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RP-HPLC method development and validation of Molnupiravir in bulk and pharmaceutical dosage form

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Abstract:

The first antiviral drug taken orally is called molnupiravir, and it is used to treat COVID-19. It inhibits human sars-cov-2 replication by increasing the frequency of viral RNA mutations. This drug has been included to the COVID-19 therapy guideline, and there have been some safety complaints about it. Utilizing RP-HPLC and UV-Visible spectroscopy, simple, precise techniques have been devised for the determination of Molnupiravir in bulk and solid dosage forms. The 200 mg MOBIFLUE tablet dosage form was utilized. The ICH Q2 (R2) recommendations were followed for all validation parameters. An easy-to-use, precise, and accurate RP-HPLC technique was created. The ideal chromatographic conditions were as follows: Phenomenex C18 (150 mm × 4.6 nm, 3µ) for the stationary phase; HPLC grade distilled water (70:30) for the mobile phase, with formic acid added and pH adjusted to 3; the flow rate was kept at 0.4 ml/min; a UV detector with a detection wave length of 236 nm was employed; the column temperature was set at 30 °C; and methanol was used as the diluent. All the results of the validated method were found to be within the limits as specified in the ICH Q2 (R2) Guidelines.

Keywords: Molnupiravir, RP-HPLC Method, Mobiflue tablets.

Introduction:

In 2019, an outbreak of illness originating in China was determined to be caused by a novel virus called SARS-CoV-2 (severe acute respiratory syndrome corona virus 2). This illness is commonly referred to as COVID-19 or corona virus disease 2019. The World Health Organization (WHO) officially declared COVID-19 as a pandemic in March 2020. The

WHO, along with other public health organizations, is actively monitoring the epidemic and providing information on their respective websites. These organizations have also issued guidance on preventive measures to halt the spread of the COVID-19 virus(Lee C.C et al.,).
The virus primarily spreads through respiratory droplets released when an infected person breathes, speaks, sings, coughs, or sneezes. Close proximity to an infected individual may lead to inhalation of these droplets or their entry into the mouth, nose, or eyes. In certain circumstances, airborne transmission can occur when individuals are exposed to tiny droplets or aerosols that remain suspended in the air for extended periods. It is also possible for the virus to spread if a person touches a contaminated surface and subsequently touches their mouth, nose, or eyes, although the risk is minimal(Pourkarim F et al., 2022).
Several COVID-19 vaccines have received emergency use authorization from the U.S. Food and Drug Administration (FDA). Vaccination can help prevent infection or reduce the severity of illness if infection does occur. Furthermore, vaccination against COVID-19 may offer greater protection compared to natural infection. Recent research indicates that individuals previously infected with COVID-19 who are not fully immunized face more than twice the risk of re-infection. Unfortunately, vaccination rates remain low in many low- and middle-income countries. Conversely, oral medications are preferred by some patients due to their ease of administration(Sumardika IW et al., 2021).
Molnupiravir (C13H19N3O7) is an antiviral medication with a molecular weight of 329. 1g/ml. Its IUPAC Name is (2R, 3S, 4R, 5R)-3,4-Dihydroxy-5-[(4Z)-4-(hydroxyimino)-2-oxo-3,4-dihydropyrimidin-1(2H)-yl]oxolan-2-yl}methyl 2-methylpropanoate (as given in Figure 1). This antiviral drug, known as Molnupiravir or Lagevrio, inhibits the replication of several RNA viruses. It is used to treat individuals with SARS-CoV-2 infection and COVID-19. The medication is taken orally(Imran M et al., 2021).

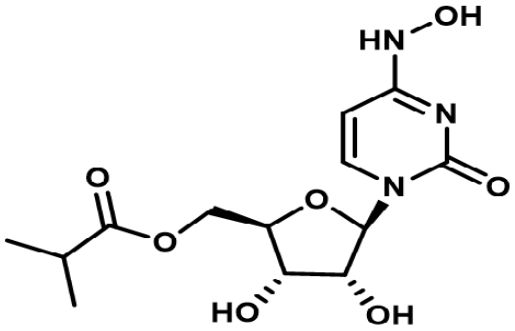

Figure 1. Structure of Molnupiravir

The mechanism of action involves inducing extensive mutations in viral RNA replication through RNA-directed RNA polymerase. By doing so, Molnupiravir hinders the spread of viruses. It undergoes conversion into -D-N4-Hydroxycytidine 5′-triphosphate, also referred to as EIDD-1931 5′-triphosphate or NHC-TP. This compound is a ribonucleosideanalog that imitates cytidine. Instead of utilizing actual cytidine during replication, the virus's enzyme incorporates NHC-TP into newly synthesized RNA (as given in Figure 2)(Kabinger F et al., 2021; Amara A et al., 2021; Gordon CJ et al., 2021).

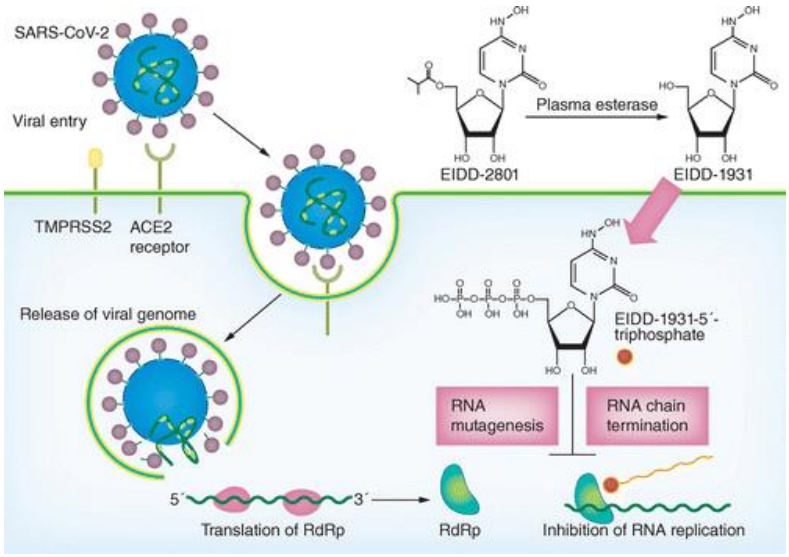

Figure 2. Mechanism of Action of Molnupiravir

Materials and Method:

Chemicals:

Molnupiravir standard drug, MOLFLU Tablet of strength 200mg, Formic Acid, Methanol and Double distilled water of HPLC Grade.

Instrumentation:

A double beam UV Visible spectrophotometer, model ELICO SL-210 and the wavelength range selected for scanning was 200-400nm, Shimadzu RP-HPLC apparatus model LC-20AD were used.

Standard Solutions Preparation:

Preparation of Molnupiravir Standard Solution:

A volumetric flask (10 ml) was filled with 10 mg of Molnupiravir after it had been weighed. After adding a small quantity of MeOH, the mixture was brought up to the flask's mark. The prepared solution concentration is 1000 ppm.

Preparation of Molnupiravir Working Standard Solution:

A clean volumetric flask (10 ml) was filled with 1 ml of a 1000 ppm solution, and enough solvent was added to bring the solution's concentration to 100 ppm.

Preparation of Molnupiravir 10ppm Solution:

A volumetric flask (10 ml) containing a 100-ppm solution was transferred and brought up to the required level using solvents in order to get the 10 ppm solution. After that, 20µl of the mixture was used to inject into the RP-HPLC.

Molnupiravir Sample Stock Solution (1000 µg/ml solution) Preparation:

Ten tablets were weighed in order to determine the average weight of each one. Subsequently, 10 milligram of powdered tablet was added to a 10-milliliter volumetric flask. Next, the diluent was added to get 1000µg/ml Molnupiravir solution.

Preparation of Sample Working Solutions (100 µg/ml solution):

A volumetric flask containing 1 ml of the previously produced solution i.e. sample stock solution was filled with diluent to get 100 µg/ml concentration of solution.

Selection of Diluent:

Molnupiravir's solubility was examined in a range of solvents. In the RP-HPLC procedure, MeOH was used as the diluent for Molnupiravir.

Working Wavelength Determination:

Using MeOH as a blank, the standard Molnupiravir 10 µg/ml solution was prepared and scanned in the UV range between 200 and 400 nm. It was found that 236 nm was absorption maximum. After repeated trails with various combinations and amounts of MeOH and water, a trail utilizing a mobile phase mixture of MeOH: Water with Formic Acid (70: 30 ratio) at a 0.4 ml/min flow rate produced prominent peaks (Optimised chromatographic conditions are given in figure 3).

HPLC Method Validation:

Specificity:

When the blank was injected into the system and peaks were not seen at the t_R of the drug Molnupiravir (given in figure 4 and figure 5). Thus, it was claimed that this procedure was specific.

Linear response:

Dilutions of 10, 30, 60, 90, 120, and 150 ppm were made from the stock solution. After all concentrations were injected into the RP-HPLC, the calibration curve was plotted. Once the concentrations and their peak regions have been tabulated, the calibration curve is created (as given in figure 6,7 and linearity data of Molnupiravir is shown in Table 1).

Working Range:

A 1000 ppm concentration stock solution was prepared. Ten milliliters of the diluent were added to one milliliter of the stock solution to bring the volume to ten milliliters. This is the working solution of concentration 100 ppm. The working standard solution was used to prepare the dilutions of concentration range from 10–150 µg/ml.

Detection Limit (DL):

The detection limit (DL) was estimated using the formula

$$DL = \frac{3.3\sigma}{S}$$

Here,S=slope of the calibration curve

σ=SD of the response.

The values obtained were tabulated.

Quantification Limit (QL):

The quantification limit was estimated using the formula

$$QL = \frac{10\sigma}{S}$$

Here, S=slope of the calibration curve

σ=SD of the response.

The values obtained were tabulated.

Precision:

There are various degrees of precision: repeatability and intermediate precision(Anonymous 2005,Anonymous 2022).

Repeatability:

At the preferred wavelength of 236 nm, 6 measurements at 100% of the test concentration were determined i.e. the peak area of a 60 µg/ml standard solution was scanned for six determinations(as given in figure 8,9,10,11,12,13). From the collected data, the RSD was estimated and tabulated (as shown in Table 3).

$$\sigma = \frac{\sqrt{\sum(xi - \mu)^2}}{N}$$

$$\%RSD = \frac{Standard\ deviation\ of\ the\ measurement}{Mean\ value\ of\ measurement} \times 100$$

Intermediate precision:
Variations within laboratories are expressed by intermediate precision, including various days, analysts, equipment, etc(Jain P et al.,2022;Annadi et al., 2022).
Intraday:
Measurements taken of the sample's peak area (60µg/ml) at 236 nm by different analysts on the same day were used to compute it. %RSD was calculated and recorded (as shown in Table 4 and Table 5).
Inter-day:
It was computed by having multiple analysts measure the sample's peak area (60µg/ml) at 236 nm on different days. %RSD was estimated and recorded (as shown in Table 6 and Table 7).
Accuracy:
A 30 ppm sample solution was chosen as the constant concentration, and it was spiked with 30 ppm, 60 ppm, and 90 ppm standard drug solution (i.e., 50%, 100%, and 150%, respectively). Three measurements were done. After calculating the mean recovery, the percentage recovery was determined (as shown in Table 2).
Robustness:
An analytical procedure's robustness, which measures its ability to be unaffected by little but intentional changes in method parameters, gives a clue as to how reliable it will be in typical conditions(Sharaf YA et al., Ali SN et al.,2022; Bhumika P et al.,2022; Deshpande et al., 2023).
The system was run at 0.3ml/min & 0.5ml/min (as shown in Table 8).
Assay:
Molnupiravir Sample Stock Solution (1000 µg/ml solution) Preparation:
Ten tablets were weighed, and the average weight of each pill was determined. Next, the 10 milligram of powdered tablet was added to a 10 ml volumetric flask. A sample solution was made using the diluent. The solution was injected into HPLC (as shown in Table 9).
%Assay was calculated by using the formula:

$$\%Assay = \frac{Absorbance\ of\ sample}{Absorbance\ of\ standard} \times \frac{Concentration\ of\ standard}{Concentration\ of\ sample} \times 100$$

Results and Discussion:

Column	PhenomenexC18, 150mm x 4.6 mm, 3µ.
Injection Volume	20.0µL
Detector	UV 236nm
Mobile Phase	Methanol: Distilled Water with Formic Acid(70:30)
Flow Rate	0.4 ml/min
Pump Mode	Gradient
Retention Time	2.193min
Run Time	10min

Figure 3. Optimised Chromatographic Conditions

VALIDATION PARAMETERS:

Specificity:

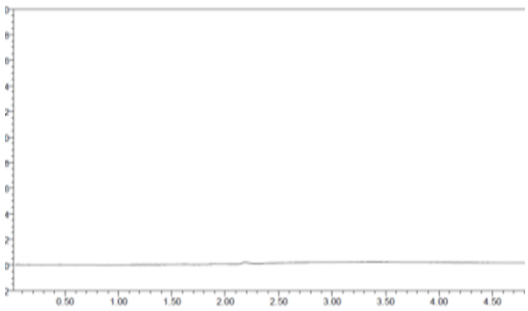

Figure 4 . Chromatogram of Blank

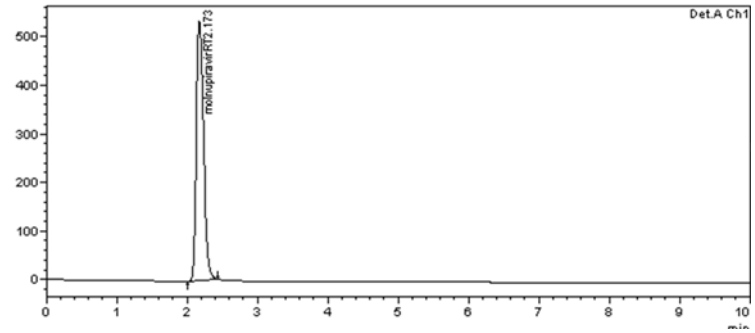

Figure 5 . Chromatogram of standard

Inference: No peaks were observed at the R_t of Molnupiravir when blank was injected.

Linear Response:

Table 1. Linearity Data of Molnupiravir

Injections	Concentration	Area	Ret. time	T. Plate Count	Tailing Factor
1	10	2177951	2.165	1959.335	0.925
2	30	3033048	2.173	1893.310	0.944
3	60	4134173	2.171	1972.632	0.989
4	90	5254623	2.177	1937.483	0.935
5	120	6275020	2.193	2193.843	0.952
6	150	7494900	2.160	1873.780	0.981

Ret.time: Retention Time, T.Plate Count: Theoretical Plate Count

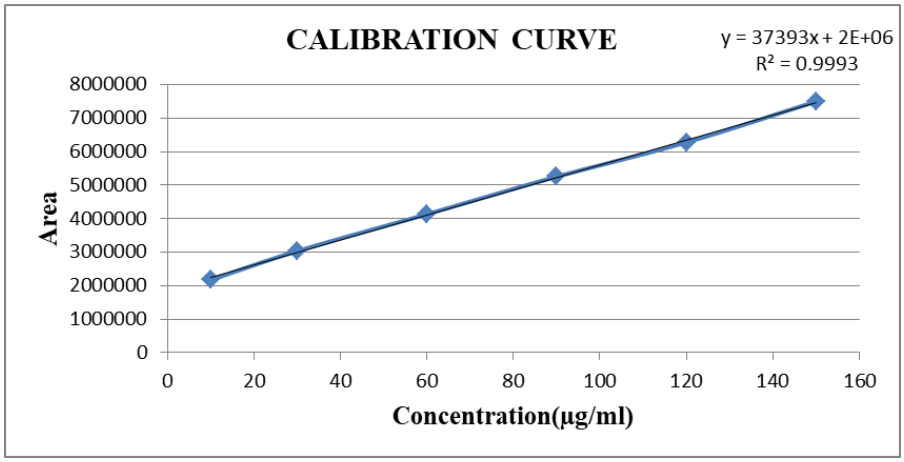

Figure 6. Calibration Curveof Molnupiravir

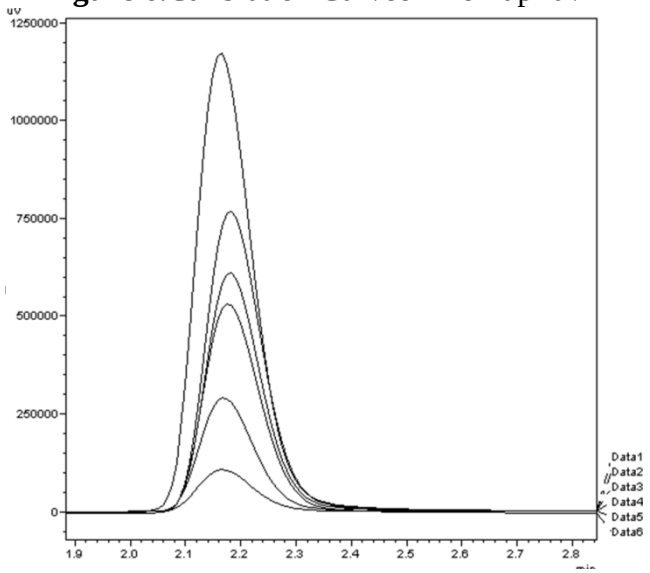

Figure 7. Linearity Chromatograms of Molnupiravir

Limits: Correlation coefficient (r^2) should be ≥ 0.999.

Result: Correlation coefficient (r^2) was found to be 0.9993 which is within the limits.

Lower Range Limits

Detection Limit:

$$DL = \frac{3.3(\sigma)}{S}$$

=0.185168218µg/ml

Inference: The detection limit was found to be 0.185168218µg/ml.

Quantitation Limit:

$$QL = \frac{10(\sigma)}{S}$$

=0.561115812µg/ml

Inference: The quantitation limit was found to be 0.561115812µg/ml.

Accuracy:

Table 2. Accuracy of Molnupiravir

Concentration Level (%)	Standard Concentration (µg/ml)	Spiked Sample Amount (µg/ml)	% Recovery	Mean% Recovery
50%	30*	30*	98.4%*	99.1%
100%	60*	30*	99.3%*	99.6%
150%	90*	30*	98.5%*	99.8%

***-** three determinations at each concentration level were performed
Limits: % Recovery should be 98-102%.
Result: The % Recovery was found to be 99.1%, 99.6% & 99.8% which is within limits.
Precision
Repeatability:

Table 3. Repeatability Data of Molnupiravir

Injections	Concentration (ppm)	Area	Mean	SD	%RSD	Retention time	T. Plate Count	Tailing Factor
1	60	4134173	4135751	2098.18035	0.05073275	2.172	1959.335	0.994
2	60	4135142				2.173	1893.310	0.992
3	60	4136242				2.171	1972.632	0.990
4	60	4133554				2.173	1937.483	0.993
5	60	4135894				2.171	1953.843	0.996
6	60	4139501				2.173	1963.780	0.998

SD: Standard Deviation, %RSD: % Relative Standard Deviation, T. Plate Count: theoretical Plate Count

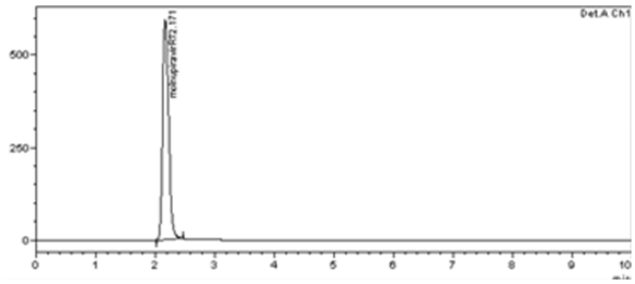

Figure 8. Injection 1 of 60ppm Molnupiravir Solution

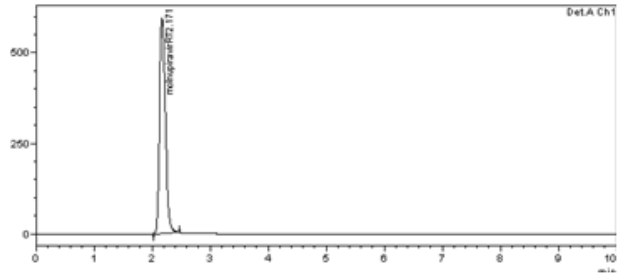

Figure 9. Injection 2 of 60ppm Molnupiravir Solution

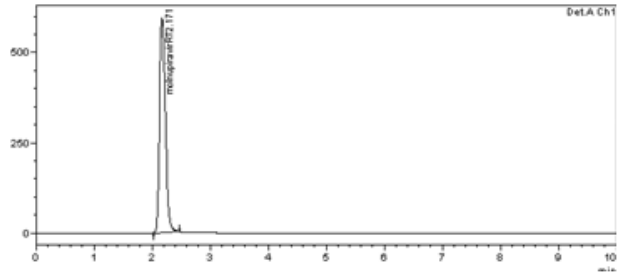

Figure 10. Injection 3 of 60ppm Molnupiravir Solution

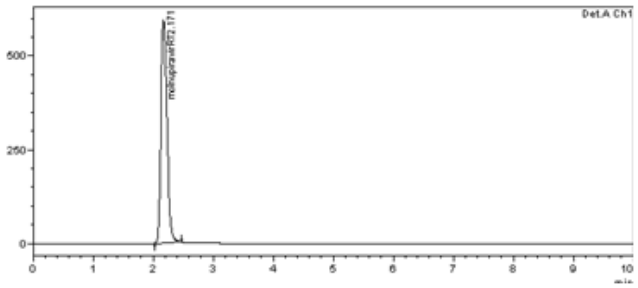

Figure 11. Injection 4 of 60ppm Molnupiravir Solution

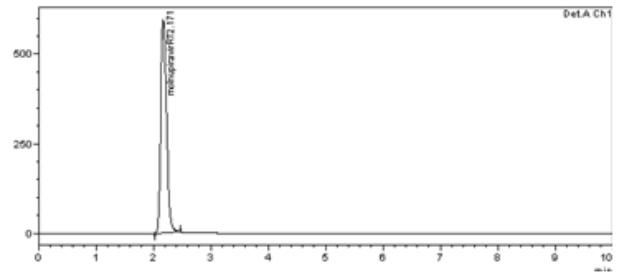

Figure 12. Injection 5 of 60ppm Molnupiravir Solution

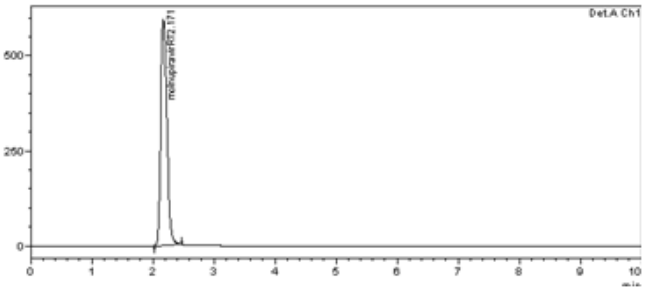

Figure 13. Injection 6 of 60ppm Molnupiravir Solution

Limits: %RSD should be less than 2.
Result: %RSD was found to be 0.05073275.
Intermediate Precision
Intra Day:

Analyst 1

Table 4. Precision Data of Analyst 1

Injections	**Concentration (ppm)**	**Area**	**Mean**	**SD**	**% RSD**	**Retention time**	**T. Plate Count**	**Tailing Factor**
1	60	4134158	4134203.8	69.179236	0.0016573	2.172	1959.335	0.994
2	60	4134173				2.173	1893.310	0.992
3	60	4134105				2.171	1972.632	0.990
4	60	4134257				2.173	1937.483	0.993
5	60	4134285				2.171	1953.843	0.996
6	60	4134245				2.173	1963.780	0.997

SD: Standard Deviation, %RSD: % Relative Standard Deviation, T. Plate Count: theoretical Plate Count

Limits: %RSD should be less than 2.

Result: %RSD was found to be0.0016573.

Analyst 2:

Table 5. Precision Data of Analyst 2

Injections	**Concentration (ppm)**	**Area**	**Mean**	**SD**	**% RSD**	**Retention Time**	**T. Plate Count**	**Tailing Factor**
1	60	4134357	4134259.8	87.923641	0.0021267	2.172	1959.335	0.994
2	60	4134104				2.173	1893.310	0.992
3	60	4134247				2.171	1972.632	0.990
4	60	4134273				2.173	1937.483	0.993

5	60	4134251	2.171	1953.843	0.996
6	60	4134327	2.173	1963.780	0.998

SD: Standard Deviation, %RSD: % Relative Standard Deviation, T. Plate Count: theoretical Plate Count

Limits: %RSD should be less than 2.

Result: %RSD was found to be0.0021267.

Inter Day

Day 1:

Table 6. Day 1 Precision Data

Injections	**Concentration (ppm)**	**Area**	**Mean**	**SD**	**% RSD**	**Retention Time**	**T. Plate Count**	**Tailing Factor**
1	60	4134158	4134203.8	69.179236	0.0016733	2.172	1959.335	0.994
2	60	4134173				2.173	1893.310	0.992
3	60	4134105				2.171	1972.632	0.990
4	60	4134257				2.173	1937.483	0.993
5	60	4134285				2.171	1953.843	0.996
6	60	4134245				2.173	1963.780	0.998

SD: Standard Deviation, %RSD: % Relative Standard Deviation, T. Plate Count: theoretical Plate Count

Limits: %RSD should be less than 2.

Result: %RSD was found to be0.0016733.

Day 2:

Table 7. Day 2 Precision Data

Injections	Concentration (ppm)	Area	Mean	SD	% RSD	Retention time	T. Plate Count	Tailing Factor
1	60	4134254	4134221.3	84.535594	0.0020448	2.172	1959.335	0.994
2	60	4134234				2.173	1893.310	0.992
3	60	4134151				2.171	1972.632	0.990
4	60	4134184				2.173	1937.483	0.993
5	60	4134138				2.171	1953.843	0.996
6	60	4134367				2.173	1963.780	0.998

SD: Standard Deviation, %RSD: % Relative Standard Deviation, T. Plate Count: theoretical Plate Count

Limits: %RSD should be less than 2.

Result: %RSD was found to be 0.0020448.

Robustness:

Table 8. Robustness of Molnupiravir

Injections	0.35ml/min	0.4ml/min	0.45ml/min
1	4134164	4134173	4134175
2	4135138	4135142	4135141
3	4136240	4136242	4136241
4	4133553	4133554	4133555
5	4135895	4135894	4135893
6	4139502	4139501	4139503
MEAN	4135748.67	4135751	4135751.33
SD	2100.25557	2098.18035	2098.38316
RSD	0.05078296	0.05073275	0.05073765

SD: Standard Deviation, RSD: Relative Standard Deviation

Limits: %RSD should be less than 2.

Result: It was found to be within the limits.

Assay:

Table 9. Assay of Molnupiravir

	Concentration	Peak Area
Standard	60ppm	4134173

Sample	59.7921132	4119849

$$\%\boldsymbol{ASSAY} = \frac{\boldsymbol{Peak\ Area\ of\ Sample}}{\boldsymbol{Peak\ Area\ of\ Standard}} \times \frac{\boldsymbol{Concentration\ of\ Standard}}{\boldsymbol{Concentration\ of\ Sample}} \times \mathbf{100}$$

$$= \mathbf{100\%}$$

Limits: % Recovery should be 98-102%.
Result: The % recovery was found to be 100%.

Conclusion:
It was discovered that the recommended RP-HPLC method for determining the dosage form of MOLNUPIRAVIR was simple, precise, quick, and reasonably priced. The established approach's statistical validation was conducted in accordance with ICH guidelines. The claims stated on each label were closely matched by the sample recovery in the formulation. For the recommended process, the mobile phase was methanol:water (70:30), pH 3 adjusted with formic acid; the phenomenex-C18 column (150x4.6mm) fpm, flow rate 0.4 ml/min, and the eluents were scanned using a UV detector in the system, indicating maximum absorbance at 236 nm. MOLNUPIRAVIR was shown to have retention of 2.17 minutes. It was found that this process performed better than previously published methods.

Acknowledgment:
I would like to thank our beloved Principal, Prof. M. Sumakanth ma'am and faculty of Department of Pharmaceutical Analysis for helping me to carry out the study**.**

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