Research Article

Reverse Phase-Liquid Chromatography Assisted Protocol for Determination of Molnupiravir Medication Used to SARS-CoV-2 Infection: An Investigative Approach

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Received: 13-12-2023; **Revised:** 26-02-2024; **Accepted:** 02-03-2024; **Published on:** 15-03-2024.

ABSTRACT

In recent years as a result of the SARS-CoV-2 infection antiviral drugs have received more attention and many potential drug molecules are currently under investigation to end the epidemic. Molnupiravir, a prodrug is one of the assuring candidates for SARS-CoV-2. Therefore, the current research work was having the aim to investigate the easy, defined, sensitive and robust avenue as a liquid chromatography for estimation of molnupiravir from pure blend and its dosage form. The reversed-phase chromatographic appropriate and efficient separation for molnupiravir has been attained with Hypersil BDS C_{18} column along with Water: acetonitrile (70: 30 % v/v) solvent system. The determination was performed at 30 oC at 1 mL/min rate for flow of solvent system through column. The eluents of column were monitored using Photodiode Array detector (PDA) at 235 nm. The investigated reversed-phase chromatographic appropriate and efficient separation for molnupiravir revealed admirable retention time i.e. 4.009 min. The developed investigation molnupiravir was show linear response in the concentration range of 10-50 µg/mL with better coefficients of determination was above than (r^2 0.999). The estimable liquid chromatography was successfully validated as per to ICH guidelines and all method validation parameters of estimable in compliance with ICH guidelines (Technical Requirements for Pharmaceuticals for Human use standards). The developed liquid chromatographic avenues were easy, defined, sensitive, robust and rugged and have admirable potential to estimate molnupiravir from pure blend and its dosage form. Thus, the projected admirable reversed-phase chromatographic method has high prospect to adopt in the pharmaceutical industry.

Keywords: SARS-CoV-2; Molnupiravir; Liquid Chromatography; Robustness.

INTRODUCTION

The pandemic situation of COVID-19 (coronavirus disease 2019), create by SARS-CoV-2, in worldwide has seen almost 270 million confirmed cases and over 5.2 million reported deaths. [1], and there still has been an attempt for effective and better therapeutic options to control the pandemic of COVID-19. Here, the antiviral candidate could be one of the treatments of coronavirus disease, mostly for high-risk patient groups. [2]

In December 2021 an EUA- emergency use authorization has been issued for molnupiravir by the U.S.FDA -Food and Drug Administration (FDA) for the treatment of both mild as well as moderate COVID-19 in adults also molnupiravir is used for who are at greater chance for development to severe coronavirus disease 2019(COVID-19), hence molnupiravir become important drug candidate to cure COVID-19 pandemic [3-4].

Molnupiravir is ribonucleoside prodrug of N-hydroxycytidine (NHC). This small-molecule, which shows higher activity toward SARS-CoV-2 and various other RNA viruses'. Molnupiravir has a high potential barrier to development of resistance against viruses[5-7]. When molnupiravir administrated orally, systemic circulation and intracellular phosphorylation of N-hydroxycytidine (NHC), NHC triphosphate is formed after to NHC triphosphate is integrating into viral RNA via viral RNA polymerase and subsequently viral polymerase to incorporate either adenosine or guanosine in stage of viral replication. This incorporation leads to the virus noninfectious and non-replicating [7, 9-11].

Pharmaceutical analyses are vital within the pharmaceutical industry due to the requirement for accurate and precise analytical methods to investigate drugs, drug metabolites and impurities in drug development processes.[12] HPLC is an widely used analytical approach in pharmaceutical drug analysis because it enables fast, appropriate, precise, accurate, and low-cost determination of analytes [13]. Within the literature, there's an LC-MS/MS approach for the detection of molnupiravir and its metabolite from sample of saliva and human plasma [14-15].

Therefore, in proposed investigation, it was aimed to develop an easy, accurate, rapid, appropriate selective, precise, rugged and robust RP-HPLC Avenue for the estimation of molnupiravir from pure as well as marketed sample. The developed method was fully validated according to the ICH guidelines

MATERIALS AND METHODS

Chemical and reagents

Molnupiravir pure sample and the capsule matrix of Molflu consisting of 200 mg of molnupiravir were procured from local Indian Market. while Acetonitrile (ACN), (HPLC Grade), methanol (HPLC Grade), Water (HPLC Grade) and other reagent and chemicals of AR Grade were acquired from Merck, Mumbai, India.

Instrumentation

Chromatographic resolution was executed with the help of LC prominence system (Shimadzu Corporation, Japan) which composed of LC-20 AD (binary solvent delivery pump) connected to 20 µL injection loop (a Rheodyne injector). SPD-M20A –PDA and thermostated column oven compartment (CTO 10 AS vp). The data were collected and analyzed with LC-solution software (Shimadzu Corporation, Japan).Ultrasonication of samples was conducted by means of an Ultrasonicator; ENERTECH Electronics Pvt. Ltd., India.

Chromatographic conditions

The chromatographic determination of present analysis was studied on Hypersil BDS C_{18}(250 mm × 4.6 mm) particle size 5 µm. Hypersil BDS column was equilibrated with solvent system comprised of Water: acetonitrile (70: 30 % *v/v*). A 20 µL solution of standard was injected. The analysis was established at 30 °C at flow rate 1 mL/min for of mobile system through Hypersil BDS column. The analytes of column were monitored using PDA at 235 nm.

Preparation of standard solution

1 mg/mL standard stock solution of molnupiravir was workout separately in 10 mL capacity of calibrated flask in methanol. Calibration standard solution (10 ug/mL, 20 ug/mL,30 ug/mL, 40ug/mL and 50 ug/mL) were prepared by diluting stock solution with water.

Preparation of sample of molnupiravir in marketed formulation

The present validated investigational RP-LC method was employed for quantification of molnupiravir in capsule formulation. The capsule of Molflu consisting of 200 mg of molnupiravir was procured from local Indian market. To estimating analyte in the marketed matrix; were weighed precisely ground capsule content. Accurately measured capsule content equivalent to 50mg of molnupiravir from marketed sample was moved into a 50 mL of flask containing 30 mL methanol, sonicated for 20 min and volume of calibrated flask was made using methanol up to the given calibrate mark to obtained 1000 µg/mL, the resulting solution was then filtered using 0.45 µm filters. The above prepared stock solution was further diluted by taking appropriate volume of above prepared solution in10mL volumetric flask and make up volume by using HPLC grade water to get sample 20 µg/mL. A twenty µl volume of prepared sample solution was injected into HPLC (six times), under the optimized appropriate chromatographic conditions described above and the measurement of peak areas detected at 235 nm.

Validation of investigated reversed phase-liquid chromatographic approach

The present RP-LC method has been validated according to ICH procedures for the confirmation of molnupiravir in bulk as well as marketed product. The investigated RP-LC method has been successfully validated with various parameters in terms of system suitability, accuracy, and precision, sensitive as well as robustness and ruggedness as mention in the Q2R1 procedure of International Council for Harmonization (ICH)[16].

System suitability

System suitability assessment is typically performed with the aimed to avoid the perceived instability of chromatographic elements such as type of column, detector, pump etc. from negatively affecting official methods [17]. The 10 µg/mL concentration of molnupiravir solution was introduced and examined as six replicates for estimation of the elements like Theoretical plate number, tailing factor, and % RSD of peak area values and retention time (R_t).

Calibration curve

Calibration curve for present developed investigation have been performed by using optimized chromatographic parameters. For calibration curve of molnupiravir, suitable and appropriate volumes were moved from the prepared stock solution of 10 µg/mL in concentration range of 1-5 mL was moved to series of volumetric flask of 10 mL and volume was make up to the mark using HPLC water to give concentration at the range of 10-50 µg/mL for molnupiravir. By using of Hamilton Syringe's a fixed volume of 20 µL is injected into the LC. All measurements for every single concentration were replicated 6 times. In general consideration value of correlation coefficient (r^2) > 0.998 is as the evidence of an acceptable fit for the data.

Accuracy

To evaluate the nearness of the measured value to the exact value, the accuracy of an analytical method is established. A method's accuracy is generally assessed through by calculating the drug candidate's percentage recovery [16-17]. The % recovery of the commenced investigation of molnupiravir has been performed at various levels 80, 100 and 120 %. It was done by the addition to the pre-studied sample a known amount of standard drug and further it was re-examined through the same investigation. The mean recovery within 90–110% should be accepted.

Precision

Precision as degree of agreement among individual tests of the commenced investigation of molnupiravir was accomplished through the intra-day precision and inter-day precision and repeatability (system precision) and was assessed as RSD percentage. According to procedure mention in ICH Q2R1 RSD % value must be not more than 2 %. Three distinct concentrations 10, 30, 50 µg/mL were selected for performing degree of agreement in terms of precision and 30µg/mL solution use for repeatability of present investigation.

Sensitivity

The commenced investigation was evaluated sensitivity in terms of LOD and LOQ[16]. LOD and LOQ for molnupiravir

have being estimated by injecting the 10 - 30 µg/mL low concentrations solution of the molnupiravir through the investigated method. The formulae's were used to determine sensitivity the LOD= 3.3 × ASD/S and LOQ= 10 × ASD/S.

Robustness

The concept of robustness of is an appraisal potential to remain unaffected of developed method due to minor changes in the analytical protocol parameters, as per ICH procedure[16,17], To check out the strength of the developed investigation; various independent variables were selected such as column oven temperature and flow rate of solvent system. The impact of independent variable like responses i.e. tailing factor R_t, and theoretical plates was checked. The strength of method has been investigated using 20µg/mL concentration of solution.

Ruggedness

The level of repetition is the ruggedness performed by injecting sample with interest of two. Ruggedness evaluation has been investigated with concentration of 20 µg/mL.

RESULTS

Optimization of proposed investigation

In present investigation, our endeavored is to established and validated easy and appropriate RP-LC investigation for quantification of molnupiravir in capsule form used to control SARS-CoV-2 infection. To established effective and easy RP-LC investigation concentrated on the gathering of detailed knowledge of molnupiravir inclusive of chemical structure, molecular weight, solubility, absorption UV-spectrum. The molnupiravir are soluble into methanol, acetonitrile and water, hence methanol and water selected for stock preparation of molnupiravir due to its low viscosity and high chemical stability. Chemically molnupiravir is [(2R, 3S, 4R, 5R)-3, 4-dihydroxy-5-[4-(hydroxyamino)-2-oxopyrimidin-1-yl] oxolan-2-yl]methyl 2-methylpropanoate show in Figure 1;

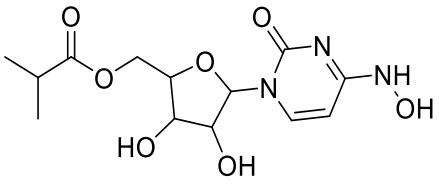

Figure 1: Chemical structure of Molnupiravir

Molecular weights: 329.31 gm/moL. UV absorption spectrum was observed at 235 nm which is further used as detection and monitoring wavelength for molnupiravir. The method was initiated and optimized after collecting this above information by modifying different LC parameters as studied in the method section. For finalized chromatographic parameters, various combination with different ratios of mobile systems like methanol: acetonitrile, methanol: water, acetonitrile: water, were check out for estimation of the analytes but it was observed that this above solvent system unable for resolving the peaks of analytes to obtained appropriate system suitability test. Therefore, in order to obtain the appropriate and excellent estimation of molnupiravir various attempted were performed effectively. Hence lastly, a solvent system comprised of Water: acetonitrile (70: 30 % *v/v*). The excellent chromatographic estimation of molnupiravir has been achieved on Hypersil BDS C_{18} (250 mm × 4.6 mm) particle size i.e. 5µm. The analysis was established at 30 °C at flow rate 1 mL/min of mobile system through Hypersil BDS column. The analytes of column were monitored using PDA at 235 nm. The standard chromatogram for molnupiravir is depicted in Figure 2.

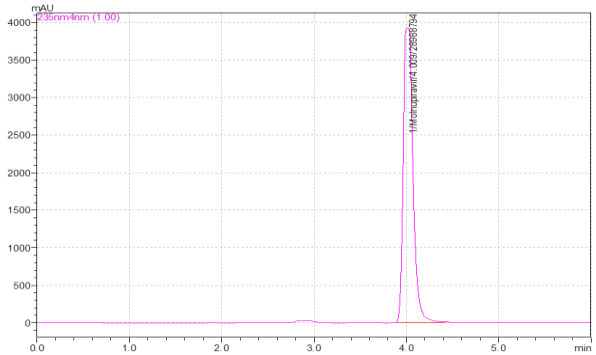

Figure 2: Standard chromatogram for Molnupiravir

Validation of method

These optimized chromatographic methods were selected for validation of proposed research.

System suitability

Parameters of system suitability like tailing factor, capacity factor, R_t and peak area, theoretical plate number, resolution, were studied using injecting the 10 µg/mL solution of molnupiravir (20 µL) for six times. The observed parameters of system suitability parameters are in the within acceptable limit as per ICH and given in Table 1.

Table 1: System suitability test

Tests/parameters	Retention time (Rt)	Theoretical plates	Tailing factor
Analytes	**Molnupiravir**		
Average (n=6)	4.009 min	7164	1.393

n=number of determinations

The analytes i.e. molnupiravir were continuously well retained at 4.009 min with RSD % less than 1 percent depicting strong reproducibility of the duplicate injections used on the integral LC system according to USP. In all chromatographic cycles TP number still crossed over 2000 maintaining strong column efficacy across the completed

separation process of developed investigation with tailing factors were 1.39 and tailing factor haven't ever crossed 1.5; with an outstanding peak symmetry.

Calibration curve

The linearity response of investigated method the calibration curve well reflects a straight line. To construct calibration curve, five drug concentrations in the range of 10-50 µg/mL for molnupiravir were prepared and constant volume of 20 µL is injected into the system. The calibration plot is linear over the range at 10-50 µg/mL. The calibration plot straight line equation is based on the peak response of drug were Y=48478X+ 9664.4 with a (r^2) determination coefficient 0.9997 for molnupiravir. The findings of the linear regression study revealed the importance of the approach proposed. The calibration plot for molnupiravir is depicted in Figure 3 and outcomes are shown in Table 2.

Accuracy

Accuracy of proposed LC investigation is assessed using the % recovery. Percent recovery of the developed investigation has been carried out at different at level 80, 100 and 120 %. For accuracy determination three various concentrations of standard molnupiravir were prepared with placebo samples. Three times every concentration is injected and recovery was calculated. The percentage recovery of proposed investigation ranged from the 99.19-100.05 % with RSD % in the ranged of 0.20–0.71 percentage indicates the accuracy of the investigation. The % recovery outcomes are tabulated in Table 3.

The precision of the conducted investigation was carried out through intra-day and inter-day precision and repeatability was calculated as a % of RSD. For intra-day and inter-day precision three distinct concentrations 10, 30, 50 µg/mL were prepared through diluting the stock solution of molnupiravir using solvent system and every concentration is injected for three times in the same day and for the successive days, accordingly the RSD % values were determined. While, repeatability of present investigation was assessed by injecting 20 µL solution six times of a concentration of 20 µg/mL, accordingly the RSD % values were determined. RSD percentage level for precision study of investigated method less than 2 % thus according ICH Q2R1 protocol, precision of the present developed investigation and the repeatability of present investigation was performed using 20 µg/mL. The precision studies outcomes are tabulated in Table 4.

Table 2: Investigation of Linearity parameters (regression analysis of calibration curves)

Analyte	Tests/parameters	Results
Molnupiravir	Linearity	10-50 µg/mL
	Regression Equation	y = 48478x + 9664.4
	Correlation coefficient (r^2)	0.9997

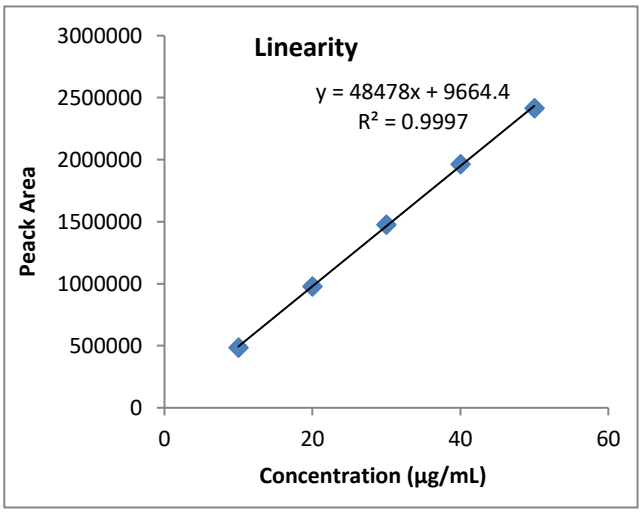

Figure 3: Linearity graph

Table 3: Investigation of accuracy study

Initial amount [µg/mL]	Amount added [µg/mL]	Amount found [µg/mL]	% Recovery	% RSD
Molnupiravir				
Level of recovery study 80 %				
20	16	35.95	99.68	
20	16	36.10	100.62	0.49
20	16	35.98	99.87	
Mean ± SD 100.05 ± 0.49				
Level of recovery study 100 %				
20	20	39.68	98.40	
20	20	39.96	99.80	0.71
20	20	39.87	99.38	
Mean ± SD 99.19 ± 0.71				
Level of recovery study 120 %				
20	24	44.05	100.2	
20	24	44.00	100	0.2
20	24	43.95	99.79	
Mean ± SD 99.99 ± 0.20				

*SD= standard deviation, %RSD= percent relative standard deviation

Precision

Table 4: Precision analysis

Intra-day Precision			Inter-day Precision		
Concentrations	**% Amount Found [n=3]**	**% RSD**	**Concentrations**	**% Amount Found [n=3]**	**% RSD**
Molnupiravir					
10	98.05	0.26	10	98.85	
	98.02			99.05	0.10
	99.01			98.99	
Mean ± SD	**98.36 ± 0.25**		**Mean ± SD**	**98.96 ± 0.10**	
30	100.56	0.66	30	100.78	
	101.88			100.24	0.28
	100.98			100.68	
Mean ± SD	**101.14 ± 0.67**		**Mean ± SD**	**100.56 ± 0.28**	
50	98.56	1.64	50	98.25	1.15
	101.8			100.54	
	99.65			99.34	
Mean ± SD	**100.00 ± 1.64**		**Mean ± SD**	**99.37 ± 1.14**	

*n=number of determinations, SD= standard deviation, %RSD= percent relative standard deviation

Table 5: Robustness evaluation

Independent variables	Explored range	Molnupiravir			Optimized value
		Tailing factor	Retention Time (R_t)	Theoretical plates	
Flow rate (mL/min)	0.9 - 1.1	1.37	4.067	7146.904	1 mL/min
		1.39	4.009	7164.909	
		1.41	4.062	7426.774	
Column oven temperature (°C)	25 – 35	1.36	4.117	7140.802	30 °C
		1.39	4.090	7150.909	
		1.37	4.062	7360.364	

Sensitivity

Sensitivity of present investigation is calculated in terms of LOD and LOQ. To estimated LOD and LOD; five distinct concentrations 10, 15, 20, 25 and 30 µg/mL have been working out through diluting the stock solution of molnupiravir using solvent system and injecting 20 µL solution to LC system six times for each concentration. The LOD and LOQ were estimated 0.10 and 0.32, for molnupiravir.

Robustness

Robustness of proposed investigation was studied by using the concentration of 30 µg/mL. It was investigated by changing the rate of flow of mobile system (0.9-1.1 mL/min), temperature of column oven (25-35 °C) according their impact was studied on tailing factor, R_t, theoretical plates of molnupiravir. From it was observed that there is no outstanding effects were observed on the responses. Hence, suggested that proposed and developed investigation was. The robustness evaluation observations are tabulated in Table 5.

Ruggedness

Ruggedness of performed investigational protocol was executed through30 µg/mL concentration. The results were obtained acceptable through view of two independent researchers under same analytical conditions. and environmental conditions. Thus, it was concluded that method was rugged. The ruggedness study outcomes are given in Table 6.

Table 6: The ruggedness study outcomes

Ruggedness	
Analysts-I (Mean ± SD)	99.45 ± 0.19
Analysts-II (Mean ± SD)	99.69 ± 0.27

Selectivity and specificity

Specificity is a step for detecting of analytical sample, specificity of the present investigation evaluated using

checking the resolution factor of molnupiravir chromatographic peaks were assessed though the UV spectra generated by a UV detector. The evaluation observations are tabulated in Table 7. Validation parameters for proposed investigation are summarized in Table 7.

Table 7: Summary of validation parameters for proposed investigation

Parameters	**RP-HPLC**
	Molnupiravir
Linearity	
Range (µg/mL)	10 – 50
Determination coefficient (r^2)	0.9997
Accuracy	
Mean percent recovery (%)	99.19 - 100.05
RSD %	0.20 - 0.72
Precision	
Intra-day precision (RSD %)	0.26 - 1.64
Inter-day precision (RSD %)	0.10-1.15
Repeatability (RSD %)	0.57
Sensitivity	
LOD (µg)	0.1
LOQ (µg	0.32
Robustness	Robust
Ruggedness	
Analysts-I (Mean ± SD)	99.45 ± 0.19
Analysts-II (Mean ± SD)	99.69 ± 0.27

Application of investigational approach for estimation of analyte in marketed preparation

The established method was effectively explored for the analysis of molnupiravir in Molflu marketed preparation. The assay of molnupiravir in Molflu was observed to be 99.78 ± 0.24 % presented in **Table 8.** Optimally, when establishing analytical protocol an analyte assay is needed; since, it encourages other researcher to analyze the same drug molecule, as well as its estimation in routine analysis, or in different type of pharmaceutical formulation.

Table 8: The Assay of Drug

Assay	
% Assay of drug	99.78 ± 0.24

DISCUSSION

It is extremely important to validate a developed analytical approach of analysis for molnupiravir in pure sample and pharmaceutical matrix to check out the accurate chromatographic recovery as well as separation. The proposed RP-LC method meant for the analysis in molnupiravir capsule dosage form using Hypersil BDS column with PDA detector to monitored analytes. The mobile phase consists of Water: acetonitrile (70: 30 % *v/v*) was employed for separation analysis with 1 mL/min rate of flow from column. The results from the developed investigation that accepted and validated RP-LC approach is easy, precise, appropriate, sensitive and robust. The study assessing specificity demonstrated that there was complete absence of any interruption of the components of capsule matrix and no other components eluting at the R_t of molnupiravir. Subsequently, the investigated method was validated as per with ICH guidelines and all the parameters of validation were recorded within the acceptable limit as per the ICH. Moreover, the superiority of the proposed liquid chromatographic approach can also be confirmed on the basis of its high sensitivity (LOD and LOQ). In detail, the findings confirm the high level of use of the investigated avenue for estimating molnupiravir in pharmaceutical matrix.

CONCLUSION

The proposed research leads to the application of RP-LC approach for estimation of molnupiravir is developed and validated for linearity, precision, accuracy, sensitivity, robustness, and ruggedness for enormously estimation in marketed preparation .This investigation implicates the easy single step for sample and stock preparation molnupiravir ; moreover, direct introduction of solution into the system. The total analysis time of method was less than 8 min; which demonstrated the minimum wastage solvent system. In addition, selected solvent system does not prepare with and ion-pairing agents as well as a buffer solution which allow low-cost analysis, which makes the developed investigation advantageous. Considering all these attribute, it is recognize that the developed HPLC method is more applicable for the routine analysis of molnupiravir compared to the LC-MS/MS method. Moreover, the availability of the regular HPLC systems compared to LC-MS systems makes our study much more relevant for routine analysis. Consequently, the investigation is quite sensitive to detect and quantify the analytes microgram quantity. In consequence the proposed investigation may be carried out for routine molnupiravir analysis in various marketed preparation as well as for estimation of metabolites of molnupiravir.

ACKNOWLEDGMENTS

The authors express their gratitude to Principal, Dr. Prashant Deshmukh, Dr. Rajendra Gode College of Pharmacy Malkapur, Maharashtra, India, for their great vision and support.

Source of Support: The author(s) received no financial support for the research, authorship, and/or publication of this article

Conflict of Interest: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ABBREVIATIONS

SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2;

COVID-19: coronavirus disease 2019;

PDA: Photodiode Array detector

ACN: Acetonitrile;

RP-HPLC: Reverse-phase high-performance liquid chromatography;

NHC: N-hydroxycytidine

ICH: International Conference on Harmonization;

LOD: Limit of detection;

LOQ: Limit of quantification

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