DRUG ANALYTICAL RESEARCH
ISSN: 2527-2616
www.seer.ufrgs.br/dar

Full factorial design assisted RP-UHPLC method development and study of stressed degradation of molnupiravir

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To combat the SARS-CoV-2 addition of molnupiravir into the current therapeutic regimen has added new hopes. But currently, not so many analytical methods are available for analyzing molnupiravir. The current study aimed at developing a suitable and efficient RP-UHPLC method for molnupiravir capitalizing full factorial design. The optimized method involved the use of trifluoroacetic acid (0.1%): methanol at a ratio of 65:35 (%, v/v) with fortis diphenyl HPLC column (150 mm x 4.6 mm i.d. [internal diameter], 5 µm particle size). The method was found highly specific (purity index <1.5), linear (0.5-100 µg.mL^{-1}), accurate (~100%), precise, rugged, and robust (%RSD<2). A stress degradation study exhibited that 0.1N HCl at 100˚C (±1˚C) for 2 hours caused drug degradation crossing its limit (>10%), the drug is extremely susceptible to basic hydrolysis and 0.1N NaOH at room temperature for 1 min caused 65.43% degradation, exposure in 3% H_2O_2 at 100˚C (±1˚C) for 1 hours caused the drug degradation over 10%, and 5 days of exposure to short wavelength UV light caused 13.16% of drug degradation. The research work successfully developed an efficient and validated method for analyzing molnupiravir in the RP-UHPLC system. Moreover, the degradation study performed will aid in adding integral information related to the stability of the drug molecule.

Keywords: Molnupiravir; RP-UHPLC; factorial; COVID-19; degradation.

Article received at 07/31/2023 and accepted at 10/24/2023.
https://doi.org/10.22456/2527-2616.134441

Introduction

SARS-CoV-2 is the name of the nightmare the world has seen. The COVID-19 disease caused by this virus has affected 690,857,685 populations worldwide with the death of 6,894,561 patients (1). Many of the affected patients required to be admitted to hospitals due to the severity of symptoms mostly those who were accompanied by risk factors like obesity, diabetes mellitus, and serious cardiac complications (2–5). To combat the pandemic several vaccines have received emergency use authorization certificates yet there was a need for an effective oral antiviral agent (6). And molnupiravir here comes with the solution. FDA issued an emergency use authorization letter for using molnupiravir in patients older than 18 years on December 23, 2021. The company reported that using molnupiravir on non-hospitalised adults with mild to moderate COVID-19 reduced the risk of admission to the hospital or death by 50% (7).

Molnupiravir, upon entry into the patient's bloodstream, gets converted to its active form N-hydroxycytidine (NHC). This NHC is responsible for providing anti-viral action against SARS-CoV-2. The NHC molecules form NHC triphosphate and are being used as substrate by viral RNA polymerase instead of cytidine and uridine. As a result, viral RNA gets misdirection with accumulation of unhealthy errors which results in loss of infectivity and replication capacity of the virus (5,8,9).

In response to the available safety as well as effectiveness data and authorization by the FDA, physicians have started using molnupiravir as an oral antiviral therapy to treat adults affected by COVID-19 (9–11). With the advent of usage, it has become equally important to analyze the drug in the pharmaceutical dosage form as well as to know its degradation characteristics. But till now there has been only a single reported method for quantification of molnupiravir in the HPLC system which used acetonitrile and water as mobile phase (12).

Product development and analytical method establishment for the developed product work hand in hand throughout the whole life cycle of any pharmaceutical product. Developing analytical methods using the conventional method is rather laborious. The QbD-based methodology has been gradually ingrained into the mindset of analytical scientists to remove the hitches experienced during method development. Quality by design (QbD) based approach in pharmaceutical development was pioneered first by Dr. Joseph M. Juran (13). Later on, the FDA recognized that increased testing doesn't ensure product quality rather it needs to be built into the process. ICH Q8 (R2) (Pharmaceutical Development), ICH Q9 (Quality Risk Management), and ICH Q10 (Pharmaceutical Quality System) were the last three documents to initiate the concept of QbD (14–17). Pharmaceutical Quality by Design (QbD) is a methodical approach to development that begins with predetermined objectives, emphasizing product and process comprehension, rooted in science and quality risk management (14).

In many previous studies, the QbD approach has been used for analytical method development including HPLC

(18–21). Among several approaches, factorial design is one of the response surface methods (RSM) which is helpful in the systematic development of analytical methods and has been adopted by many of the scientists for the development of analytical methods in their studies (21–23). Factorial designs are predominantly employed to ascertain the significance of factors in relation to the process. For experiments involving the study of the effects of two or more factors factorial designs are most efficient (24). Earlier this approach has been adopted by many of the analytical scientists to develop analytical methods (19,22,23).

In this study, the aim is to develop an easy and efficient RP-UHPLC method using factorial design for analyzing molnupiravir in pharmaceutical formulations as well as validation of the developed method using ICH Q2 (R1) guidelines (25). Apart from method development degradation of molnupiravir in several stressed conditions i.e. acid, base, heat, and photo was also studied (26,27).

Experimental section

Chemicals and reagents

Molnupiravir (Potency: 98.9%, Halolife Technology Co. Ltd.) was provided as a generous gift by Incepta Pharmaceuticals Ltd. (Dhaka, Bangladesh). HPLC grade methanol procured from Merck, Germany, was utilized for preparing the mobile phase. Hydrochloric acid (37%) and sodium hydroxide pellets were purchased from Merck, Germany, and Merck, India, respectively. Trifluoroacetic acid (TFA) was sourced from Panreac AppliChem, Germany, and hydrogen peroxide from Scharlab S.L., Spain. Purified water was generated using the LaboStar® RO DI system (Evoqua, Germany).

Chromatographic conditions

The HPLC setup utilized in the study was a Perkin Elmer Flexar series equipped with an auto-sampler, FX-15 binary pump, vacuum degasser, column oven, and PDA plus detector. The separating column was the Fortis diphenyl HPLC column (150 mm x 4.6 mm i.d., 5 µm particle size). The column temperature was upheld at 30˚C, and the injection volume utilized was 10 µL. The detection was performed at a wavelength of 240 nm. Elution was obtained through the isocratic elution method with a mixture of methanol and 0.1% trifluoroacetic acid.

Preparation of standard stock solution

An accurate weight of 10 mg of molnupiravir working standard was taken in a 100 mL volumetric flask, and a suitable volume of a mixture of methanol and 0.1% TFA in water (75:25, v/v) was added to dissolve the drug. After sonication, the volume was adopted to the designated level with the same solvent. The concentration of stock solution thus obtained was 100 µg.mL^{-1}.

Preparation of mobile phase

A mixture of HPLC grade methanol and 0.1% TFA in water at a ratio of 35:65, % v/v was used as a mobile phase to develop the RP-UHPLC method for molnupiravir. Both the components were filtered through 0.45 µm filter paper, mixed thoroughly, and degassed by sonicating before use.

Design of experiment

The experimental design employed a 3^2 full factorial design using Design Expert® software (Version 13, Stat-Ease Inc., USA) considering two independent variables and their three responses. The covariates selected were the percentage of methanol (A, % v/v) and the flow rate of the mobile phase (B, mL.min^{-1}). Both were considered at three levels, i.e., low, mid, and high (Table 1). The responses were retention time (RT) (R1, min), tailing factor (TF) (R2), and theoretical plate count (TP) (R3, N). The optimization constraints were set to attain a lower RT, lower TF, and a higher TP, as the lower RT value ensures a faster method, a lower TF confirms the symmetry of the chromatogram, and a higher TP denotes higher efficiency of the column. The data was gathered from operating Chromera® software and further processed by employing Design Expert® software.

Table 1. Variables and their levels for designing the experiment.

Variable	Unit	Type	Level	Coded Value	Actual value
Methanol composition in mobile phase (A)	% v/v	Numeric	Low	-1	30.0
			Medium	0	32.5
			High	+1	35.0
Mobile phase flow rate (B)	mL.min^{-1}	Numeric	Low	-1	1.1
			Medium	0	1.2
			High	+1	1.3

Method validation

The developed method's linearity, accuracy, precision, specificity, system compatibility, ruggedness, and robustness were all validated in accordance with ICH Q2 (R1) requirements.

System suitability

The working standard of molnupiravir at a concentration of 10 µg.mL^{-1} (made by dilution from the standard solution of 100 µg.mL^{-1}) was utilized, and percentage relative standard deviation (%RSD) values were

determined in each case to evaluate the system suitability of the suggested HPLC approach.

Linearity

Analyzing eight working solutions of molnupiravir generated in triplicate and ranging in concentration from 0.5 to 100 µg.mL^{-1} allowed for the evaluation of the linearity of the HPLC approach. The following concentrations were used to generate the calibration curve: 0.5, 1, 5, 10, 15, 20, 50, and 100 µg.mL^{-1}. First, linear regression analysis was used to evaluate the linearity, and then least square regression analysis was used to confirm it. Y = mX + C is the formula for calculating the regression line, where Y is the response (peak area), X is the molnupiravir concentration in µg.mL^{-1}, m is the regression line's slope, and C is the line's intercept.

Specificity

The specificity of the approach was determined by means of a placebo analysis. Molnupiravir tablet formulations that included all the components except API were used to generate a placebo for this investigation. After that, the placebo received the same care as the test samples.

Accuracy

Drug solution recovery studies were used to test the accuracy of the proposed techniques. Molnupiravir standard solutions ranging in concentration from 0.5 to 100 µg.mL^{-1} were tested in HPLC against a reference standard solution that was known to contain pure molnupiravir. Next, the percentage recoveries for molnupiravir in its pure form were calculated, expressed as the mean ± RSD of three replicates.

Precision

Molnupiravir solution at nominal standard concentration (10 µg.mL^{-1}) was analysed in six replicates on the same day (intra-day precision) and daily for six times over a period of three days (inter-day precision) to determine the repeatability (intra-day precision) and intermediate precision (inter-day precision) of the method. The findings were given as the measurement's %RSD.

Sensitivity

The HPLC technique required a preliminary evaluation of the baseline parameters in order to determine the Limit of Detection (LOD) and Limit of Quantitation (LOQ). Highly diluted standard solutions of molnupiravir were analysed under the same chromatographic circumstances when the conditions satisfied the predetermined specifications. The concentration of the molnupiravir standard solution at which the chromatogram showed a peak area around 3 times larger than the blank was determined to be the LOD. When the peak area of the sample was about 10 times larger than the blank, the limit of quantification (LOQ) was established (28).

Ruggedness

The ruggedness of the proposed HPLC technique was assessed by the examination of six molnupiravir test sample solutions, each with a concentration of 10 µg.mL^{-1}. To evaluate the repeatability of the test findings, these analyses were performed in two distinct labs by two different analysts. In both cases, the percentage recovery and the %RSD were computed (19, 29).

Robustness

The HPLC method's robustness a measure of the method's ability to withstand small changes in its parameters was assessed. The influence of mobile phase composition was tested at (Methanol: 0.1% TFA = 30:70 v/v) and (Methanol: 0.1% TFA = 32.5:67.5 v/v) instead of (Methanol: 0.1% TFA = 35:65 v/v). The impact of flow rate was investigated at 1.1 and 1.2 mL.min^{-1} instead of 1.3 mL.min^{-1}. For robustness testing, the percentage RSD was calculated in each of these scenarios.

Stressed degradation study

The degradation behavior of molnupiravir was studied under different experimentally set stress conditions following ICH Q1A and ICH Q1B. The drug was forced to undergo hydrolytic (acidic and basic), oxidative, thermal, and photolytic degradations, and then analyzed for drug content.

Results and Discussion

Analysis of responses

Analysis of responses has revealed that response 1 (retention time) tends to follow a quadratic mathematical model showing its dependence on the selected variables, as the difference between the adjusted and predicted R^2 values is less than 2 (adjusted R^2-0.998 and predicted R^2-0.993). Interestingly, both response 2 (tailing factor) and response 3 (theoretical plate count) followed linear models depicting their association with the independent variables (R2: adjusted R^2-0.981 and predicted R^2-0.968; R3: adjusted R^2-0.984 and predicted R^2-0.976). The response surface modeling indicated that an increase in both factors (A & B) decreases the retention time (Figures 1.a & 1.b). Similarly, the tailing factor also decreases with the increase of both the percentage of methanol in the mobile phase and mobile phase flow rate, although the impact of methanol composition appeared to be less prominent (Figures 1.c & 1.d). In contrast, theoretical plate count increases with the increase of both factors A and B (Figures 1.e & 1.f). Data gathered during analysis are presented in Table 2 along with the developed equations for response.

Table 2. ANOVA and regression equation of responses

ANOVA for the responses									
	R1			R2			R3		
Source	SS*	F	P	SS	F	P	SS	F	P
Model	5.4100	1128.27	$< 0.1^4$	0.0513	205.61	$< 0.1^4$	8.336E+05	240.95	$< 0.1^4$
A	3.3800	3526.47	$< 0.1^4$	0.0023	18.61	0.0050	1.332E+05	77.00	0.1^4
B	1.9900	2070.58	$< 0.1^4$	0.0490	392.62	$< 0.1^4$	7.004E+05	404.89	$< 0.1^4$
AB	0.0150	15.64	0.0288						
A^2	0.0191	19.96	0.0209						
B^2	0.0084	8.72	0.0599						
Residual	0.0029			0.0007			10379.33		
Cor Total	5.4100			0.0520			8.440E+05		
Fit statistics				Regression equation					
Source	R1	R2	R3	R1 =	+38.84944-1.61180A+1.80417B+0.245000AB+0.015653A^2-6.46667B^2				
Std. Dev.	0.031	0.01	41.59	R2 =	+1.48089-0.007867A-0.903333B				
Mean	5.430	0.14	2952.67						
C.V. %	0.570	7.91	1.41	R3 =	-3084.33333+59.60000A+3416.66667B				
AP**	104.887	34.12	40.86						

*SS-Sum of squares; **AP-Adequate precision.

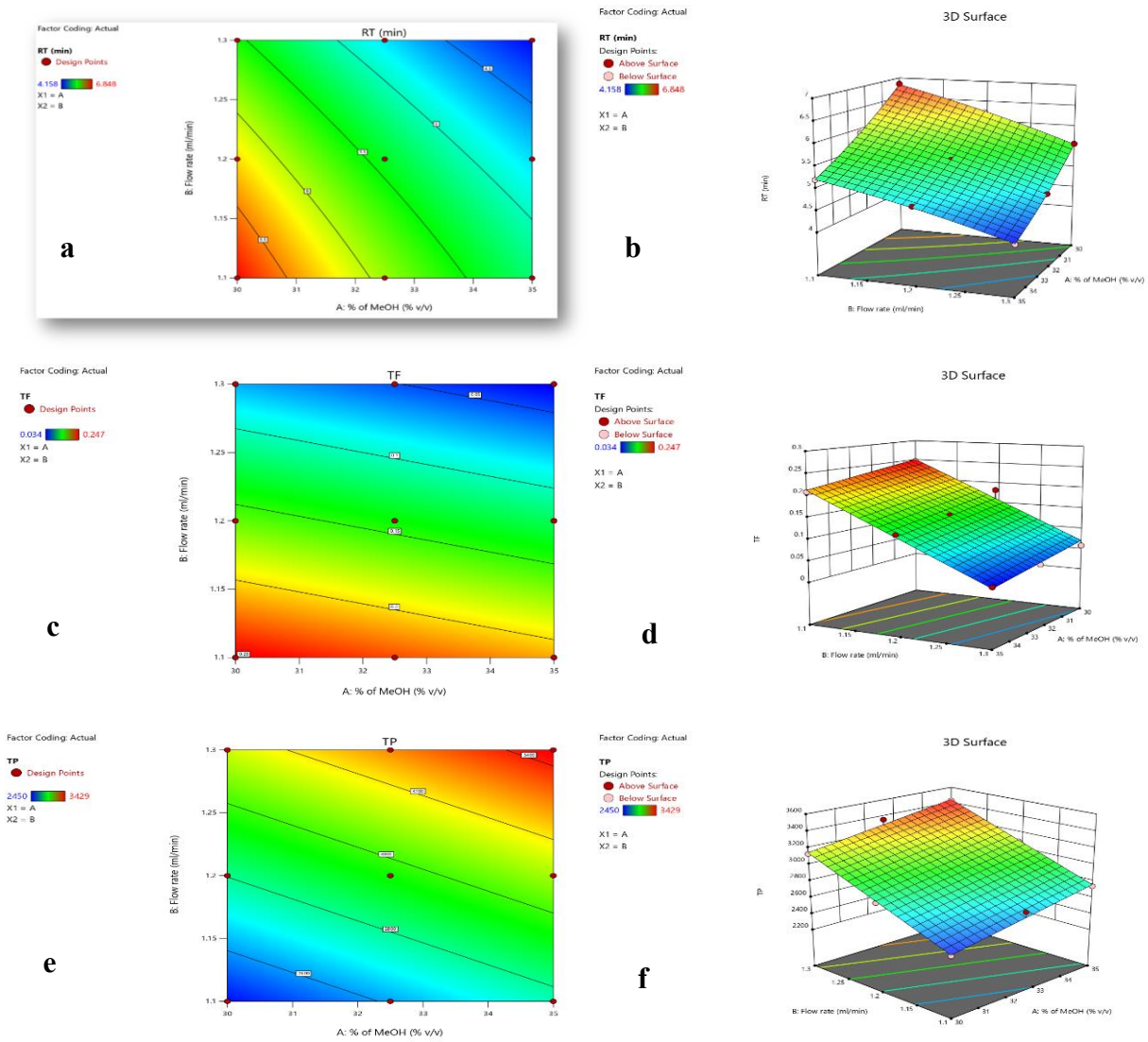


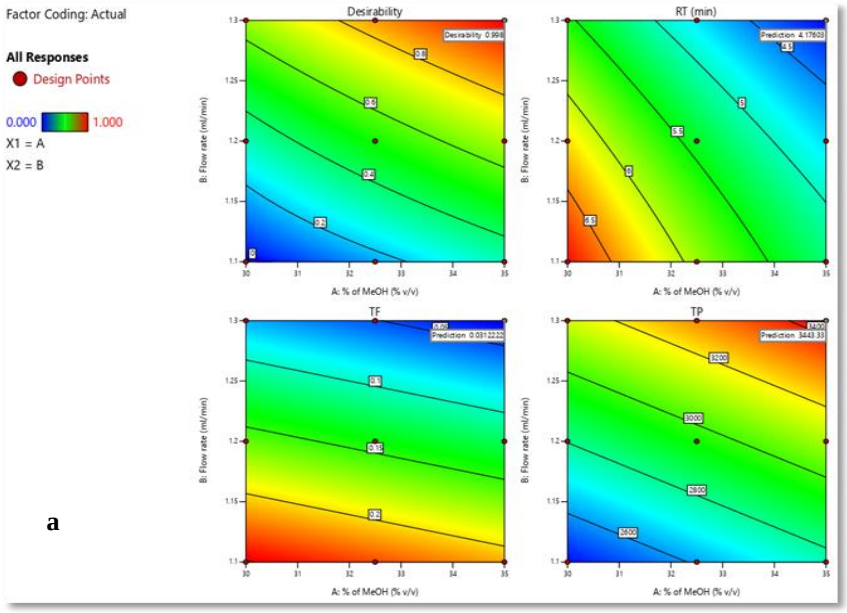
Figure 1. Impact of change of factors (A & B) on (a, b) retention time (RT); (c, d) tailing factor (TF); (e, f) theoretical plate count (TP).

From the analysis of responses, it has been observed that the retention time of the molnupiravir in the utilized system changes quadratically with the change of concentration of methanol in the mobile phase and flow rate. An increase in factor A affects the retention time negatively, whereas the squared term of factor A affects it positively. An increase in factor B causes a decrease in the retention time for molnupiravir. The tailing factor of the chromatograms also depends on both factors. An increase in factor A and factor B causes to decrease in the tailing factor. And theoretical plate count increases linearly with the increase of both factors.

Optimization of method

Factors affecting the responses of the study were optimized to obtain desirable responses with the aid of Design Expert® software (Version 13, Stat-Ease Inc., USA).

Among the eight different suggestions, the one with maximum desirability (0.998) was selected initially as an optimized method (Figure 2). The selected method involved the use of 35% factor A and factor B as 1.3 $mL.min^{-1}$. Interestingly, the predicted errors calculated from experimental data exploiting the optimized method were found to be -0.00072% for R1, 0.573% for R2, and -0.126% for R3 (Table 3). All these errors were within the set tolerance of ±2%, indicating the robustness of the prediction. Figure 2.a represents a contour plot for the optimization of the method and 2.b represents a chromatogram obtained through the optimized method. Moreover, the utilization of methanol in the current method will surely reduce the cost significantly as acetonitrile is a very expensive solvent. Apart from that when methanol gets mixed in the mobile phase, it will cause the emission of heat due to interaction with water which will aid in degassing the dissolved air bubbles



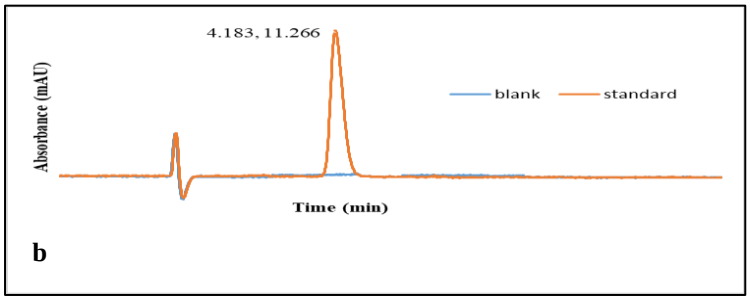


Figure 2. (a) Contour plot for the optimization of the method. (b) Overlay chromatogram of blank sample & molnupiravir standard at 10 $\mu g.mL^{-1}$ concentration using optimized condition.

Table 3. Predicted errors (%) of the responses compared to the experimental values.

Method	Factor A (%, v/v)	Factor B (mL.min^{-1})	R1 (min)	R2	R3
Predicted values	35	1.3	4.17603	0.03122	3443.333
Experimental values	35	1.3	4.176	0.0314	3439
Predicted error (%)			-0.00072	0.573	-0.126

Predicted error (%) = (Experimental value-Predicted value)*100/Experimental value
Tolerance: ±2%

Method validation

System suitability

System suitability of the proposed method was performed by analyzing peak area and all three responses for six replicate injections of molnupiravir at 10 µg.mL^{-1} concentration. Values were expressed as mean±%RSD, and the derived values were 147048.39±0.24%, 0.0376±1.37%, 3391.33±0.46%, and 4.163±0.07% for peak area, tailing factor, theoretical plate count and retention time, respectively indicating good performance of the system (figure 3).

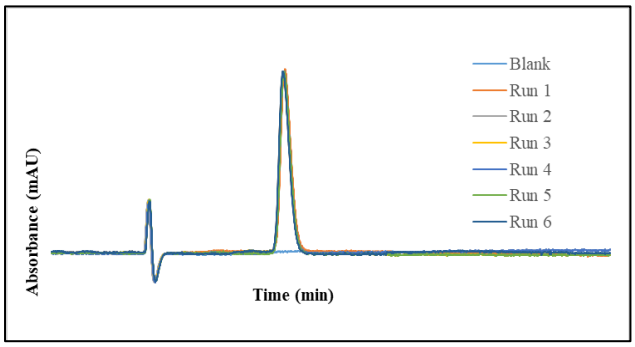


Figure 3. Overlay chromatograms of blank and six replicate molnupiravir standard at 10 µg.mL^{-1} concentration.

Specificity

Chromatograms acquired for molnupiravir working standard and blank divulged there was no interference within the retention time of the drug molecule, indicating a good specificity of the method (Figure 2.b). Moreover, peak purity analysis showed that peak purity index found from the method was less than 1.5 which indicates the acceptable purity of the chromatograms (Figure 2.c).

Linearity

The linearity study for molnupiravir was carried out within the concentration range of 0.5-100 µg.mL^{-1} and the correlation coefficient (R^2) value of 0.9998 indicated good linearity of the method (Figure 4). Furthermore, residual plot shows the data are randomly dispersed around horizontal axis which affirms that linear model is the best fit for the data (Figure 5).

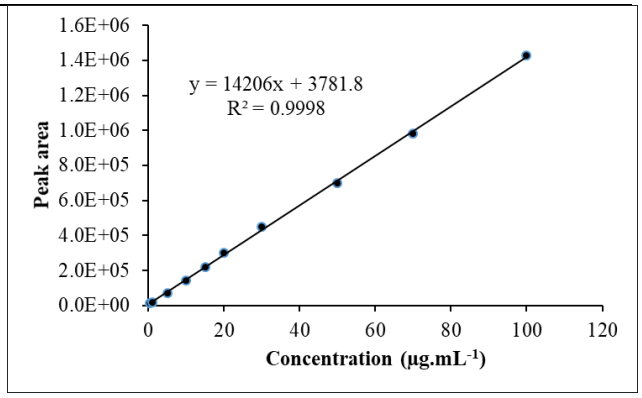


Figure 4. Linearity of molnupiravir quantitation (range: 0.5-100 µg.mL^{-1}.

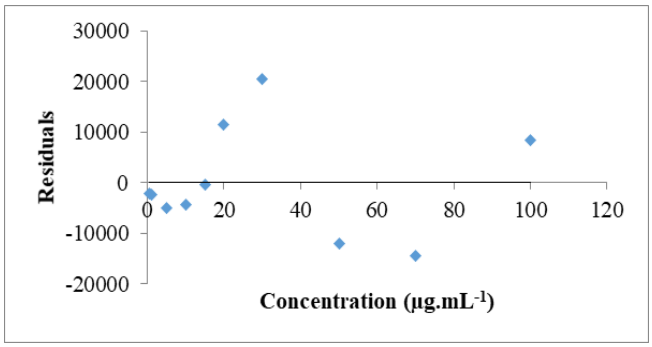


Figure 5. Residual plot of molnupiravir quantitation (range: 0.5-100 µg.mL^{-1}.

Accuracy

Recovery experiments were used to assess the method's accuracy. The recovery amount of molnupiravir ranged from 98.18±0.11% to 102.87±0.29% in molnupiravir standard solutions, demonstrating that the proposed method is accurate (Table 4).

Precision

Precision study analysis presented in Table 4 reveals that there were no considerable discrepancies among assays of standard solution and sample solution within or between days (% RSD less than 1%), stipulating a high level of precision of the method (Table 4).

Sensitivity

The LOD and LOQ were found to be 0.04 µg.mL^{-1} and 0.10 µg.mL^{-1} of molnupiravir, respectively (Figure 6).

Thus, the proposed method could be used for the identification and quantitation of traces of molnupiravir in pharmaceutical products.

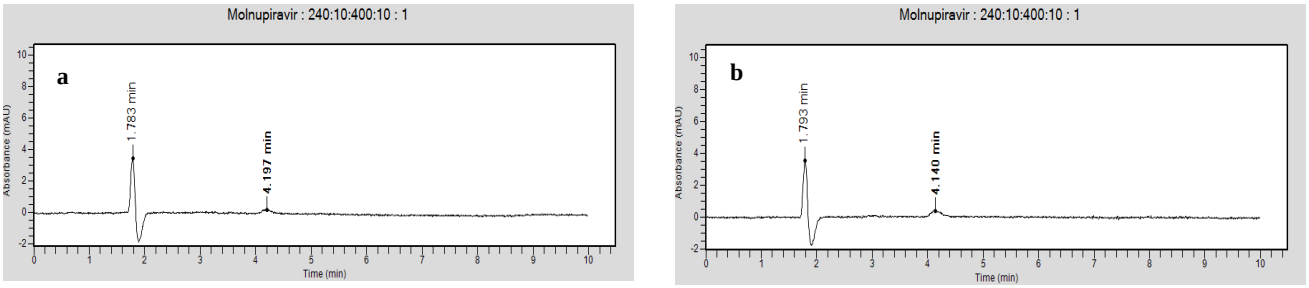


Figure 6. (a) Chromatogarm of LOD of molnupiravir (0.04 µg.mL^{-1}). (b) Chromatogarm of LOQ of molnupiravir (0.10 µg.mL^{-1}).

Table 4. Results of validation parameters for analysis of molnupiravir.

Type of Study	Amount added (µg.mL^{-1})			Mean % recovery±%RSD
Accuracy	0.5			102.87±0.29
	1			100.86±0.34
	5			98.62±0.44
	10			98.27±0.25
	15			100.54±0.5
	20			102.44±0.38
	50			98.18±0.11
	100			100.29±0.07
	Spike level (%)	Type of precision		
Precision	10	Intra-day		100.57±0.15
		Inter-day	Day 1	100.12±0.09
			Day 2	100.73±0.19
			Day 3	101.13±0.16
	Type of ruggedness	Amount added (µg.mL^{-1})		
Ruggedness	Analyst 1	10		100.30±0.23
	Analyst 2	10		100.24±0.31
Type of Study	Parameters	Variations	Amount added (µg.mL^{-1})	
Robustness	Mobile phase flow rate (mL.min^{-1})	1.1	10	101.72±0.14
		1.3	10	100.07±0.11
		1.5	10	99.35±0.31
	Mobile phase (% Methanol)	30	10	101.80±0.15
		35	10	100.05±0.15
		40	10	99.07±0.18

Ruggedness

The ruggedness of the method was measured by evaluating the %RSD values of six replicate quantitations of molnupiravir by different analysts. The values ranged from 100.24±0.31% to 100.30±0.23%, indicating that the proposed method is rugged (Table 4).

Robustness

Robustness was determined by varying the composition of the mobile phase and its flow rate. The %RSD of the robustness study under different altered conditions varied from 99.07±0.18% to 101.80±0.15%, demonstrating that the proposed UHPLC method is appreciably robust (Table 4).

Stressed degradation study

Hydrolytic Degradation

Results of the hydrolytic degradation study in acidic conditions showed that stressing in 0.1N hydrochloric acid at 100˚C (±1˚C) for 2 hours caused the degradation of molnupiravir at an extent of approximately 33%, which is much higher than the set limit for drug product shelf life determination (i.e., 10% degradation). Interestingly, molnupiravir tends to be less prone to acidic hydrolytic degradation at room temperature, 40˚C (±1˚C), 60˚C (±1˚C), and 80˚C (±1˚C), as all these conditions caused the extents of molnupiravir degradation much below 10% (Table 5, Figure 7).

Alkaline hydrolytic degradation showed that molnupiravir is extremely susceptible. The drug substance exhibited an approximately 65% degradation extent when stressed in 0.1N sodium hydroxide at RT (room temperature) for 1 min. Accordingly, basic hydrolysis for 1 hour at RT, 40˚C (±1˚C), and 60˚C (±1˚C) was found to cause complete degradation of molnupiravir (Table 5, Figure 8).

Oxidative degradation

Oxidative degradation studies pointed out that molnupiravir is less sensitive to oxidative degradation, as treatment with 3% H_2O_2 solution at room temperature, 40˚C (±1˚C), 60˚C (±1˚C), and 80˚C (±1˚C) for 1- or 2-hour caused as maximum as ~6% drug degradation. However, stress with 3% hydrogen peroxide at an extremely elevated temperature condition (100˚C ±1˚C)

for 1- or 2-hour caused over 10% degradation of molnupiravir (Table 5, Figure 9).

Photolytic degradation

An exposure of molnupiravir to UV light at a short wavelength caused only 2.5% degradation after three days. However, prolonged exposure to UV light appeared to cause degradation at higher extents, as an exposure for 5 days was shown to degrade approximately 13% of molnupiravir (Table 5, Figure 10).

Thermal degradation

Molnupiravir was found to be stable for 2 hours at 80°C (±1˚C). However, a further elevation of temperature to 100°C (±1˚C) tended to cause a significant degradation of molnupiravir, with an approximately 21% degradation by 2 hours (Table 5, Figure 11).

A forced degradation study of molnupiravir revealed that the drug is highly susceptible to degradation in basic conditions which suggests that during handling the drug in solution maintaining an acidic environment is desirable. In the case of photolytic degradation, it has been observed that short UV light exposure also causes the drug to be degraded crossing the acceptable limit, thereby suggesting the requirement of an appropriate packaging material while storing the drug to protect against photolytic degradation. And results of thermal degradation also postulate that the drug should not be exposed for a longer duration at high temperatures.

Analysis of commercial drug product

The method is applicable for the analysis of molnupiravir in marketed capsule dosage forms of 200 mg. The mean percentage assay results were found to be 99.75%. The results are summarized in Table 6.

Table 5. Degradation of molnupiravir in various stressed conditions.

Temp.	**Added ($\mu g.mL^{-1}$)**	**T (hr.)**	**Mean % degradation±%RSD**				
			0.1 N HCl	**0.1 N NaOH**	**3% H_2O_2**	**Thermal degradation**	**Photolytic degradation**
RT	10	1	0.011±0.57	Complete degradation	0.043±0.35	-	-
40˚C (±1˚C)		1	0.161±0.62		0.322±0.25	-	-
60˚C (±1˚C)		1	0.933±0.27		1.021±0.44	-	-
80˚C (±1˚C)		1	1.281±0.51		2.554±0.47	0.452±0.34	-
80˚C (±1˚C)		2	2.331±0.34		6.148±0.32	1.622±0.25	-
100˚C (±1˚C)		1	1.745±0.38		13.109±0.06	6.685±0.14	-
100˚C (±1˚C)		2	33.344±0.47		66.407±0.42	20.820±0.45	-
RT		1 day					0.621±0.25
		3 day					2.536±0.38
		5 day					13.158±0.57

Table 6. Assay of molnupiravir commercial product using the proposed RP-UHPLC method.

Type of Solution	**Amount Added**	**Peak Area**	**Amount Found ($\mu g.mL^{-1}$)**	**Drug Content (mg)**	**% Drug Content**	**% Drug Content (Mean±%RSD)**
Sample	10 $\mu g.mL^{-1}$	145719.9	9.991	199.382	99.69	99.75±0.32%
		146289.2	10.031	200.180	100.09	
		146003.3	10.011	199.781	99.89	
		144982.7	9.940	198.364	99.18	
		145696.4	9.989	199.342	99.67	
		146110.2	10.019	199.941	99.97	

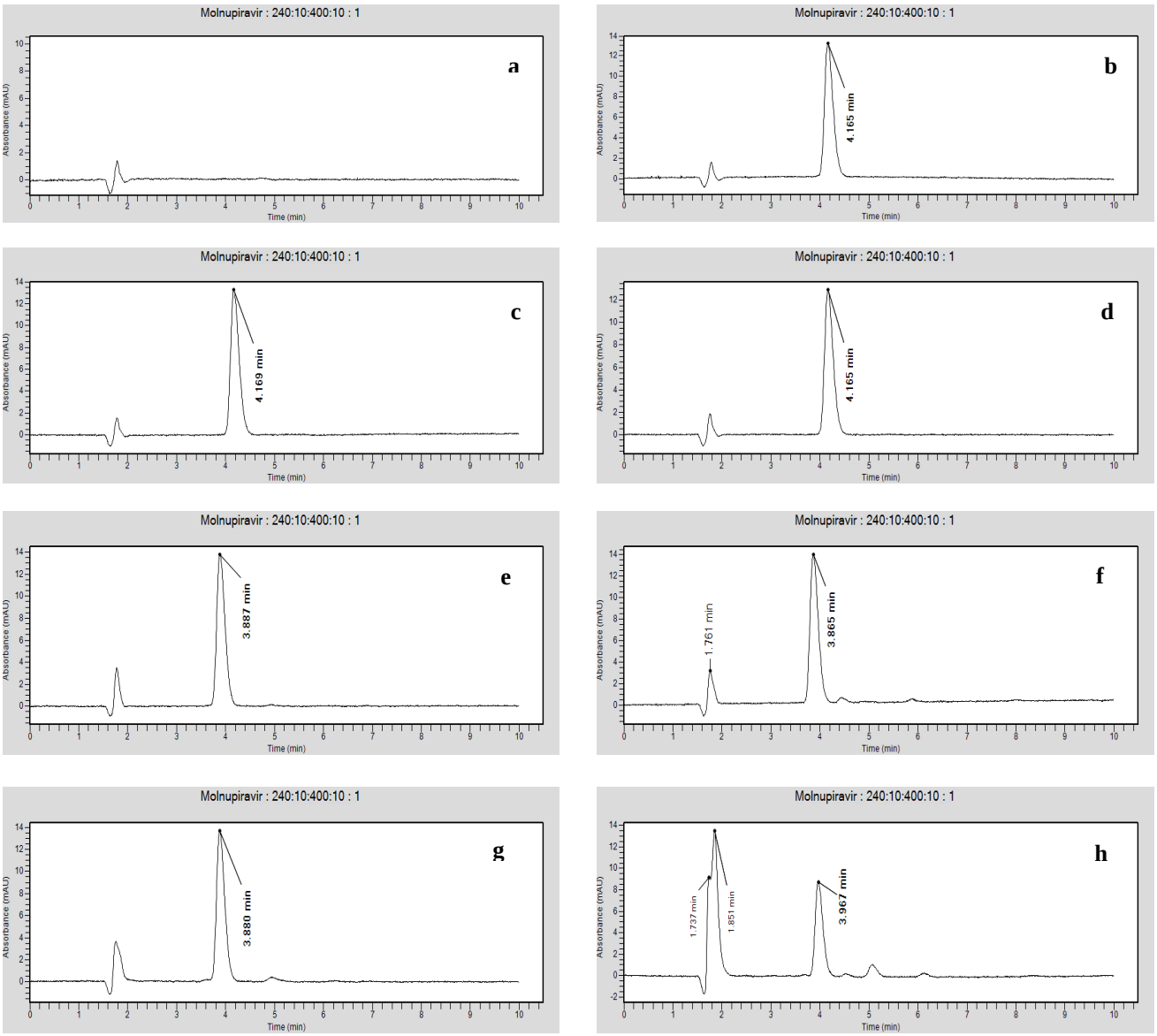


Figure 7. Hydrolytic degradation of molnupiravir in acidic conditions. (a) Chromatogram of 0.1 N HCl stressed blank sample; (b-h) Chromatogram of 0.1 N HCl stressed molnupiravir (10 µg.mL^{-1}) at (b) RT (± 1°C) for 1 hour, (c) 40°C (± 1°C) for 1 hour, (d) 60°C (± 1°C) for 1 hour, (e) 80°C (± 1°C) for 1 hour, (f) 80°C (± 1°C) for 2 hours, (g) 100°C (± 1°C) for 1 hour, and (h) 100°C (± 1°C) for 2 hours.

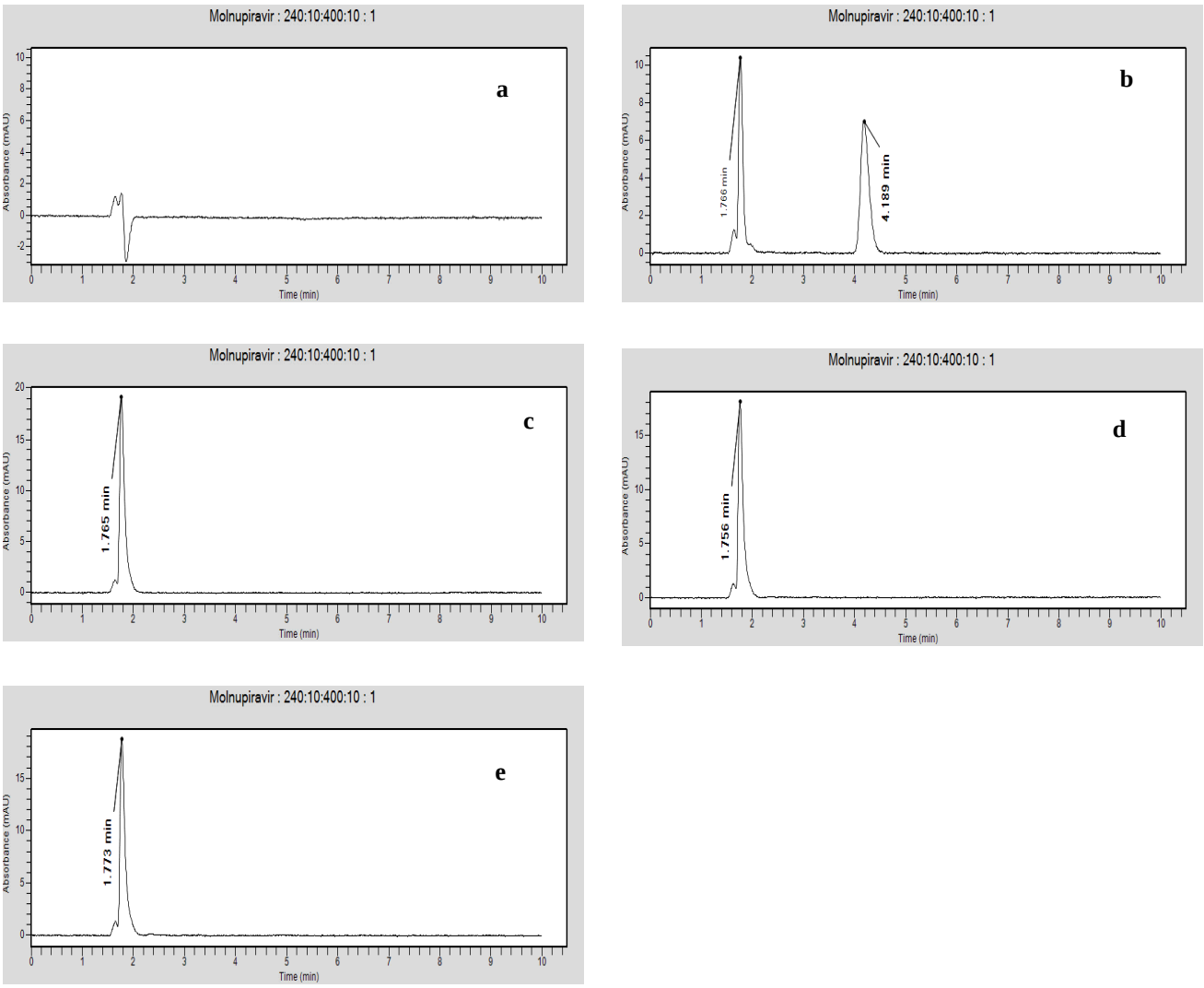


Figure 8. Hydrolytic degradation of molnupiravir in alkaline conditions. (a) Chromatogram of 0.1 N NaOH stressed blank sample; (b-e) Chromatogram of 0.1 N NaOH stressed molnupiravir (10 µg.mL^{-1}) at (b) RT (± 1°C) for 1 min, (c) RT (± 1°C) for 1 hour, (d) 40°C (± 1°C) for 1 hour, and (e) 60°C (± 1°C) for 1 hour

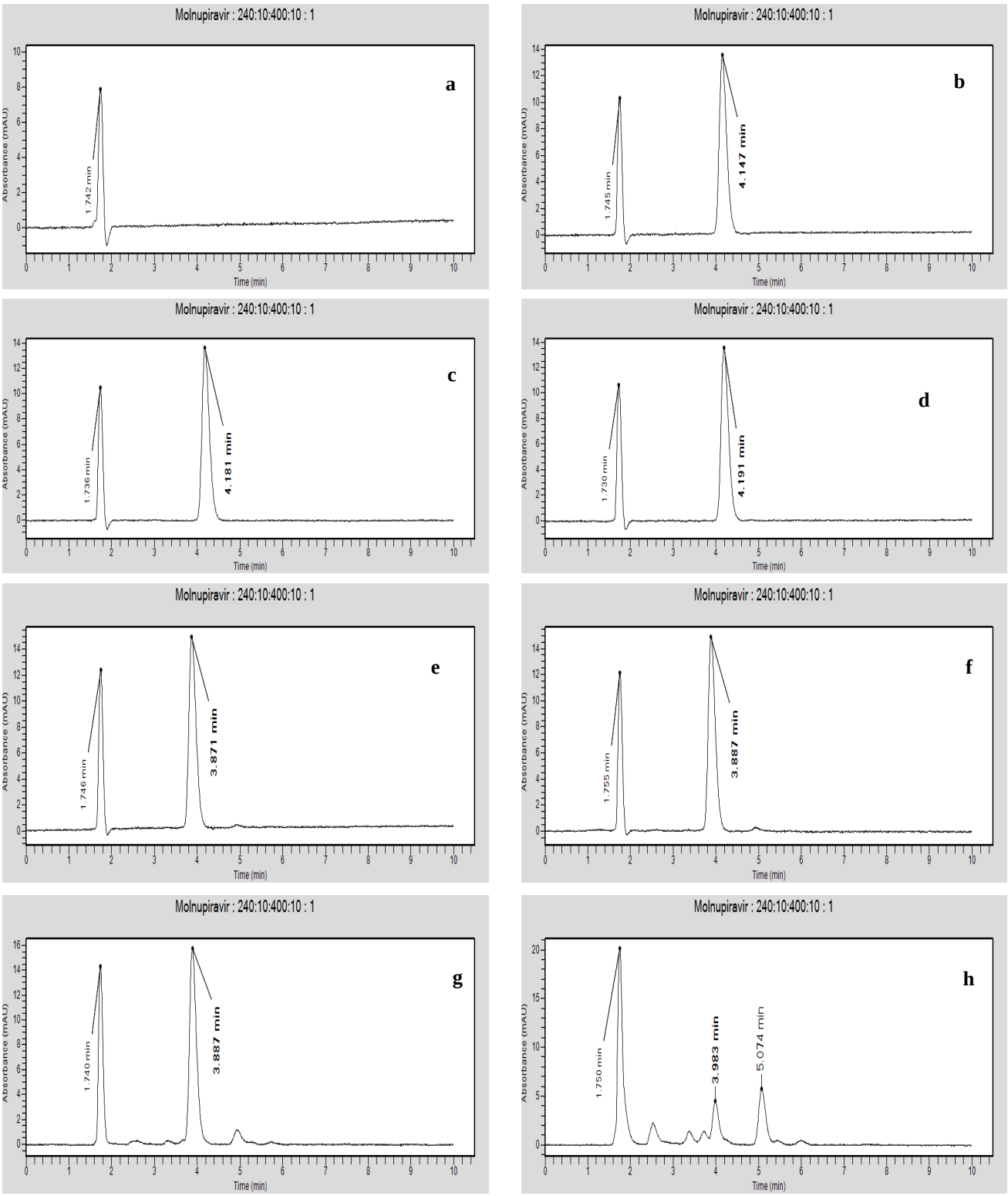


Figure 9. Oxidative degradation of molnupiravir in hydrogen peroxide solution. (a) Chromatogram of 3% hydrogen peroxide blank sample; (b-h) Chromatogram of 3% hydrogen peroxide stressed molnupiravir (10 µg.mL^{-1}) at (b) RT (± 1°C) for 1 hour, (c) 40˚C (± 1°C) for 1 hour, (d) 60˚C (± 1°C) for 1 hour, (e) 80˚C (± 1°C) for 1 hour, (f) at 80˚C (± 1°C) for 2 hours, (g) 100˚C (± 1°C) for 1 hour, and (h) 100˚C (± 1°C) for 2 hours.

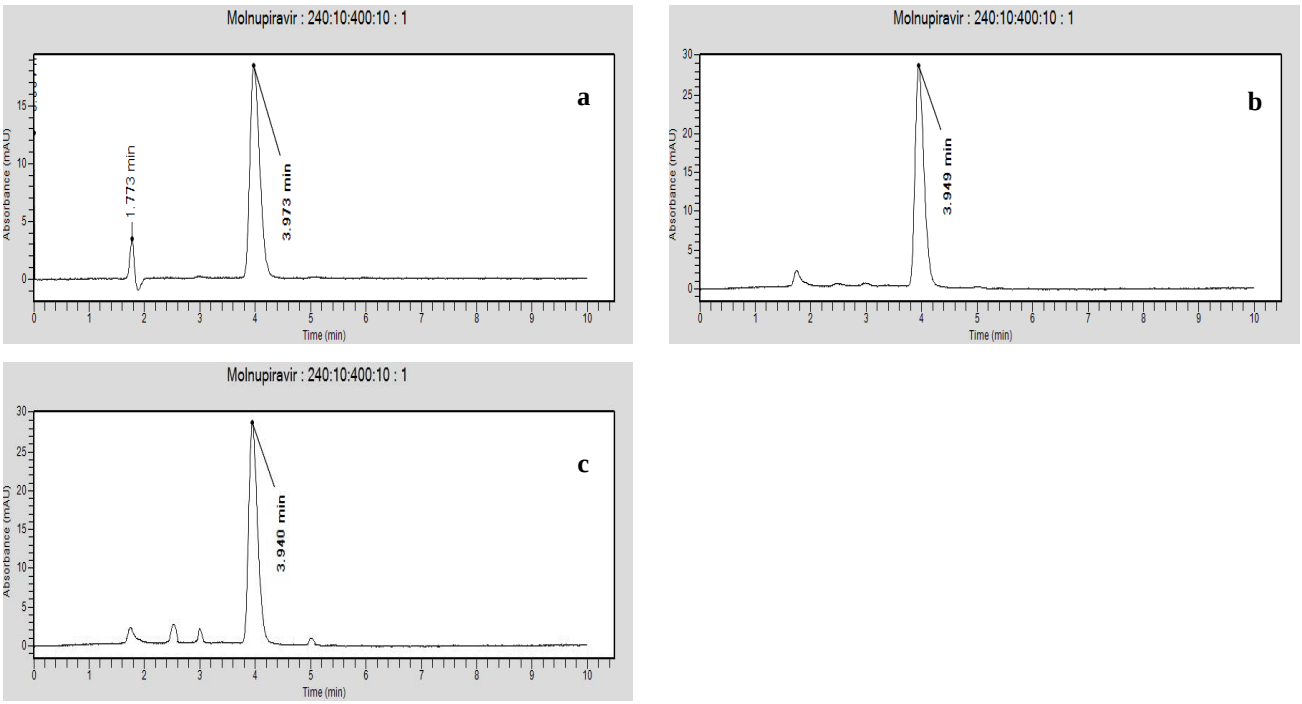


Figure 10. Photolytic degradation of molnupiravir under UV stress. Chromatogram of molnupiravir (10 µg.mL^{-1}) after exposure to UV light for (a) 1 day, (b) 3 days, and (c) 5 days.

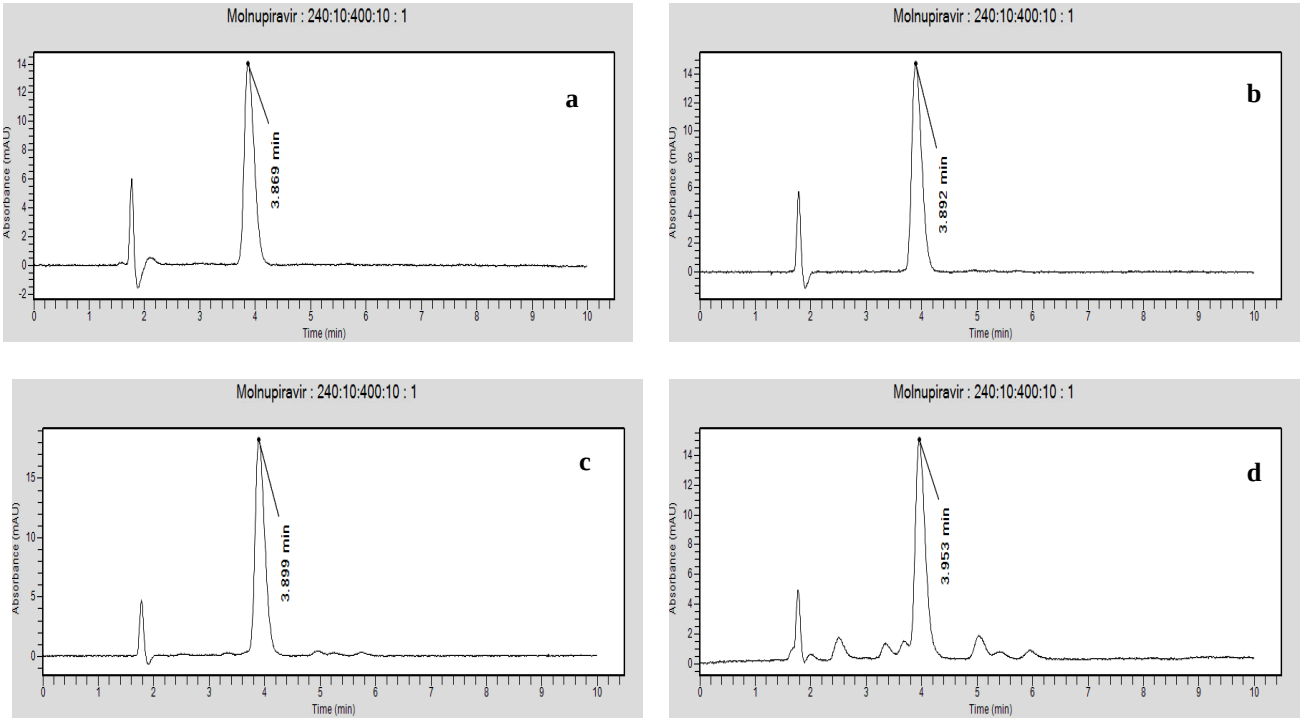


Figure 11. Thermal degradation of molnupiravir. Chromatogram of thermal stressed molnupiravir (10 µg.mL^{-1}) at (a) 80°C (± 1°C) for 1 hour, (b) 80°C (± 1°C) for 2 hours, (c) 100°C (± 1°C) for 1 hour, and (d) at 100°C (± 1°C) for 2 hours.

Conclusions

A rapid, sensitive, specific, and robust RP-UHPLC method has been developed employing a quality-by-design-based approach. The optimized method consisted of trifluoroacetic acid (0.1%) and methanol at a ratio of 65:35 (%, v/v) in the mobile phase with a flow rate of 1.3 $mL.min^{-1}$. Errors calculated from the experimental run and optimized method were within tolerance limits. So, this can be inarguably postulated that the studied analytical outcome can be utilized effectively for analyzing the content of molnupiravir in bulk drug and dosage forms. Moreover, the study also focused on the degradation behavior of molnupiravir in stressed conditions which will assist in handling and storing the drug to impede the degradation. As the data are very promising this developed method can be further recommended for studying molnupiravir content in an *in-vivo* model.

Acknowledgment

The authors acknowledge the contribution of Incepta Pharmaceuticals Limited (Dhaka, Bangladesh) for their kind donation of materials for conducting the research work.

Conflict of interest

The authors declare no conflicts of interest.

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