RESEARCH PAPER

Development of QbD Driven liquid chromatographic Method for Quantification of Molnupiravir API Capsule Formulation

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ABSTRACT

Molnupiravir is an antiviral drug used to treat moderate-to-severe Coronavirus Disease-2019 (COVID-19) infection. The current research article presents a novel method that applies the principle of quality by design (QbD) to the development of a robust High Performance Liquid Chromatography (HPLC) method for estimating Molnupiravir in Active Pharmaceutical Ingredient (API) and its capsule formulation. Using this QbD concept, we have defined the Quality Target Method Profile (QTMP), identified Critical Quality Attributes (CQA), including Critical Analytical Attributes (CAA), Critical Material Attributes (CMA), and applied risk assessment to optimize CMA. Through Central Composite Design (CCD), a design space was obtained, ensuring the robustness of the proposed method. Mobile phase of the developed method was optimized to 80:20 ratio for Water: ACN (Acetonitrile) with flow rate of 1.0 ml/min, measured at 235 nm wavelength. The chromatographic run time of the proposed analytical method is 10 minutes. The optimized HPLC method was validated as per the International Council of Hormonisation (ICH) Q2 (R1) guidelines. The study highlights the effectiveness of QbD strategy in achieving a reliable and optimized liquid chromatographic method for Molnupiravir quantification.

Keywords: Robust, Central Composite Design, ICH (Q2) R1

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INTRODUCTION

Molnupiravir is orally bioavailable isopropyl ester cytidine analogue, is being investigated to treat COVID-19. Molnupiravir is an orally administered prodrug that undergo in vivo metabolism to N4-hydroxycytidine. Inside cells, the metabolite is converted into its active triphosphate form, which becomes incorporated into viral Ribonucleic Acid (RNA) during replication; this causes an error catastrophe and thereby inhibits viral replication. The science-based and risk-driven approach of Analytical QbD used for the establishment of robust HPLC method that consistently meets predefined performance requirements. The Analytical Quality by design (AQbD) strategy involved defining QTMP, identifying critical quality attributes, selection of dependent response factor (CAA) and independent experimental parameter (CMA) applying systematic risk assessment. Through design of experiment CMA are optimised to obtain quality method. Based on these studies, a Method Operable Design Region (MODR) is established, within which the method demonstrates reliable performance throughout its lifecycle.

In the present study, a QbD-guided chromatographic (HPLC) method utilises 80% water for the Quantitative measurement of Molnupiravir in capsule dosage form, the method was then validated. Method optimization was carried out using response surface methodology through a CCD to achieve robust chromatographic performance with minimal environmental impact.

Fig. 1: Overview of AQbD

Very limited RP-HPLC and UV spectroscopic methods have been stated for quantification of Molnupiravir, relying on conventional optimization approaches without applying the Quality by Design (QbD) concept. A single HPLC method is developed using QBD approach using box behnken design but the present developed method utilises central composite design to ensure method robustness and regulatory compliance.

MATERIALS AND METHODS

Chemicals & reagents:

Pure sample of Molnupiravir was gifted by Cipla Ltd., Mumbai, India. The Solvents of Acetonitrile, Formic Acid, of HPLC grade and water of HPLC grade were purchased from Advent Chem Bio Pvt ltd, Navi Mumbai, India.

Instruments:

Chromatographic analysis was executed using a HPLC, Alliance (Model 2695) Configured with a quaternary solvent delivery pump and a photodiode array (PDA) detector (Model 2996). C18 reversed-phase column Zorbax XDB, 150 × 4.6 mm, 5 µm particle size were used.

Software

Data acquisition, system control was carried out using Empower 2 software. Experimental design, optimization, and statistical analysis were conducted using Design-Expert (DOE) software Version 13.

Mobile Phase:

Mobile phase optimized for proposed method was Water: ACN in the ratio of 80:20.

Standard Stock Solution (1000ppm):

The precisely weighed 100mg of Molnupiravir was dissolve in some quantity of diluent in previously calibrated 100ml of volumetric flask, volume was make-up to 100ml using diluent.

Working Standard Solution (200ppm):

20 mL of standard stock solution of Molnupiravir (1000 ppm) was accurately transferred in100 mL of volumetric flask and diluted using the same diluent to get a working standard solution of 200 ppm.

QbD approach for method development:

1. Define QTMP:

According to ICH Q2(R1) recommendations, preliminary study Quality Target Method Profile (QTMP) was established. The QTMP is the intended quality features of the method to be developed is summarized in Table number 1. To achieve the objectives outlined in the QTMP, critical analytical attributes (CAAs), specifically the tailing factor and retention time of Molnupiravir, were systematically evaluated using a DoE approach.

Table 1: QTMP Table

QTMP	Objectives	Explanation
System	HPLC	Need a Development of HPLC technique and measure Molnupiravir
Type	RP-HPLC	High Retention of Molecules will observe
Purpose	Accurate quantification of Molnupiravir	Applicable for quantification of Molnupiravir in capsule.

2. Risk assessment studies

In this work a risk assessment was carried out for identification of relation of Critical Quality Attributes (CQA) with Critical Analytical Attributes (CAA) and Critical Process Parameters (CPP) of the analytical method which is to be developed. From risk assessment we have found that Flow rate, percent of organic are key important factors influence validation parameter of analytical method.

Experimental design was employed to enhance method understanding and achieve precise optimization. The effects of the percent of organic and flow rate, were evaluated to develop a reliable chromatographic database for method optimization.

3. Experimental Design

The CCD was applied so as to optimize various effects including main, factor interaction and quadratic effects of the input variable. The output variables are then evaluated from second order multinomial equation obtained from DOE. To get the design percent of organic, flow rate was carefully chosen as independent variables and Retention time (RT), tailing factor of Molnupiravir as an input or dependent variable.

RESULT & DISCUSSION:

Statistical Data: ANOVA

The matrix of experimental design are shown in Table no. 2

Table 2: Layout of Design Matrix

		Factor one	Factor two	Response one	Response two
Std	Run	A: Percent of Organic	B: Flow Rate	Retention Time	Tailing factor
		%	ml/min	min.	
9	1	20	1	2.465	1.14
10	2	20	1	2.48	1.15
6	3	24.2426	1	2.032	1.14
7	4	20	0.717157	3.405	1.12
13	5	20	1	2.38	1.14
3	6	17	1.2	2.475	1.14
11	7	20	1	2.52	1.13
4	8	23	1.2	1.781	1.15
1	9	17	0.8	3.732	1.11
12	10	20	1	2.42	1.12
2	11	23	0.8	2.66	1.12
8	12	20	1.28284	1.924	1.15
5	13	15.7574	1	3.271	1.11

Response 1: Retention Time

ANOVA was implemented to estimate the statistical importance of selected factors influencing the responses.

ANOVA for Quadratic model

ANOVA result for the Retention Time of Molnupiravir is as following Table 3.

Table 3. ANOVA for Quadratic model for retention time

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3.95	5	0.7904	459.10	< 0.0001	significant
A-Percent of organic	1.55	1	1.55	898.69	< 0.0001	
B-flow rate	2.24	1	2.24	1299.38	< 0.0001	
AB	0.0357	1	0.0357	20.75	0.0026	
A^2	0.0699	1	0.0699	40.61	0.0004	
B^2	0.0793	1	0.0793	46.05	0.0003	
Residual	0.0121	7	0.0017			
Lack of Fit	0.0003	3	0.0001	0.0307	0.9917	not significant
Pure Error	0.0118	4	0.0029			
Cor Total	3.96	12				

The model terms are significant as the P-values obtained is less than 0.05. In above model, the terms AB, A, A^2, B^2, B is significant. In the above model 99.17% chance of failure is occur due to noise. The Lack of fit is non-significant as F-value is 0.03 which indicate that the model is good.

Fit Statistics

Table 4: Fit Statistics Data

Standard Deviation	0.0415	R^2	0.9970
Average	2.58	**R^2 Adjusted**	0.9948
Percent C.V.	1.61	**R^2 Predicted**	0.9949
		Adequate Precision	68.7211

Predicted and Adjusted R^2 was 0.9949 and 0.9948 as shown in table 4, the difference between them is less than 0.2, and this indicates that the model has excellent predictive ability, which also confirms good agreement between experimental data and model predictions.

Model assessment for retention time:

Polynomial equation with actual factors was obtained from study of above Quadratic model.

Retention Time =+18.30318-0.749648 Percent of organic-11.13153 flow rate+0.157500 Percent of organic * flow rate +0.011139 Percent of organic^2 + 2.66875 flow rate^2

Final equation with coded factors

Retention Time = +2.45-0.4398A-0.5288B+0.0945AB+0.1002A^2+0.1068B^2

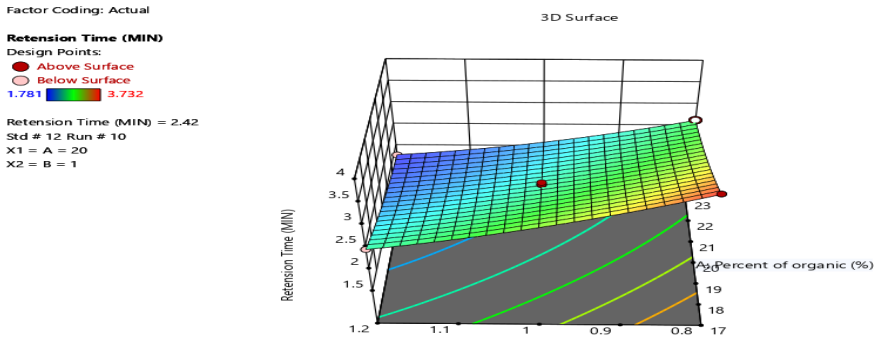

Fig. 2 Response plot for Retention Time

Response 2: Tailing Factor

The ANOVA for identification of the significant and non-significant factors.

ANOVA for Quadratic model: ANOVA data for the tailing factor of Molnupiravir are as following Table 5.

Table 5. Quadratic model of ANOVA for Tailing Factor

Source	SS	df	Average Square	F-statistic	Probability value	
Model	0.0020	5	0.0004	4.53	0.0367	Significant
A-Percent of organic	0.0005	1	0.0005	5.49	0.0517	
B-FR	0.0013	1	0.0013	14.77	0.0063	
AB	0.0000	1	0.0000	0.0000	1.0000	
A^2	0.0002	1	0.0002	2.37	0.1676	
B^2	1.739E-06	1	1.739E-06	0.0196	0.8926	
Residual	0.0006	7	0.0001			
Lack of Fit	0.0001	3	0	0.2602	0.8512	Not significant
Pure Error	0.0005	4	0.0001			
Cor Total	0.0026	12				

The model F-statistic is 4.53 with a probability (p-value) less than 0.05 confirms that the observed variation in the response is not due to random error, and therefore the model is reliable and suitable for further analysis and optimization**.** Fit Statistics data shown in table 6.

Table 6. fit statistics data for Retention Time

Standard Deviation	**0.0094**	**R^2**	**0.7638**
Mean value	1.13	R^2 Adjusted	0.5950
Percent C.V.	0.8321	R^2 Predicted	0.4169
		Adeq Precision	6.8288

Model assessment for tailing factor:

Equation 1

Tailing factor=+0.763017+0.027046 Percent of organic+0.089017 flow rate+6.77488E-16 Percent of organic * flow rate-0.000611 Percent of organic²-0.012500 flow rate²

Equation 2

Tailing factor =+1.14+0.0078A+0.0128B+0.0000AB-0.0055A²-0.0005B²

Response plot and graphical presentation of all responses is shown in fig. 3,4.

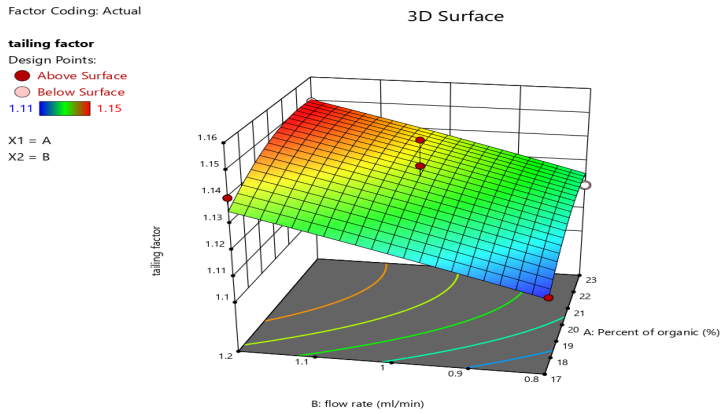

Fig. 3 Response plot for tailing factor

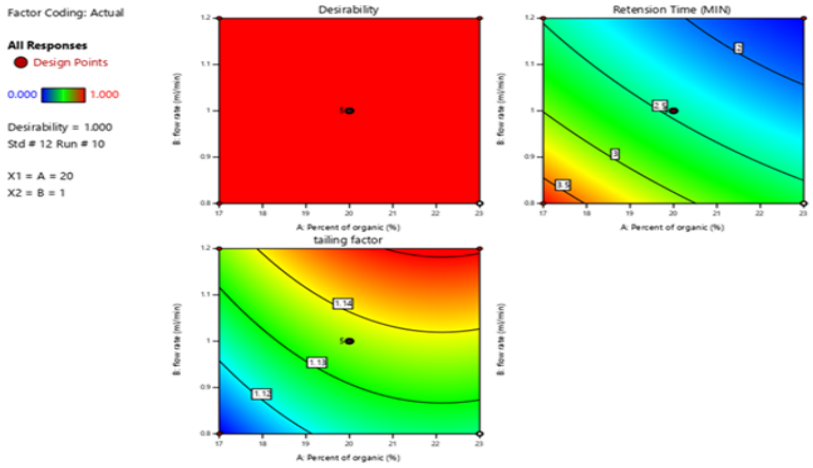

Fig. 4 Graphical Presentation of all responses on the factors percent organic and flow rate

Numerical optimization:

The response surface derived from the CCD was optimized using numerical optimization techniques. The desirability approach, where a value of 1 denotes an ideal response, was employed to identify the optimal combination of variables yielding the highest overall desirability.

Graphical optimization

The graphical optimization provides the basic information to define the design space that is shown in Fig.5

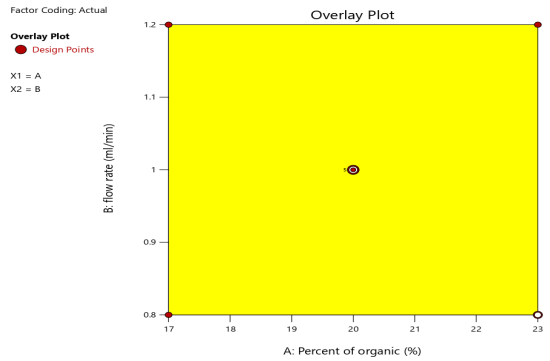

Fig.5 Method Operable Design Region

The optimised method is percent of organic is 20 ml, FR of 1ml/min. The fig 6 is optimised chromatogram for developed HPLC method for estimation of Molnupiravir.

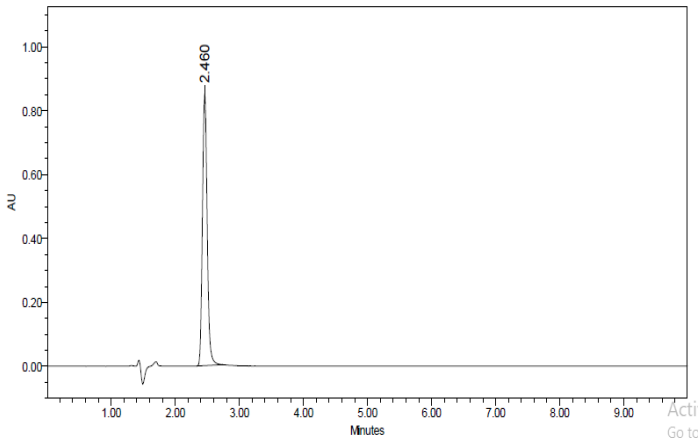

Fig 6: Chromatogram of optimized method

Method Validation:
The QbD- driven developed HPLC method was evaluated for linearity, interday and intraday precision, accuracy, LOD, LOQ to ensure its reliability and suitability for routine analysis.

Linearity (range)
Linearity of proposed method was assessed by making various dilutions of Molnupiravir solutions within the range of 100–300 ppm. A calibration curve was plotted as peak area versus concentration, from this data the regression equation and correlation coefficient confirmed excellent linearity across the studied range.

Precision and system suitability:
System suitability and precision were evaluated by six replicate injection of the standard solution of Molnupiravir. The % recovery and % RSD were calculated.

Accuracy
Accuracy was resolute by employing addition of standard at three concentration level of 80, 100, and 120 of the target concentration. Each level was analysed in triplicate. The mean percentage recovery of Molnupiravir fell within the acceptable range of 98–102%, confirming the accuracy of the QbD- driven developed HPLC method.

LOD/LOQ:
The LOD for Molnupiravir were examined chromatographically by injecting progressively lower concentrations of the analyte until the minimum detectable responses were obtained. The LOQ was examined through a chromatogram where minimum concentration of Molnupiravir was quantified. These values confirm the method's high sensitivity under optimized chromatographic conditions.

RESULT AND DISCUSSION
System suitability:
The peak asymmetry, % RSD and column efficiency were calculated with the help of standard solution of Molnupiravir. The results of suitability of method are reported in Table 7.

Table-7: System suitability parameters of Molnupiravir

Parameters	Results
Retention time	2.480 min
Theoretical plates count	5390
Tailing factor	1.14
Peak area	4404110
% RSD	< 2

Linearity:

The calibration curve for 100-300 µg/ml range was linear & range is 100-300 µg/ml. (as shown in Figure 7). The correlation coefficient was 0.9987.

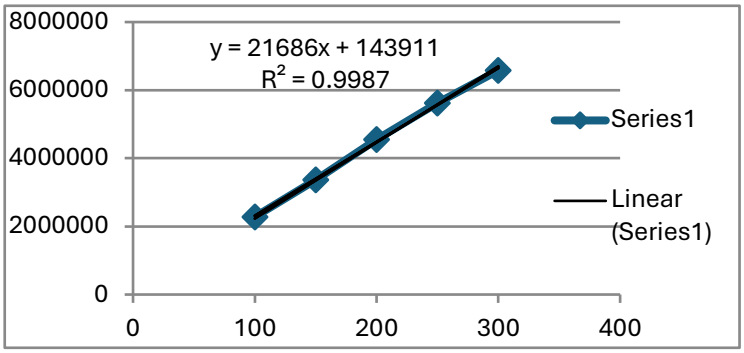

Fig. 7: Calibration curve of Molnupiravir

Precision

The developed HPLC method was precise The results are reported in Table 8.

Table: 8 Precision data with percent RSD

Molnupiravir				
Sample	RT (min)	Area	Plate Count	TF
Standard 1	2.525	4630905	4544.27	1.44
Standard 2	2.529	4604464	4641.4	1.42
Standard 3	2.521	4650050	4607.7	1.45
Standard 4	2.522	4659462	4535.11	1.47
Standard 5	2.527	4643757	4621.6	1.45
Standard 6	2.524	4650838	4661.62	1.44
MEAN	2.5247	4639912.667	4601.95	1.445
SD	0.003	19782.97	51.63	0.016
%RSD	0.119	0.426	1.122	1.137

LOD and LOQ

The LOD and LOQ for Molnupiravir were measured chromatographically and the Chromatogram for LOD and LOQ shown in fig. 8 and 9 respectively. LOD is 0.1PPM and LOQ

is 10 ppm.

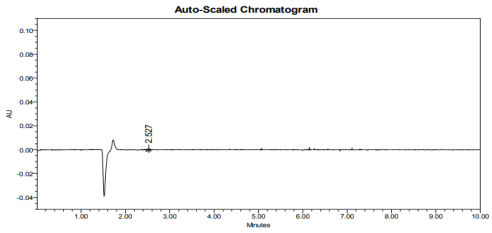

Fig 8. LOD (0.1 ppm)

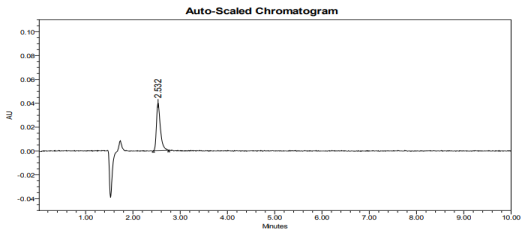

Fig 9. LOQ (10 ppm)

Analysis of Marketed Formulation

Molnupiravir Tablet was measured using predicted HPLC method and the assay results was found to be 99.65 % for Molnupiravir capsule.

Accuracy:

80%, 100% and 120% of solution 0f 100ppm was accessed for accuracy study. The % recovery of Molnupiravir was found to be 99.68.

Robustness

The developed method is robust as it was found that MODR of the design shows yellow region all over the range, shown in fig 10.

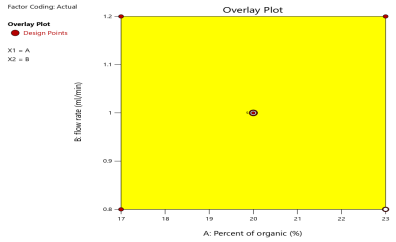

Fig 10: MODR for molnupiravir HPLC method

CONCLUSION

A Quality by Design–driven analytical HPLC method was successfully developed for the measurement of Molnupiravir, and thereafter the method is validated. Application of a face-centred experimental design enabled systematic optimization of critical method parameters, resulting in a robust and reliable method. Owing to its simplicity, rapidity, and low reagent consumption, the method is good for routine analysis or measurement of Molnupiravir in bulk drug, capsules. Notable advantages include cost-effective reagents, short analysis time, operational simplicity, and consistent precision and accuracy.

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