.98533% S.D. = 0.183045 % R.S.D.= 0.183071
S_5 : 100 %	50	49.887	612451	99.774	
S_6 : 100 %	50	50.047	614254	100.094	
S_7 : 120 %	60	60.192	728547	100.32	Mean= 100.311% S.D. = 0.408574 % R.S.D.= 0.407308
S_8 : 120 %	60	59.939	725698	99.898	
S_9 : 120 %	60	60.429	731211	100.715	

2. Precision

2.1. Repeatability

The exactitude [15] of every technique was determined one by one from the height areas & retention times obtained by actual determination of six replicates of a set quantity of drug. Molnupiravir (API). The % relative variance[16] was calculated for Molnupiravir square measure bestowed within the table-6.

Table 6: Repeatability Readings

HPLC Injection Replicates of Molnupiravir	Retention Time (Minutes)	Peak Area
Replicate – 1	3.649	5674158
Replicate – 2	3.684	5654715
Replicate – 3	3.687	5665841
Replicate – 4	3.688	5654578
Replicate – 5	3.688	5652284
Replicate – 6	3.687	5641487
Average		**5657177**
Standard Deviation		**11369.72**
% RSD		**0.200979**

2.2. Intermediate Precision

2.2.1. Intra-assay & inter-assay

The intra & inter day variation[17] of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Molnupiravir revealed that the proposed method[18] is precise.

Table 7: Results of Intra-Assay & Inter-Assay

Conc. of Molnupiravir (API) (µg/ml)	Observed Conc. of Molnupiravir (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
40	40.05	1.09	39.89	1.08
50	50.08	0.95	49.54	0.76
60	60.09	0.97	59.86	0.94

3. Linearity & Range

The calibration curve showed good linearity in the range [19] of 0-70µg/ml, for Molnupiravir (API) with correlation coefficient (r^2) of 0.999 (Fig-5). A typical calibration curve[20] has the regression equation of y = 11266.x + 50416 for Molnupiravir.

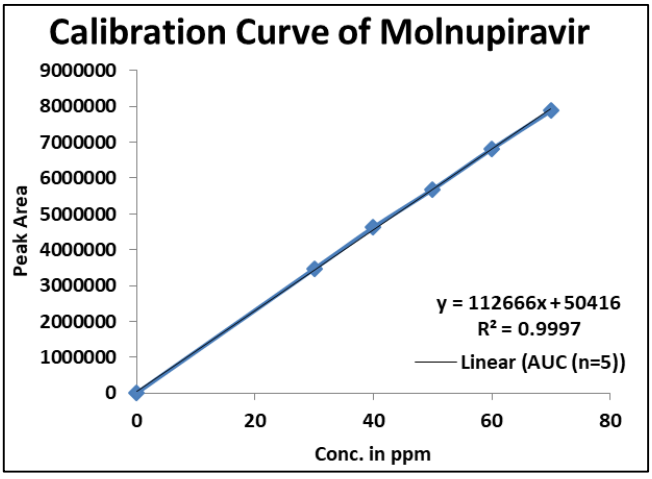

Fig 5: Calibration Curve of Molnupiravir (API)

Table 8: Linearity Results

CONC.(µg/ml)	MEAN AUC (n=6)
0	0
30	3465974
40	4626478
50	5682284
60	6815478
70	7878721

4. Method Robustness

Influence of small changes in chromatographic conditions [21] such as change in flow rate (± 0.1ml/min), Temperature

(±2^{0}C), Wavelength of detection (±2nm) & Acetonitrile content in mobile part (±2%) studied to work out the strength of the tactic also are in favour of (Table-9, and RSD < 2%) the developed RP-HPLC technique [22] for the analysis of Molnupiravir (API).

Table 9: Result of Method Robustness Test

Change in Parameter	% RSD
Flow (1.1 ml/min)	0.56
Flow (0.9 ml/min)	0.87
Temperature (27^{0}C)	0.72
Temperature (23^{0}C)	0.53
Wavelength of Detection (257 nm)	0.61
Wavelength of detection (253 nm)	0.96

5. LOD & LOQ

The Minimum concentration level at which the analyte can be reliable detected[23] (LOD) & quantified [24] (LOQ) were found to be 0.09 & 0.27 µg/ml respectively.

6. System Suitability Parameter

System quality testing [25-26] is associate degree integral a part of several analytical procedures. The tests square measure supported the idea that the instrumentation, physics, associate degree analytical operations and samples to be analyzed represent an integral system that may be evaluated intrinsically. Following system quality check parameters were established. The info square measure shown in Table-10.

Table 10: Knowledge of System quality Parameter

S.No.	Parameter	Limit	Result
1	Resolution	Rs > 2	8.54
2	Asymmetry	T ≤ 2	Molnupiravir =0.98
3	Theoretical plate	N > 2000	Molnupiravir =4782
4	Tailing Factor	T<2	Molnupiravir =1.49

7. Estimation of Molnupiravir in Pharmaceutical Dosage Form

Twenty pharmaceutical dosage forms[27] were taken and the I.P. method was followed to work out the typical weight. On top of weighed tablets/capsules were finally pulverized and triturated well. A amount of powder cherish twenty-five mg of medicine was transferred to a twenty-five cc meter flask, built, and the resolution was sonicated for a quarter-hour, there one volume was created up to twenty-five cc with the same solvent. Then ten cc of the on top of a resolution was diluted to a hundred cc with the mobile part. The answer was filtered through a membrane filter (0.45 µm) and sonicated to remove. The answer ready was injected in 5 replicates into the HPLC system and therefore the observations were recorded.

A duplicate injection of the quality resolution was conjointly injected into the HPLC system[28] and therefore the peak areas were recorded. The info square measure is shown in Table-11.

Assay

Assay % =

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Avg. Wt} = \text{mg/tab}$$

Where:

AT = Peak space of drug obtained with check preparation
AS = Peak space of drug obtained with normal preparation
WS = Weight of operating normal taken in mg
WT = Weight of sample taken in mg
DS = Dilution of normal resolution
DT = Dilution of sample resolution
P = proportion purity of operating normal

Table 11: Recovery Data for Estimation Molnupiravir

Brand Name of Molnupiravir	Labelled amount of Drug (mg)	Mean (± SD) amount (mg) found by the Proposed Method (n=6)	Assay % (± SD)
Molflu Capsule (200mg) (Dr. Reddy's)	200mg	199.685 (± 0.685)	99.698 (± 0.763)

Result & Discussion: The amount of drugs in Molnupiravir Capsule was found to be 199.685 (± 0.685) mg/tab for Molnupiravir & % assay [29] was 99.698%.

Summary and Conclusion

The analytical method was developed by studying different parameters. First of all, maximum absorbance was found to be at 246nm and the peak purity was excellent. The injection volume was selected to be 20µl which gave a good peak area. The column used for the study was Symmetry C18 ODS (4.6mm × 250mm) 5µm particle size because it was giving a good peak. Ambient temperature was found to be suitable for the nature of the drug solution. The flow rate was fixed at 1.0ml/min because of the good peak area and satisfactory retention time. The mobile phase is Acetonitrile: Phosphate buffer (0.01M, pH-3.2) (30:70 v/v) was fixed due to a good symmetrical peak. So this mobile phase was used for the proposed study. Methanol was selected because of maximum extraction sonication time was fixed to be 10min at which all the drug particles were completely soluble and showed good recovery. Run time was selected to be 10min because analyze gave a peak around 5.453min 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 found linearity over the range of 6-14ppm of the Molnupiravir target concentration. The analytical passed both robustness and ruggedness tests. On both cases, relative standard deviation was well satisfactory.

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