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STABILITY INDICATING ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR MOLNUPIRAVIR IN HUMAN PLASMA

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ABSTRACT:

This study presents a robust and validated reverse-phase high-performance liquid chromatography (RP-HPLC) method for the quantification of molnupiravir, an antiviral drug used in the treatment of COVID-19. The method was developed and optimized using a C18 column with a mobile phase consisting of methanol and sodium acetate buffer. System suitability tests demonstrated the appropriateness of the chromatographic system, ensuring precision, resolution, and peak symmetry. The method exhibited excellent specificity, as evidenced by the matching retention times of standard and test samples. Linearity was confirmed over a concentration range of 15-45 µg/ml, with a correlation coefficient (R^2) of 0.9995. Accuracy studies revealed recoveries ranging from 99.81% to 101.91%, indicating the method's reliability. Precision assessments, including intra-day and inter-day variations, demonstrated reproducibility with %RSD values within acceptable limits. Additionally, the method showed adequate sensitivity, with limits of detection (LOD) and quantification (LOQ) of 1.04 µg/ml and 3.17 µg/ml, respectively. Robustness studies indicated that small variations in method parameters did not significantly affect assay results. Stability studies under various stress conditions demonstrated the drug's susceptibility to degradation under acidic conditions while showing stability under other conditions.Overall, the validated RP-HPLC method offers a simple, accurate, and precise means for the routine analysis of molnupiravir, contributing to the effective management of COVID-19 treatment protocols.

Keyword:- RP-HPLC, Molnupiravir, Method Development, Validation

1. INTRODUCTION

Drugs that combat viral infections are known as antivirals. There are no effective antiviral drugs for many viral infections. A number of antiviral drugs work by stopping viruses from proliferating. This is how the great majority of HIV infection treatments function. Since viruses are tiny and replicate inside of cells by using the metabolic processes of the host cell, antiviral drugs are only able to target a limited number of metabolic processes. On the other hand, bacteria are large organisms that often grow outside of cells and have a range of metabolic functions that antibacterial drugs may target. The number of SARS-CoV-2 variants of concern (VoCs) has increased, making the COVID-19 pandemic scenario worse.

On December 20, 2021, the European Medicines Agency (EMA) approved the emergency use of MLP for the treatment of mild-to-moderate COVID-19 in people who tested positive for the SARS-CoV-2 virus and

who were at high risk of developing severe COVID-19.the giving of MLP to people who have a higher than normal risk of getting a significant case of COVID-19 in November 2021. MLP, a prodrug of the synthetic nucleoside derivative N4-hydroxycytidine, is well-known for its good safety and tolerability features and has been demonstrated to be instantly effective in decreasing viral RNA and the SARS-CoV-2 virus. Its antiviral effect is demonstrated by transcription errors added to viral RNA replication.

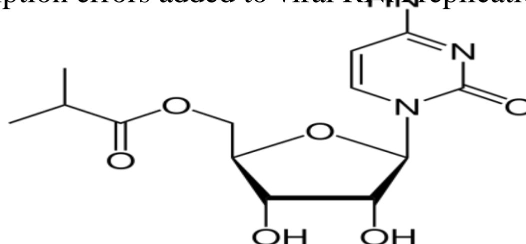

Fig. 1 chemical structure of Molnupiravir.

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.

2. MATERIALS AND METHODS: MATERIALS AND EQUIPMENTS

The drugs, chemicals, reagents, instruments and filters used during the experiment. Molnupiravir API were purchased from Bulat Pharmaceutical pvt. ltd. Gurugram, Haryana.

2.1 Instruments Used:

table no 01:- instruments used in method development

Sr.no	Name of instrument	Model
1	HPLC System	Younglin-HPLC system(ACME-9000)
2	Detector System	Detector – UV detector (730D)
3	Analytical Column	C_{18} (Hypersil BDS) (4.6 × 250mm, 5µm)
4	Software	Autochrom 3000
5	Ph Meter	M Lab
6	Injector	Manual
7	Analytical Balance	Shimadzu Model-ATX224
8	UV Spectrophotometer	Shimadzu UV1800 Spectrophotometer Japan Corporation)

2.2 Solvents And Chemicals:

- Methanol (gradient grade)
- Sodium acetate buffer
- Glacial acetic acid
- Acetonitrile (gradient grade)
- Water (HPLC grade)
- Human plasma

3. EXPERIMENTAL WORK

Optimization of chromatographic condition for the estimation of Molnupiravir.

3.1 Solubility Studies:

As a first step of method development solubility of drugs was tasted in different solvents to obtain a suitable solvent which can be used for method development.

3.2 Selection Of Wavelength:

An UV spectrum of 10 ug/ml Molnupiravir in methanol was recorded by scanning in the range of 200 nm to 400 nm. A wavelength which gives good response for the drugs to be selected. From the UV spectrum a wavelength of 239 nm was selected. Drug showed maximum absorbance at this wavelength.

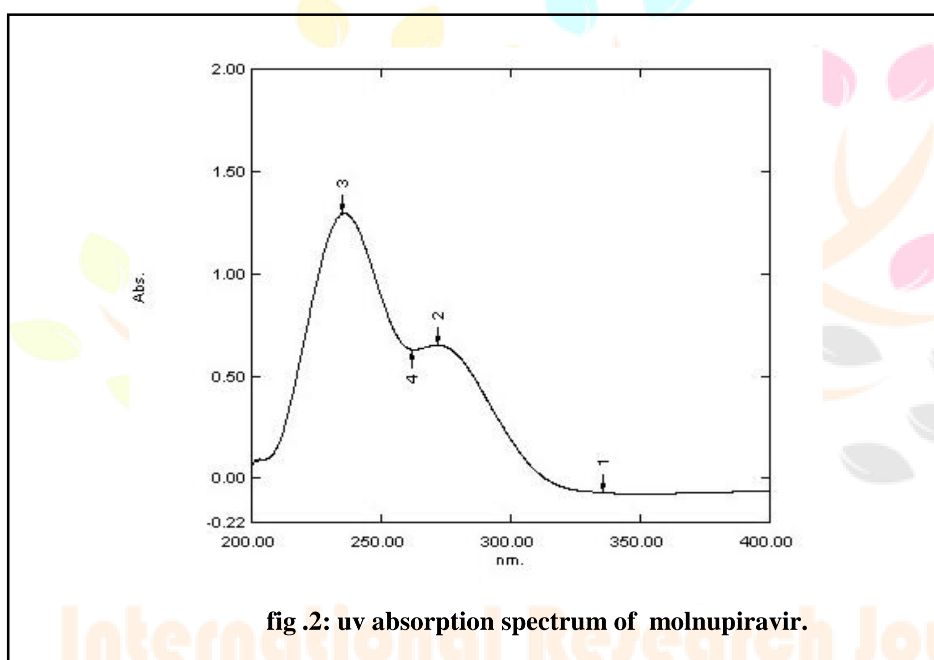

fig .2: uv absorption spectrum of molnupiravir.

3.3 Selection of stationary phase:

The C18 column is used because Molnupiravir is polar.

3.4 Selection of mobile phase:

Based on a review of the literature,different mobile phase like acetonitrile and water, methanol and water, methanol and ammonium formate, methanol and sodium acetate various proportions were tried to get a stable peak each mobile phase was filtered through 0.2µm membrane filter and sonicated on ultrasonic bath and experiments were conducted. Following the experiments, a mixture of Methanol-Sodium acetate buffer with a 70:30% V/V ratio was chosen, and more tests were run to obtain optimized chromatograms by adjusting the other parameters.

3.5 Optimization of Chromatographic Condition:

The following chromatographic conditions were established by trials and werekept constant throughout the experimentation.

table no.02 :-chromatographic conditions

HPLC system	Younglin-HPLC System
Column	Column - C_{18} (Hypersil BDS) (250 × 4.6 mm, 5µm)
pump	Pump – SP930 D
Mobile phase	Methanol:Sodium acetate(70:30)
Detection wavelength	239 nm
Flow rate	1.0 ml per minute.
Temperature	Ambient
Injection volume	20 µL.
Run time	8 minutes

3.6 Preparation of stock solutions:

weighed accurately 30mg of Molnupiravir and transferred to 100ml volumetric flasks. , dissolve and diluted upto the mark with the help of diluent, shake well sonicate for about 2 min.

3.7 Preparation of standard solution

Pipette out 2ml from stock solution and transferred to 20 ml volumetric flask, diluted upto the mark with diluent, shake well, filter through 0.2 µm syringe filter before injecting to hplc.

3.8 Preparation of test solution

Prepare homogeneous mixture of 0.2 ml of plasma and 2.0 ml of stock and 1.6ml of diluent, shake well, centrifuge for 10 min at 6000 rpm, inject from supernent layer.

4. METHOD VALIDATION:

The proposed method's linearity, range, accuracy, precision, ruggedness, and other properties were verified in accordance with ICH Q2 (R1) standards.
Validation of RP-HPLC method was done as per ICH guidelines for parameters like linearity, accuracy, precision, robustness, LOD and LOQ.

4.1 System Suitability:

This was done to make sure the chromatographic system was appropriate for the planned purpose. Precision Requirement, Theoretical Plates, and Tailing Factor are a few of the factors that can be evaluated for system applicability.

4.2 Specificity

Solutions of standard and sample were prepared and injected in to HPLC system and its respective peak area and retention time were observed.

4.3 Linearity

Suitable quantity of standard solution was transferred into a series of 10 ml volumetric flasks. The volume was made up to the mark with mobile phase to obtain the concentration of 15, 22.5, 30.0, 37.5, 45 µg/ml. Peak area of this solution was recorded and the graph was plotted against concentration. The correlation coefficient (R2) of least square linear regression of molnupiravir was calculated.

4.4 Accuracy:

The convergence of the produced concentration of the substance to be measured and the concentration of the subsequent assay represents the accuracy (recovery) of the analytical method. Studies on accuracy were conducted in triplicate using formulations with 80–120% Molnupiravir concentration. The excellent recoveries using the conventional addition technique demonstrate the methods' high degree of accuracy.

4.5 Precision

The precision of the method was determined in terms of intraday and interday precisions. Intraday and Interday precision was determined by analyzing the drugs in triplicate concentrations.

4.6 Limit of Detection and Limit of Quantification

Detection limit of molnupiravir was determined based on the standard deviation of peak area and was calculated by formula LOD = 1.04(Standard deviation/Slope). Also Quantification limit was determined based on the standard deviation of peak area and was calculated by formula LOQ = 3.17(Standard deviation/Slope).

4.7 Robustness

Few parameters were deliberately varied for study of robustness. The Robustness was carried out by changing flow rate, wavelength and mobile phase composition. Flow rate, wavelength and mobile phase composition were varied by ±2% and the %RSD was calculated.

4.8 Stability:

4.8.1 Oxidation:

30mg of molnupiravir standard drug weighed accurately and dissolved in 50ml of methanol and water (1:1). In above solution 20ml of hydrogen peroxide is added and heated the solution at 60°C for 2hrs. after heating solution was cooled at room temperature.make up the volume to 100ml with methanol.
Pipette out 2ml from above stock solution and make up volume upto 20ml with methanol. then the solutions injected into the RP-HPLC system.

4.8.2 Acid Hydrolysis:

30mg of molnupiravir standard drug weighed accurately and dissolved in 50ml of methanol and water (1:1). In above solution 20ml of 0.1M HCL is added and heated the solution at 60°C for 2hrs. after heating solution was cooled at room temperature. Then neutralized with suitable amount of 0.1M NaOH solution. make up the volume to 100ml with methanol.
Pipette out 2ml from above solution and make up the volume upto 20ml with methanol. then the solutions injected into the RP-HPLC system

4.8.3 Alkali Hydrolysis:

30mg of molnupiravir standard drug weighed accurately and dissolved in 50ml of methanol and water (1:1). In above solution 20ml of 0.1M NaOH is added and heated the solution at 60°C for 2hrs. after heating solution was cooled at room temperature. Then neutralized with suitable amount of 0.1M HCLsolution make up the volume to 100ml with methanol.
Pipette out 2ml from above solution and make up the volume upto 20ml with methanol.
then the solutions injected into the RP-HPLC system

4.8.4 Photo stability:

Exposed molnupiravir standard drug to UV-light in UV cabinet for 6hrs at 254nm.
30mg of molnupiravir weighed accurately and dissolved with 100ml methanol.
Pipette out 2ml from above solution and make up the volume upto 20ml with methanol.
then the solutions injected into the RP-HPLC system.

4.8.5 Thermal degredation:

Name	Area	RT(min)	TP (NLT 2000)	TF (NMT 2.0)
Standard _Inj_01	2021.1976	4.367	9144	1.08
Standard_Inj_02	2025.3640	4.383	9031	0.99
Standard_Inj_03	1960.7937	4.367	9069	1.06
Standard_Inj_04	1982.3488	4.367	6783	1.00
Standard_Inj_05	2031.7166	4.400	9818	1.06
Mean	2004.2841	4.377		
SD	31.0465	0.0147		
%RSD (NMT 2)	**1.55**	**0.34**		

Molnupiravir standard drug is placed in oven to elevated temperature of 80°C for 6hrs.after that cooled at room temperature. 30mg of molnupiravir weighed accurately and dissolved with 100ml methanol.
Pipette out 2ml from above solution and make up the volume upto 20ml with methanol.
then the solutions injected into the RP-HPLC system.

5. RESULT AND DISCUSSION

HPLC Method Optimization

For method optimization based on a review of the literature,different mobile phase like acetonitrile and water, methanol and water, methanol and ammonium formate, methanol and sodium acetate various proportions were tried to get a stable peak each mobile phase was filtered through 0.2µm membrane filter and sonicated on ultrasonic bath and experiments were conducted. Some of these mobile phases were unacceptable due to tailing, fronting and no sharpness in the peak. After various trials following the experiments, a mixture of Methanol-Sodium acetate buffer with a 70:30% V/V ratio was chosen, and more tests were run to obtain optimized chromatograms by adjusting the other parameter.

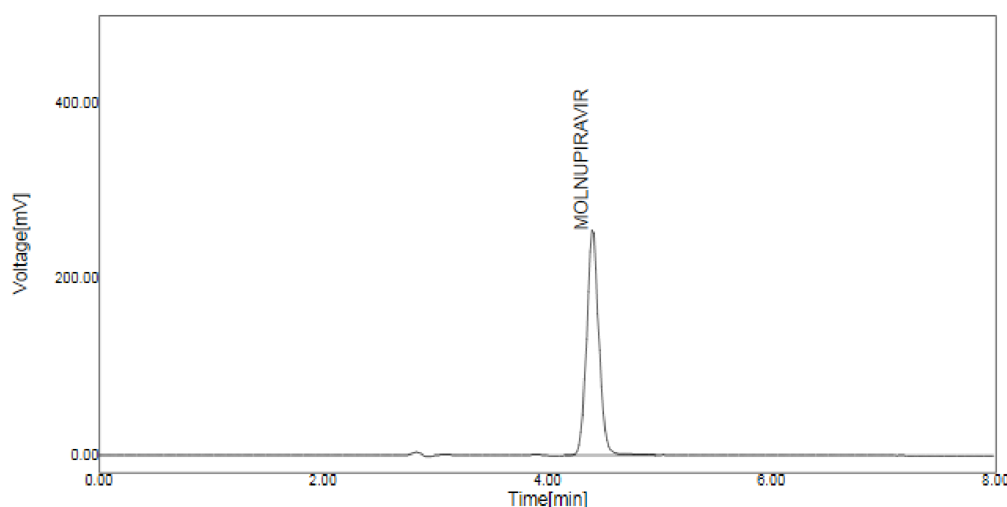

fig.no.3:- chromatogram of standard molnupiravir solution

System suitability

These parameters were shown to be within specified limits. Column efficiency (theoretical plates), resolution factor and peak asymmetry factor, tailing factor, LLOQ are the system suitability parameters. These parameters of the optimized methods were found satisfactory. The results of the system suitability studies in plasma were shown in table 8. These parameters were shown to be within specified limits.

table no .03:- system suitability studies of molnupiravir

Linearity

The linearity for molnupiravir was determined in the range of 15-45µg/ml. The regression equation was found to be $y = 65.001x + 29.915$ $R^2 = 0.9995$. Data for calibration curve was shown in Table 3 and the calibration curve was shown in Fig 3.

table no.04:-calibration data of molnupiravir

Con. (ppm or ug/ml)	Area
15.0	983.0639
22.5	1511.1653
30.0	1992.4733
37.5	2473.7666
45.0	2939.3149

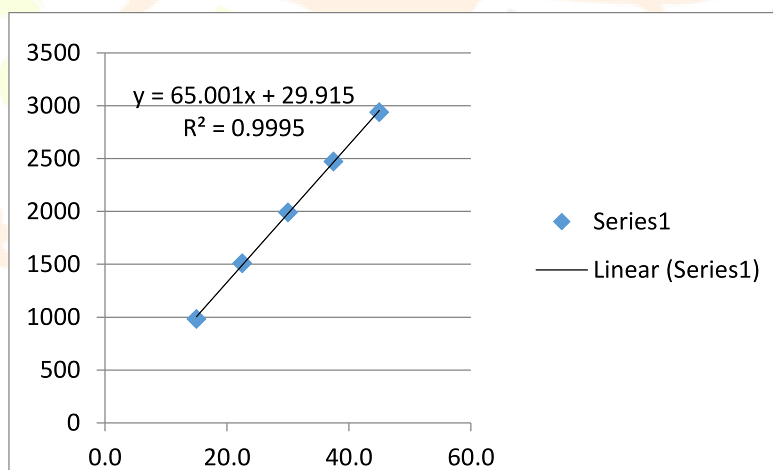

fig.no.04:- calibration curve of molnupiravir

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LOQ and LOD

LOD and LOQ were calculated from the equation and were found to be 1.04µg/ml and 3.17 µg/ml respectively shown in Table 4

table no.05 :- lod and loq of molnupiravir

Sr. no	Parameter	Result
1	LOD (µg/ml)	1.04
2	LOQ (µg/ml)	3.17

Accuracy

The accuracy of the analytical procedure for Molnupiravir was determined at 80%, 100% and 120% levels of standard solution. Results were expressed in terms of % recoveries. The % recovery was found in the range from 99.81% to 101.91% and was shown in Table 5.

table no 06:- % accuracy of molnupiravir.

Name	Preparations	Area	ug/ml	ug/ml	%
Accuracy at 80 %	Prep-1	1667.1047	24.50	24.95	101.85
Accuracy at 80 %	Prep-2	1638.8216	24.20	24.53	101.36
Accuracy at 80 %	Prep-3	1598.4682	23.80	23.93	100.53
Accuracy at 100 %	Prep-1	2007.0858	30.10	30.04	99.81
Accuracy at 100 %	Prep-2	2002.8256	30.00	29.98	99.93
Accuracy at 100 %	Prep-3	1998.9269	29.90	29.92	100.07
Accuracy at 120 %	Prep-1	2485.1741	36.50	37.20	101.91
Accuracy at 120 %	Prep-2	2461.1239	36.20	36.84	101.76
Accuracy at 120 %	Prep-3	2401.4278	35.90	35.94	100.12

Precision

The precision results (measurement of intraday, interday) showed good reproducibility and %RSD values were within limits which proved that method was highly precise. The results were shown in Table 6 and 7.

table no 07:- interday precision studies of molnupiravir.

Name	Prepparation	% ASSAY
Day-1	prep-01	**99.83**
	prep-02	**100.72**
Day-2	prep-01	**98.97**
	prep-02	**98.75**
Mean		99.5675
SD		**0.8986**
% RSD (NMT 2.0)		**0.90**

table no 08:- interday precision studies of molnupiravir

Name	Prepparation	% ASSAY
Set-1	prep-01	**99.83**
	prep-02	**100.72**
Set-2	prep-01	**98.41**
	prep-02	**100.31**
Mean		99.8175
SD		**1.0064**
% RSD (NMT 2.0)		**1.01**

Specificity

Table 9 shows that retention time for standard and test sample of molnupiravir are same. This shows that the method is highly selective.

table no 09:- specificity studies of molnupiravir

Sample Name	Area	Retention time
Standard	2004.2841	4.37
Test	2061.0051	4.37

Robustness

Robustness was carried out by deliberate modification of analytical parameters, which indicated that retention time and peak area remained unaffected by small changes in wavelength, and flow rate. %RSD calculated was with in ICH limit thus indicating that method was sufficiently robust. The results were shown in Table 10

table no 10:- robustness results of molnupiravir

Name	Preparations	%Assay
Robustness change in method parameters		
Original method parameters	Test prep-1	99.83
Original method parameters	Test prep-2	100.72
Flow rate 0.90 ml/min	Test prep	98.39
Flow rate 1.10 ml/min	Test prep	99.90
Wavelength 237 nm	Test prep	99.85
Wavelength 241 nm	Test prep	98.84
Mean		99.59
SD		0.8367
%RSD (NMT 2)		0.84

Stability:

table 8.11: stability

Name	Area	RT(min)	% ASSAY	% Degradation
Acid Degradation	1867.3264	4.367	92.24	7.76
Base Degradation	1915.0248	4.333	95.23	4.77
Oxidation Degradation	1865.3254	4.367	92.15	7.85
Photolytic Degradation	1994.8345	4.333	99.86	0.14
Thermal Degradation	1886.7534	4.383	95.41	4.59

Conclusion

The developed RP-HPLC method proved to be accurate, precise, selective, and robust for the estimation of Molnupiravir. It can be successfully applied for routine analysis of Molnupiravir in pharmaceutical formulations and biological samples. The method's stability-indicating nature ensures its suitability for assessing the drug's stability under various storage conditions. Hence, the developed method holds promise for quality control laboratories involved in the analysis of Molnupiravir-containing formulations and in pharmacokinetic studies.

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