QBD BASED ANALYTICAL DEVELOPMENT AND VALIDATION OF STABILITY-INDICATING METHOD FOR ESTIMATION OF MOLNUPIRAVIR IN PHARMACEUTICAL DOSES FROM RP–UPLC

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Abstract

A simple, Accurate, precise method was developed for the estimation of the Molnupiravir in bulk and pharmaceutical dosage form. Chromatogram was run through HSS 100 x 2.1 mm, 1.8µ. Mobile phase containing 0.1N OPA: Acetonitrile taken in the ratio 55.89 (%v/v) and 44.11 was pumped through column at a flow rate of 0.2 ml/min. Temperature was maintained at 30.66°C. Optimized wavelength selected was ACQUITY TUV ChA 260.0 nm. Retention time of Molnupiravir was found to be 1.413 min. %RSD of the Molnupiravir was found to be 0.4 %Recovery was obtained as 100.02% for Molnupiravir. LOD, LOQ values obtained from regression equations of Molnupiravir were 0.08, 0.23. Regression equation of Molnupiravir is y = 42046x + 4850.1. Retention times were decreased and that run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

Key Words: Molnupiravir, QbD Approach, Method development, RP-UPLC

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1. INTRODUCTION

The conventional approach for analytical method development involves changing one factor at a time (OFAT), commonly known as OFAT technique. The OFAT is very time consuming and lengthy process which generates voluminous data, resulting in a tedious approach. Also, once the method is developed, the method still may need additional efforts when validated[1]. The modern pharmaceutical analysis and regulatory scenario, demands to use novel chemometric tools which control many variables simultaneously and helps to provide desired results with minimum experimental trials. This can be achieved by implementing Quality-by-Design (QbD) approach in analytical method development. In International Conference on Harmonization (ICH) guideline Q8(R2) for Pharmaceutical development, QbD is defined as "A systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management"[1,2]. The elements of QbD can be extended for analytical method development. The Analytical QbD utilizes statistical modelling and design of experiments (DoE) to arrive at a method operable design region (design space), the robust area, where the developed method provides the desired results[3].

Molnupiravir 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 [4]. Chemically called as [(2*R*,3*S*,4*R*,5*R*)-3,4-dihydroxy-5-[4-(hydroxyamino)-2-oxopyrimidin-1-yl]oxolan-2-yl]methyl 2-methylpropanoate. [4].

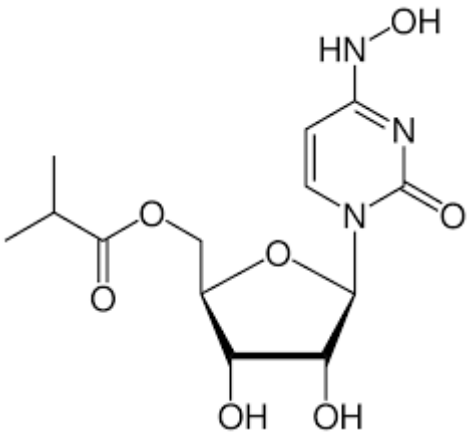

Figure1: Chemical structure of Molnupiravir

2. MATERIALS AND METHOD [5-8]

Chemicals and reagents

Pure Molnupiravir was procured from Spectrum pharma lab (Hyderabad). Hydrochloric acid AR grade (HCL) and sodium hydroxide AR grade (NAOH) were obtained from rankem, India. Hydrogen Peroxide (H_2O_2) was purchased from Qauligens. Acetic acid AR grade was purchased from Fisher scientific, India and S.D. Fine chem Ltd. Respectively. Potassium dihydrogen orthophosphate and orthophosphoric acid were obtained from S.D. Fine chem Ltd and Merck India Pvt Ltd. Respectively. UPLC grade Acetonitrile (ACN) and methanol (MeOH) were

purchased from Fischer scientific. UPLC grade water used throughout analysis was obtained from the Merck milli-Q water purification unit.

Apparatus and Equipment

UPLC studies were carried out on WATERS UPLC 2965 SYSTEM with a Photo diode array detector (PDA) set at 220 nm for uv detection. columns, viz; Agilent C18 (150×4.6 mm, 5 µm), Discovery C18(150*4.6mm,5 µm), Zodiac (150*4.6mm,5 µm) ,BDS (150*4.6mm,5 µm), Hibar 100 x 2.1 mm, 2µ and Phenomenex (150*4.6mm,5 µm) column were utilized in the study. Design Expert® (11.0.0) modeling software (Stat-Ease Inc., Minneapolis, MN, USA) was used for generation of contour plots and 3D space.

pH meter (Eutech instruments pH tutor, pH meter, India) was used to check the pH of all solutions. sonicator (ePEI ultrasonic generator), Analytical balance (Mettler Toledo), vortex meter (IKA Vortex), Hot air oven (Yorco scientific).

Preparation of buffer

0.01N Potassium dihyrogen ortho phosphate

Accurately weighed 1.36gm of Potassium dihyrogen Ortho phosphate in a 1000ml of Volumetric flask add about 900ml of milli-Q water added and degas to sonicate and finally make up the volume with water then added 1ml of Triethylamine then pH adjusted to 3.0 with dil. Orthophosphoric acid solution

0.1% Ortho phosphoric acid buffer

1ml of Ortho phosphoric acid solution in a 1000ml of volumetric flask add about 100ml of milli-Q water and final volume make up to 1000 ml with milli-Q water

Optimization of method

The method was optimized using central composite design (CCD). The initial trials are needed to optimize the final method. Total Three factors viz; % Organic concentration, Flow rate and column temperature were needed to be optimized. So, CCD was used to optimize these parameters which were varied over three level (high, mid and low). different ranges of four parameters ranging from 36.59-53.41%, 0.01N potassium dihydrogen orthophosphate, column temperature 26.64 and 33.36 ^{0}C and 0.6636-1.34ml/min flow rate respectively were taken and counter and 3D surface plot showing the effect of each parameter on Retention Time, Area, Theoretical plates and Asymmetry (CQA) were generated. A desirability function applied to the optimized conditions to predict retention time, asymmetry, theoretical plates and peak area.

Table 1. Design summary of CCD

Design Summary					
File version: DX 11.0.0 Study Type: Response surface Design Type: central composite design Design Model: Quadratic			ATP: Robustness CQA: Retention time, Area, Theoretical plates and Asymmetry Runs: 24		
CMPs	Unit	Type	Subtype	Min.	Max.
column temperature	**^{0}C**	Numeric	Continuous	26.64	33.36
Flow rate	ml/min	Numeric	Continuous	0.6636	1.34
%Org ratio	%	Numeric	Continuous	36.59	53.41

Method validation [9-19]

The final optimized chromatographic analytical method was validated as per the International Conference on Harmonization (ICH) Q2(R1) guidelines for system suitability, linearity, accuracy, precision,

limit of detection, limit of quantitation and robustness. Standard stock solution was prepared by dissolving 10mg of Molnupiravir in 50 mL of diluents to a final concentration of 200µg/ml. then 1ml stock solution is transferred into 10 v/f and made upto the volume to get 20µg/ml.

Linearity

Standard calibration curves were generated with seven different concentrations including the LOQ by making serial volume to volume dilution of stock solution I over the range of 25-150 µg/ml. Linear calibration curves were generated between peak area and drug concentration. The linearity was examined using linear regression, which was calculated by the least square regression method.

Accuracy

The accuracy of developed analytical method was analyzed by developed method, accuracy experiments were carried out using standard addition method. Three different level concentrations (50%, 100%, and 150%) of standards were added to pre-analyzed samples in triplicate. The percentage accuracy of Molnupiravir at each level and each triplicate were calculated and the mean of percentage accuracy (n=9) and the relative standard deviation was determined.

Precision

The precision of the developed analytical method was determined by repeatability (intraday) and intermediate precision (inter-day).Repeatability defines the use of the analytical procedure within a laboratory over a short period of time that was examined by assaying the samples during the same day. Intermediate precision was evaluated by comparing the assays on different days. SD and %RSD were determined.

Limits of detection and quantitation

Limits of detection (LOD) and limit of quantitation (LOQ) were determined from the signal-to-noise ratio. The detection limit was refer to as the lowest concentration level resulting in a peak area of three times the baseline noise. The quantitation limit was refer to as the lowest concentration level that provided a peak area with a signal-to-noise ratio higher than ten.

System suitability

The system suitability was determined by taking six replicates of the drug at same concentration of 100µg/ml. The acceptance criteria was ± 2% for the percent coefficient of variation (% CV) for the peak area, retention time of drug, USP Plate Count, and asymmetry.

Robustness

The Robustness is one of the validation parameter, it measure of method capacity to remain unaffected by small, deliberate changes in chromatographic conditions was studied by testing the influence of small changes in the organic content of mobile phase (±10%), flow rate (±10%) and pH (±10%) and.

Stress study[20-29]

Generation of stress samples of Molnupiravir

Acid Hydrolysis

To 1 ml of stock solution of 1ml of 2N HCl solution was added. These degradation samples were kept in Radley apparatus (Veego) with continuous stirring at 70º C for 1 hrs in 2N HCl. These samples were neutralized to pH 7, diluted and analyzed by the UPLC system.

Base hydrolysis

To 1 ml of stock solution of 1ml of 2N NAOH solution was added. These degradation samples were kept in Radley apparatus (Veego) with continuous stirring at 70º C for 1 hrs in 2N NaOH solution. These samples were neutralized to pH 7, diluted and analyzed by the UPLC system.

Neutral hydrolysis

10 mg of Molnupiravir was weighed and dissolved in 50 ml of water. These degradation samples were kept in Radley apparatus (Veego) with continuous stirring at 70º C for 24 hrs. These samples were diluted and analysed by the UPLC system.

Oxidative study

To 1 ml of stock solution of 1ml of 20% H_2O_2 solution was added. These degradation samples were kept in dark area without disturbance at room temperature for 24 hrs. These samples were diluted and analyzed by the UPLC system.

Thermal degradation

10mg Molnupiravir was kept in a Petri dish and kept in hot air oven at 70ºC for 1 day. Sampling was done at multiple time points. Samples were dissolved in methanol, diluted 10 times and analysed by the UPLC system.

Photo degradation

10mg Molnupiravir was uniformly spread in a Petri dish and was exposed to directly sunlight for 24 hrs. Sampling was done at multiple time points and analyzed by the UPLC system.

3. RESULTS AND DISCUSSION

Authentication by UV-VIS spectra

After scanning from 400 to 200nm in UV-VIS spectrophotometer, Molnupiravir was showed absorption maxima at 260.0 nm in 0.1NHCl. UV spectra of drug given in figure 3**.**

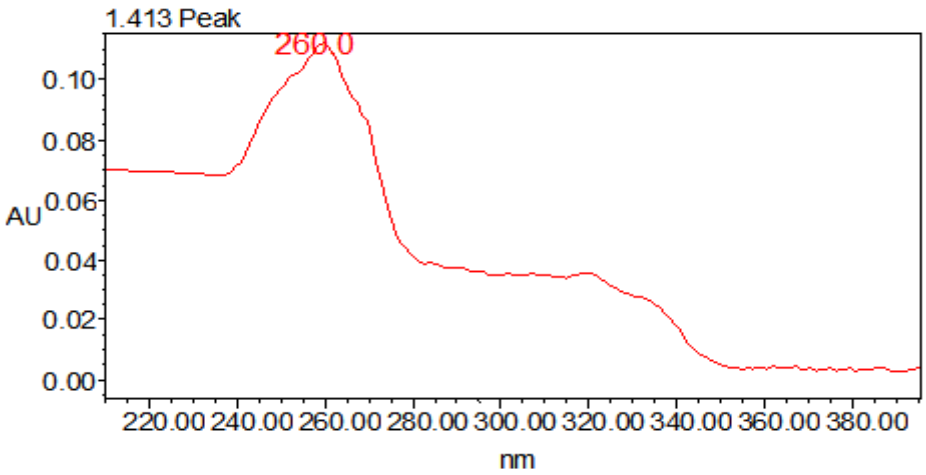

Figure1: UV spectrum of Molnupiravir

Optimization of method

The method was optimized using central composite design (CCD). The initial trials are needed to optimize the final method. Organic concentration, Flow rate and column temperature were needed to be optimized. So CCD was used to optimize these parameters which were varied over three level (high, mid and low).different ranges of four parameters ranging from 40-50%, 0.01N potassium dihydrogen orthophosphate, column temperature 28^0C and 32 ^{0}C and 0.8-1.20ml/min flow rate respectively were taken and counter and 3D surface plot showing the effect of each parameter on Retention Time, Theoretical plates and Asymmetry (CQA) were generated. A desirability function applied to the optimized conditions to predict retention time, asymmetry, theoretical plates and peak area.

Final developed UPLC method

In order to understand the results, 3D space was obtained after processing all data using the software. A composite desirability was applied to get an optimum set of conditions based on the specified goals and boundaries for the each response. This desirability function was depends on a scale of desirability function ranges between d = 0, for a completely undesirable response, to d = 1 for a fully desirable response Based on

the specified goals and boundaries for the retention time, area, Asymmetry and a composite desirability (D) of 1 was obtained, which gave the optimal flow rate of 1 ml/min. To confirm these optimum set of conditions, three replicate injections of 30 µg/ml Molnupiravir was analyzed to determine if their observed retention time, asymmetry and theoretical plates were within the predicted ranges. It was observed that the differences between the observed and predicted peak response were less than 5%.

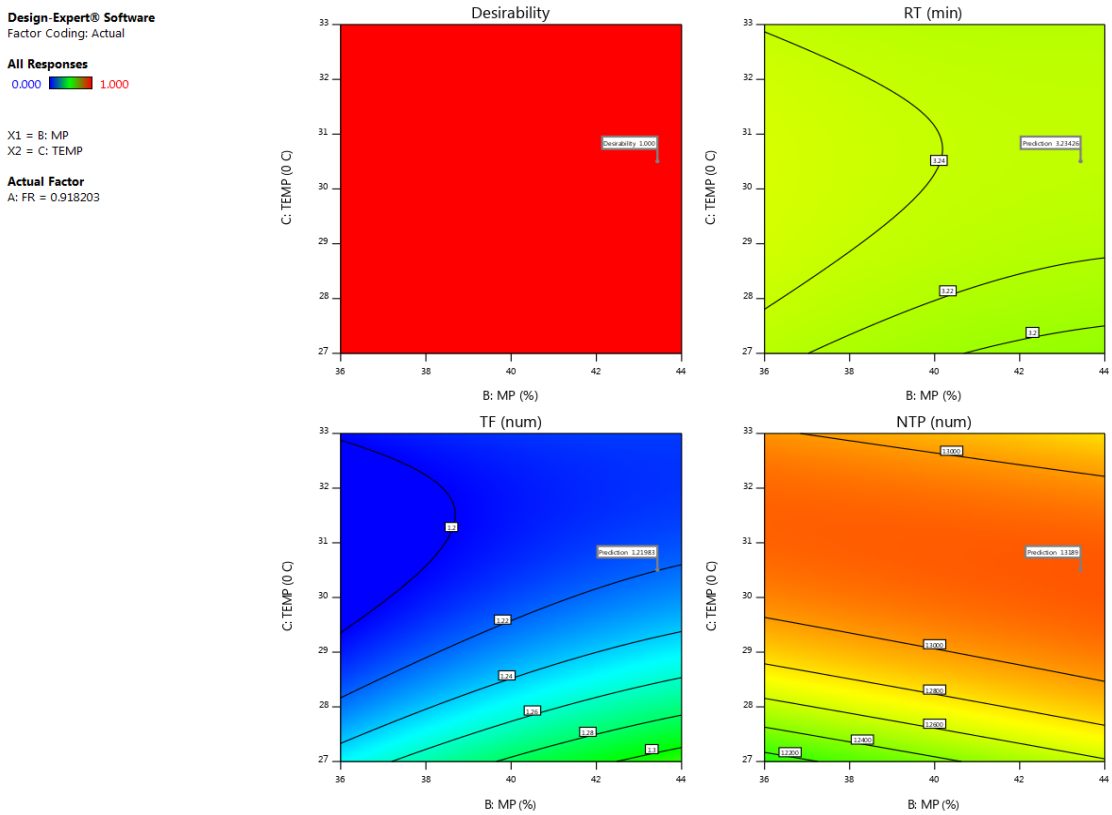

Figure 2. Overall desirability of final method

Table 1 Final developed and optimized UPLC method parameter using QbD

Parameter	Found values
Mobile phase	(56.44%) 0.1 OPA: Acetonitrile (56%)
Temp	30.7424
Flow rate	0.2

OPTIMIZED METHOD for anla
Buffer: (0.01N KH2PO4)
The buffer was prepared by adding 1.36gm of KH_2PO_4 to the solution of into 1000ml of HPLC water(Grade) and refluxed in sonicator for 10-15 min and filter with 0.45µm nylon filters and ph adjusted by adding OPA ph-4.8)

Mobile phase:

Buffer and acetonitrile: taken in the ratio 56:44

Chromatographic conditions:

Flow rate	**:**	**0.2**ml/min
Column	**:**	HSS 100 x 2.1 mm, 1.8µ.
Detector wave length	**:**	260.0 nm
Column temperature	**:**	42°C
Injection volume	**:**	1.0µL
Run time	**:**	**3.0** minutes

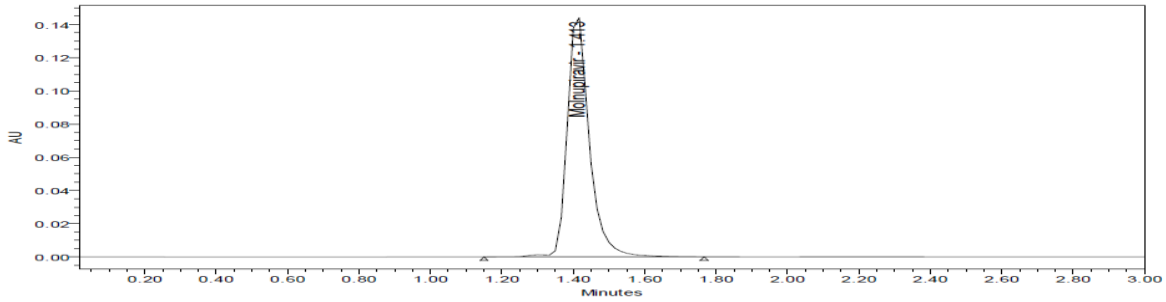

Fig 2. Chromatogram of optimized method

Method validation

Validation:

Specificity:

Retention time of Molnupiravir was 1.413 min. We did not find and interfering peaks in blank and placebo at retention times of these drugs in this method. So, this method was said to be specific.

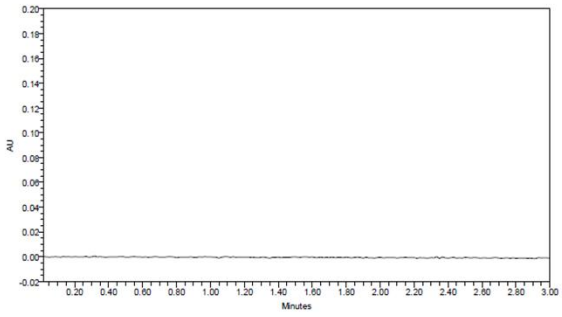

Figure 3 blank Chromatogram

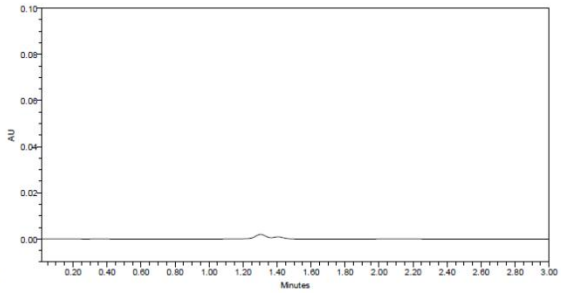

Fig 4 Placebo Chromatogram

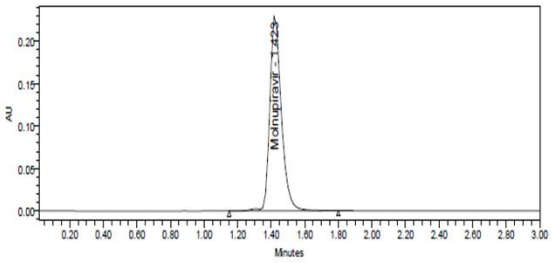

Fig 5 Standard Chromatogram

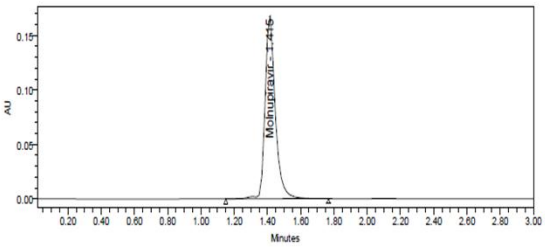

Fig 6 Sample Chromatogram

Accuracy: Three Concentrations of 50%, 100%, 150% are Injected in a triplicate manner and %Recovery was calculated as 99.95%. And chromatograms were shown in fig 6.11-6.13.

% Level	**Amount Spiked (µg/mL)**	**Amount recovered(µg/mL)**	**% Recovery**	Mean %Recovery
50%	10	10.10	101.02	100.02%
	10	10.03	100.31	
	10	10.07	100.66	
100%	20	19.90	99.52	
	20	19.70	98.49	
	20	20.00	100.00	
150%	30	29.86	99.54	
	30	30.13	100.43	
	30	30.07	100.24	

Table 2 Accuracy data

Precision:

System Precision: Six working sample solutions of 20ppm are injected and the % Amount found was calculated and %RSD was found to be 0.4 and chromatogram was shown in fig

Table 3 Repeatability data

S.No	Peak Area
1	840966
2	841682
3	843914
4	845065
5	847341
6	848315
AVG	844547
STDEV	2956.5
%RSD	0.4

Method precision: Six working sample solutions of 20ppm are injected on the next day of the preparation of samples and the % Amount found was calculated and %RSD was found to be 0.4.

Table 4 Method precision data

S.No	Peak Area
1	849976
2	845533

3	843828
4	847402
5	849286
6	841167
AVG	846199
STDEV	3365.4
%RSD	0.4

Intermediate precision: Six working sample solutions of 20ppm are injected on the next day of the preparation of samples and the % Amount found was calculated and %RSD was found to be 0.3 and chromatogram was shown in fig 6.3.

Table 5 Intermediate precision data

S.No	Peak Area
1	843281
2	843581
3	847326
4	842783
5	843880
6	849727
AVG	845096
STDEV	2787.0
%RSD	0.3

LINEARITY:
To demonstrate the linearity of assay method, inject 6 standard solutions with concentrations of about 5ppm to 30ppm of Molnupiravir. Plot a graph to concentration versus peak area. Slope obtained was y = 42460x + 4850 and Correlation Co-efficient was found to be 0.999 and Linearity plot was shown in Fig 6.15.

Table 6 Linearity Concentration and Response

Linearity Level (%)	**Concentration (ppm)**	**Area**
0	0	0
25	5	217098
50	10	422069
75	15	638836
100	20	853811
125	25	1055149
150	30	1252595

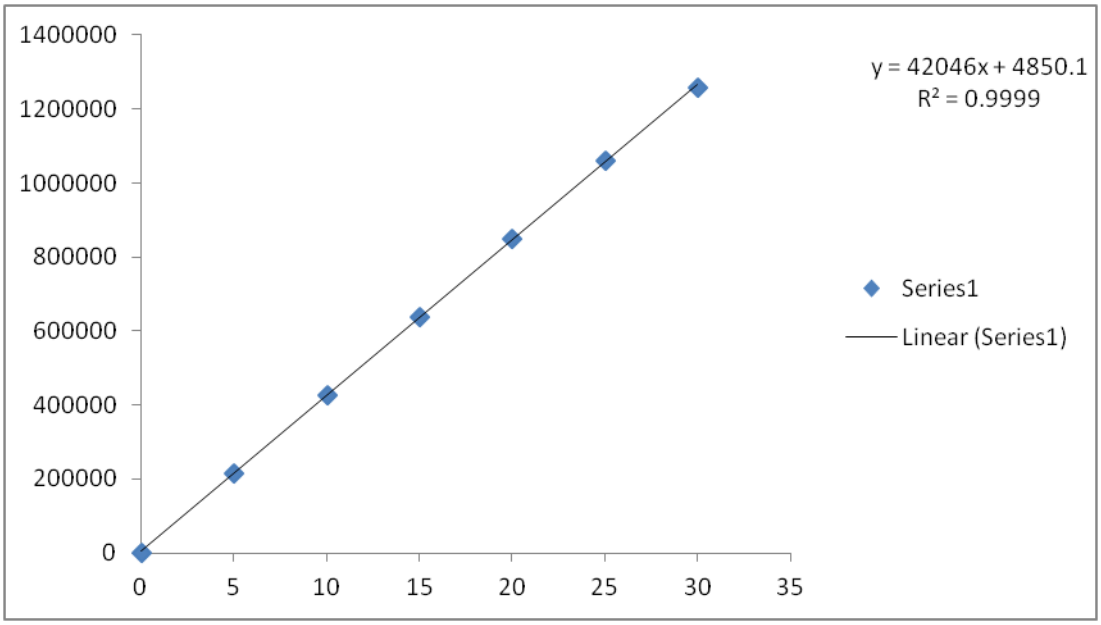

Figure 8 Linearity curve for Molnupiravir

LOD: Detection limit of the Molnupiravir in this method was found to be 0.20 µg/ml.
LOQ: Quantification limit of the Molnupiravir in this method was found to be 0.60 µg/ml.

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 is calculated.

Table 7 Robustness Data

Parameter	**%RSD**
Flow Minus	0.3
Flow Plus	0.3
Mobile phase Minus	0.4
Mobile phase Plus	0.5
Temperature minus	0.3
Temperature plus	0.4

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 9: Assay

S.No	Standard Peak Area	Sample Peak Area	%Assay
1	840966	849976	100.44
2	841682	845533	99.92
3	843914	843828	99.72
4	845065	847402	100.14
5	847341	849286	100.36
6	848315	841167	99.40
AVG	844547	846199	100.00
STDEV	2956.5	3365.4	0.40
%RSD	0.4	0.4	0.40

Stress degradation study

Acid hydrolysis

The drug substance was exposed to 2N HCl, kept for reflux in Radley apparatus at 70 ^{0}C temperature for 8 hrs, it was showing there is 4.69% of Molnupiravir degradation in acid hydrolysis. The blank solutions also subjected to stress study in the same fashion as the drug solution. The exposed stress sample and blank solutions were analyzed by UPLC system.

Base hydrolysis

The drug substance was exposed to 2N NaOH, kept for reflux in Radley apparatus at 70 ^{0}C temperature for 8 hrs, it was showing 5.21% of Molnupiravir degradation in base hydrolysis with two degradation products. The blank solutions also subjected to stress study in the same fashion as the drug solution. The exposed stress sample and blank solutions were analyzed by UPLC system.

Neutral hydrolysis

The drug substance was exposed to water, kept for reflux in Radley apparatus at 70 ^{0}C temperature for 24 hrs; it was showing there was 0.58% degradation in neutral hydrolysis. The blank solutions also subjected to stress study in the same fashion as the drug solution. The exposed stress sample and blank solutions were analyzed by UPLC system

Oxidative degradation

The drug was exposed to 20% H_2O_2, at room temperature for 24 hours. Samples were withdrawn at different time intervals and injected into the UPLC system and chromatogram was recorded 6.83% of Molnupiravir degradation in 20% H2O2 solution at the end of 24 hrs .The blank solutions also subjected to stress study in the same fashion as the drug solution. The exposed stress sample and blank solutions were analyzed by UPLC system.

Thermal degradation

The drug sample was exposed to 70°C for 1 day in a hot air oven and samples were withdrawn at different time intervals from 1day. Samples were injected into the UPLC system and chromatogram was recorded. 2.14% of Molnupiravir degradation was found at the end of 3 days of exposure. Hence the drug can be regarded as thermostable at 70°C.

Photo degradation

The drug sample was exposed to direct sunlight for 24 hours. Samples were injected into the UPLC system and chromatogram was recorded. 1.94% of Molnupiravir degradation was found at the end of 24hrs of exposure. Hence the drug can be considered as photostable.

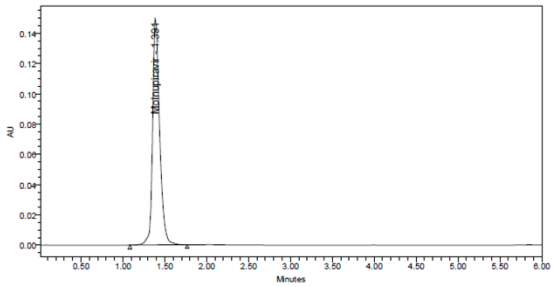

Figure 7 Chromatogram of drug in 2N HCl at 70 ^{0}C temperature for 8 hrs

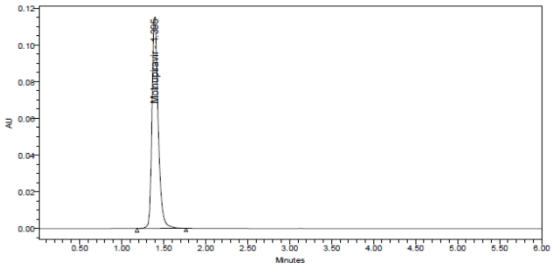

Figure 8 Chromatogram of drug in 2N NaOH at 70 ^{0}C temperature for 8 hrs

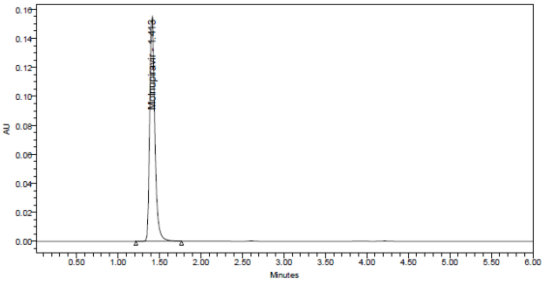

Figure9:Chromatogram of drug in water at 70 ^{0}C temperature for 24 hrs

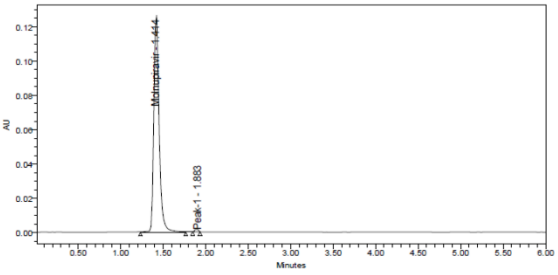

Figure10:Chromatogram of drug at 70 ^{0}C temperature for 24 hrs

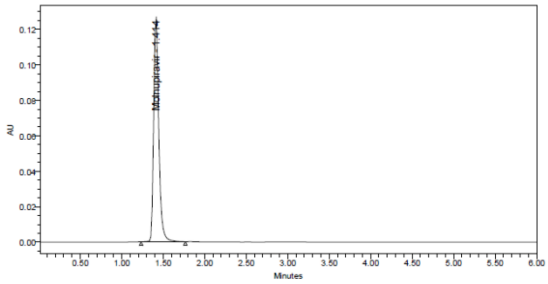

Figure11:Chromatogram of drug after 24hrs exposure in direct sunlight

Table 10. Summary of degradation study

Sr.No.	Condition of degradation study	% of drug degraded	Retention time of degradant
1.	2N HCl, 8 hrs	4.69	-
2.	2N NaOH, 8hrs	5.21	-
3.	Oxidative degradation, 24 hrs	6.83	1.883
4.	Thermal degradation, 1 days	2.14	-
5.	Photo degradation, 24 hrs	1.94	-
6.	Neutral hydrolysis, 24 hrs	0.58	-

4. CONCLUSION

A simple analytical and robust UPLC method was developed for the determination of Molnupiravir by using QbD approach using Design Expert® software. Validated stability indicating UPLC method for Molnupiravir was developed which is capable to separate drug substance from the degradation products. Stress degradation studies have been performed for drug by using various stress conditions. No degradation products were found in case of Peroxide hydrolysis, neutral hydrolysis, thermal degradation and UV degradation. One significant degradation product was found in 2N HCL, and 2N base hydrolysis.Results which were obtained from the validation of developed analytical method were within limit as per ICH guidelines.

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