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Development and Validation of UV Method for Routine Analysis of Molnupiravir

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Abstract

A simple and cost-effective UV spectrophotometric method was developed and validated for the routine analysis of molnupiravir (MLP) using 10% (v/v) methanol as the solvent. The absorption maximum (λ_{max}) of molnupiravir was determined to be 238 nm. The method was validated according to ICH guidelines, and the results demonstrated its suitability, specificity, linearity, accuracy, precision, sensitivity, ruggedness, and robustness. The method showed a linear response within the concentration range of 2-20 µg/ml, with a correlation coefficient (R^2) of 0.9972. The accuracy, expressed as % recovery, ranged from 95.92% to 104.38%, while intra-day and inter-day precision studies yielded %RSD values within acceptable limits. The limits of detection (LOD) and quantification (LOQ) were found to be 2.42 µg/ml and 7.34 µg/ml, respectively. Ruggedness and robustness studies further confirmed the reliability of the method under varied conditions. Equivalency studies comparing the developed UV method with a reference RP-UHPLC method demonstrated comparable performance in terms of linearity, accuracy, precision, ruggedness and robustness, with statistical analysis supporting the equivalence of the two methods. The developed UV method offers a viable alternative for the routine analysis of molnupiravir, providing a balance between accuracy and simplicity.

Key words: Molnupiravir, UV, method development, validation, equivalency.

Introduction

Molnupiravir (MLP), an antiviral agent, has attracted considerable interest as a potential therapeutic for viral infections, especially in the context of the COVID-19 pandemic (Bernal *et al.*, 2022; Imran *et al.*, 2021; Pontolillo *et al.*, 2022). MLP functions as a prodrug of β-D-N4-hydroxycytidine, exerting its mechanism of action by inducing errors in the viral RNA synthesis process. This ultimately hinders the replication of the virus, effectively impeding its spread (Kabinger *et al.*, 2021; Mass *et al.*, 2024; Yip *et al.*, 2022). Given its clinical importance, the accurate and reliable quantification of MLP in pharmaceutical formulations is essential to ensure both its efficacy and safety.

UV spectrophotometry is a simpler, more cost-effective method that can provide rapid and reliable quantification of drugs without the need for complex equipment or extensive training (Bulduk and Akbel, 2021; Sahu *et al.*, 2018).

Validation is a critical step in the development of any analytical method, ensuring that the method is both reliable and suitable for its intended purpose. In

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pharmaceutical analysis, method validation confirms that the procedure is capable of producing consistent and accurate results within defined parameters (Araujo, 2009; Peters *et al.*, 2007; Shabir, 2003). This process typically involves the assessment of various analytical characteristics, such as linearity, accuracy, precision, specificity, robustness etc. as specified in the ICH Q2 (R1) guideline (ICH, 2005; Kazusaki *et al.*, 2012; Kollipara *et al.*, 2011).

Materials and Methods

Chemicals and reagents: Molnupiravir standard (potency = 99.82%) was graciously supplied by Incepta Pharmaceuticals Limited, Bangladesh. Analytical grade methanol (Merck KGaA, Germany) and molnupiravir tablets were procured from the local market. All solvents and chemicals used in this study were of analytical grade.

Method development: The UV method for the analysis of molnupiravir was developed through a series of trial-and-error experiments. Various solvent systems were tested and after careful consideration, 10% (v/v) methanol was selected as the optimal solvent for method development due to its compatibility and efficacy.

Standard solution preparation: 10 mg of molnupiravir standard was weighed accurately and transferred to a 100 ml volumetric flask. Initially, 10 ml of methanol was added to dissolve the drug, followed by dilution with distilled water to achieve a final volume of 100 ml. This preparation resulted in a concentration of 10 µg/ml, which was further diluted as necessary to obtain the desired working concentrations.

Blank preparation: 10% (v/v) methanol solution was prepared and used as a blank reference throughout the analysis.

Absorption Maximum (λ_{max}) determination: The absorption maximum (λ_{max}) was determined by preparing a 10 µg/ml solution of molnupiravir in methanol. This solution was scanned within a wavelength range of 200 to 800 nm using a Shimadzu 1800 UV-visible double beam spectrophotometer.

Method validation: The developed UV method was validated following the ICH Q2 (R1) guidelines, assessing various parameters as outlined below.

System Suitability: To evaluate system suitability, photometric measurements were conducted on MLP samples with a concentration of 10 µg/ml. The % RSD of the results was calculated to ensure the method's consistency and precision.

Specificity: The specificity of the method was determined using placebo tests. Placebo MLP tablets were prepared and processed in the same manner as the test samples, excluding the active pharmaceutical ingredient.

Linearity: The linearity of the UV method was assessed by preparing ten working solutions of MLP at varying concentrations (2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 µg/ml). Each concentration was analyzed in triplicate and a calibration curve was constructed using linear regression analysis.

Accuracy: Accuracy was evaluated by calculating the percentage recovery of known added concentrations of MLP standard across the concentration range of 2 to 20 µg/ml. Each concentration level was tested in triplicate.

Precision: The precision of the method was determined by analyzing MLP solutions with a nominal concentration of 10 µg/ml. Intra-day precision was assessed through six replicate analyses performed on the same day, while inter-day precision was evaluated by repeating the analysis over three consecutive days.

Sensitivity: The suggested method's sensitivity was estimated in terms of its limit of detection (LOD) and limit of quantitation (LOQ).

$$\mathrm{LOD} = 3.3 \times \frac{\mathrm{SD}}{\mathrm{S}} \text{ and, } \mathrm{LOQ} = 10 \times \frac{\mathrm{SD}}{\mathrm{S}}$$

Here, SD is the standard deviation of the y-intercept of the calibration curves and S is the slope of calibration curves.

Ruggedness: The ruggedness of the method was assessed by analyzing six MLP assay samples (10 µg/ml) using two different analysts. The % recovery

and % RSD were calculated to evaluate the reproducibility of the results.

Robustness: Robustness was examined by varying the detection wavelength (236 nm and 240 nm) and analyzing the effect on the method's performance. The % RSD was calculated for each wavelength variation.

Drug content analysis: Six tablets of molnupiravir (200 mg each) were triturated into a fine powder. A portion of the powder equivalent to 10 mg of MLP was taken for analysis. The absorbance of the sample solutions was measured using the developed UV method and the concentration of MLP was determined using the calibration curve. The % drug content was then calculated using the following formula.

$$\%\ \text{Drug content} = \frac{\text{Actual drug content}}{\text{Total drug amount taken}} \times 100\%$$

Equivalency studies: Equivalency studies were conducted to establish the equivalency of the developed UV method with an existing RP-UHPLC method (Faruk *et al.*, 2023). The percent recovery data were analyzed using Minitab statistical software (version 21.1.0, Pennsylvania, USA). Paired t-tests ANOVA and paired equivalence tests were performed to statistically validate the equivalency of the analytical methods.

Results and Discussion

Absorption maximum (λ_{max}) determination: The UV spectrophotometric analysis revealed that the maximum absorbance (λ_{max}) of MLP in 10% (v/v) methanol occurred at 238 nm. This finding is crucial as it establishes the wavelength at which the drug exhibits optimal absorbance, facilitating accurate quantitative analysis. The obtained UV spectrum for the molnupiravir standard solution is presented in figure 1.

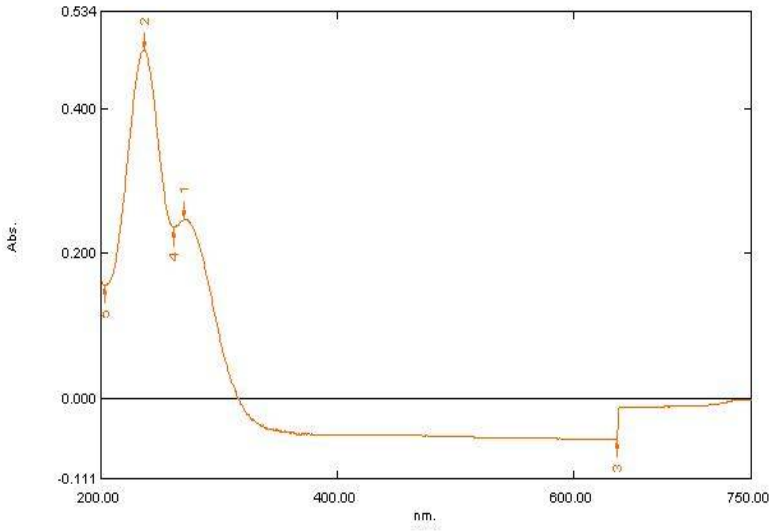

Figure 1. UV spectrum of molnupiravir standard solution (10 µg/ml).

Method validation: The developed UV spectrophotometric method was subjected to rigorous validation as per ICH guidelines. The method was found to be specific, linear, accurate, precise, sensitive, rugged and robust confirming its suitability for routine analysis.

System suitability: The results, summarized in table 1, indicate that the method exhibited consistent performance with a mean absorbance of 0.4748 and a relative standard deviation (% RSD) of 0.52%, well within the acceptable limit of % RSD ≤ 2%.

Specificity: The method specificity was validated by comparing the UV spectra of a placebo with that of the MLP standard solution. Figure 2 illustrates that there were no interfering peaks from the placebo, demonstrating that the method was specific to MLP.

Table 1. Results of system suitability study.

Parameter	Replicate	Value	Value (Mean ± % RSD)	Acceptable limit
Absorbance	R1	0.478	0.4748± 0.52%	% RSD ≤2
	R2	0.476		
	R3	0.473		
	R4	0.477		
	R5	0.472		
	R6	0.473		

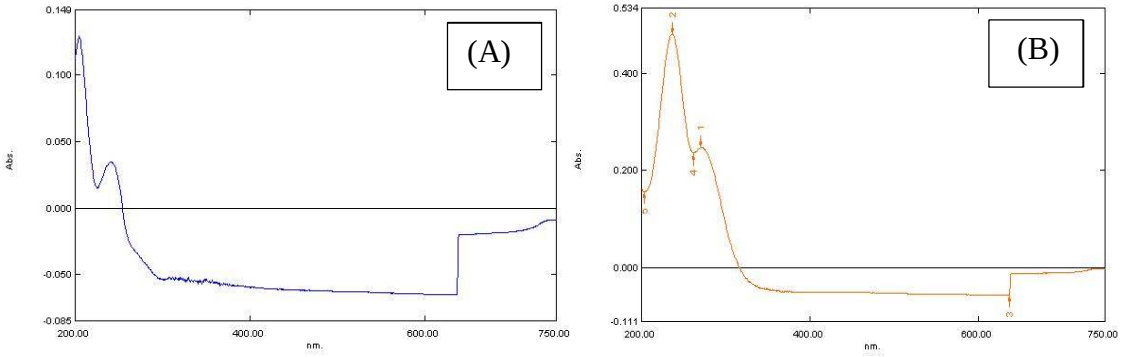

Figure 2. UV spectrum of (A) placebo and (B) molnupiravir standard solution (10 µg/ml).

Table 2. Results of linearity study.

Amount (µg/ml)	Absorbance	Absorbance (Mean ± % RSD)	Amount (µg/ml)	Absorbance	Absorbance (Mean ± % RSD)
2	0.030	0.0303±1.90	12	0.577	0.575 ± 0.20
	0.031			0.575	
	0.030			0.575	
4	0.125	0.123±1.23	14	0.661	0.661 ± 0.23
	0.122			0.660	
	0.123			0.663	
6	0.213	0.213±0.27	16	0.768	0.766 ± 0.27
	0.213			0.764	
	0.214			0.767	
8	0.375	0.374±0.31	18	0.880	0.881 ± 0.17
	0.375			0.883	
	0.373			0.882	
10	0.478	0.477±0.32	20	1.015	1.013 ± 0.19
	0.476			1.013	
	0.479			1.011	

Linearity: The method showed excellent linearity over the concentration range of 2-20 µg/ml, as evidenced by the calibration curve (Figure 3) and the linear regression equation y = 0.0542x-0.0845 with a correlation coefficient (R^2) of 0.9972. The consistent linear response across this concentration range is depicted in figure 4, confirming the method's suitability for quantitative analysis within this range.

Accuracy: The recovery values ranged from 95.92% to 104.13%, with % RSD values below 1%, indicating that the method is accurate and capable of quantifying MLP with high reliability (Table 3).

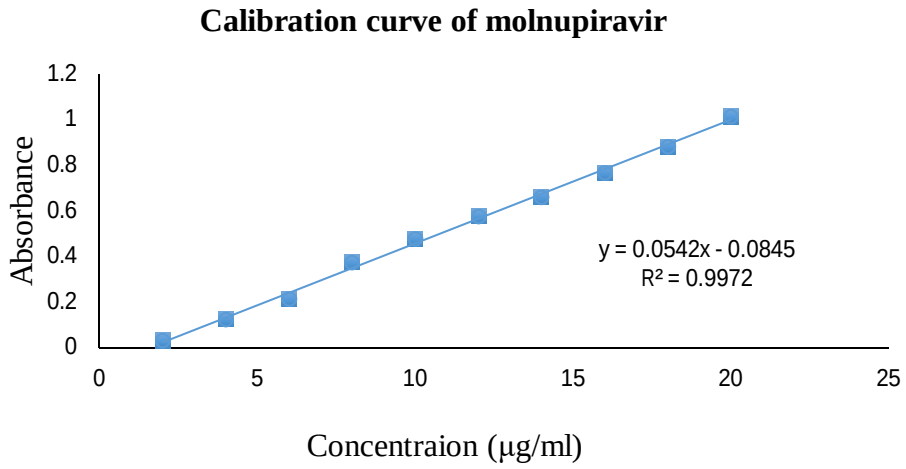

Figure 3. Standard curve of molnupiravir in the concentration range 2–20 µg/ml.

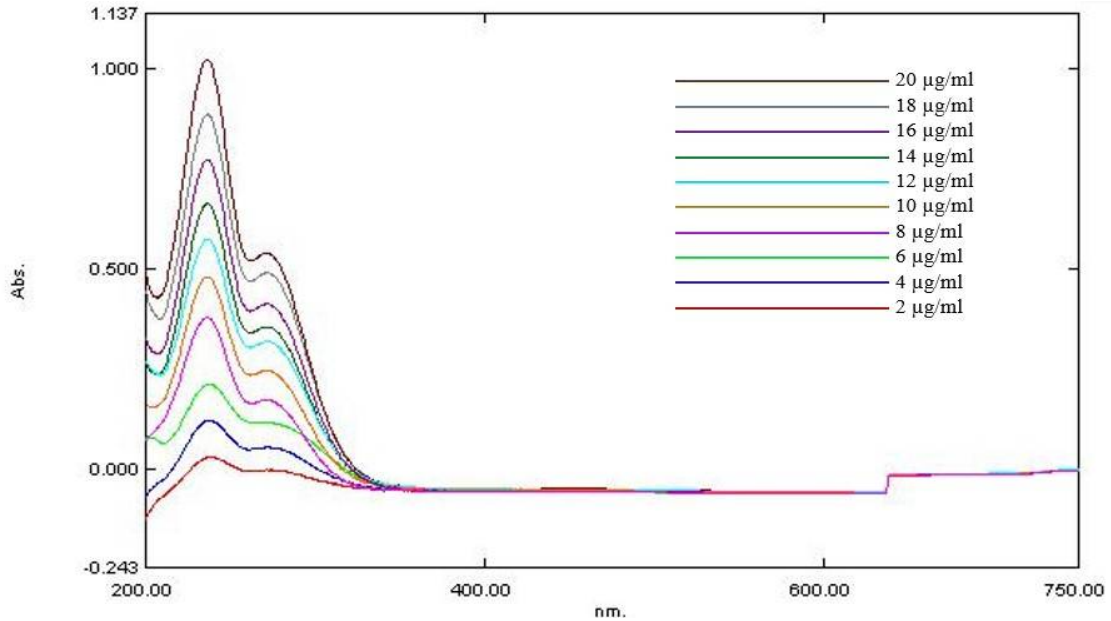

Figure 4. Overlay spectrum of molnupiravir in methanol at various concentrations.

Table 3. Results of accuracy study.

Amount added (µg/ml)	Replicate	Absorbance	Amount recovered (µg/ml)	% Recovery	% Recovery (Mean ± % RSD)
2	R1	0.026	2.04	102	102 ± 0.98
	R2	0.027	2.06	103	
	R3	0.025	2.02	101	
4	R1	0.125	3.87	96.75	95.92 ± 0.80
	R2	0.122	3.81	95.25	
	R3	0.123	3.83	95.75	
6	R1	0.227	5.75	95.83	95.94 ± 0.20
	R2	0.228	5.77	96.17	
	R3	0.227	5.75	95.83	
8	R1	0.368	8.35	104.38	104.13 ± 0.24
	R2	0.366	8.31	103.88	
	R3	0.367	8.33	104.13	
10	R1	0.478	10.38	103.8	103.72 ± 0.28
	R2	0.476	10.34	103.4	
	R3	0.479	10.396	103.96	
12	R1	0.577	12.20	101.67	101.50 ± 0.14
	R2	0.575	12.17	101.42	
	R3	0.575	12.17	101.42	
14	R1	0.661	13.75	98.21	98.26 ± 0.22
	R2	0.660	13.73	98.07	
	R3	0.663	13.79	98.50	
16	R1	0.768	15.73	98.31	98.10 ± 0.27
	R2	0.764	15.65	97.81	
	R3	0.767	15.71	98.19	
18	R1	0.880	17.79	98.83	99.02 ± 0.18
	R2	0.883	17.85	99.17	
	R3	0.882	17.83	99.06	
20	R1	1.015	20.29	101.45	101.25 ± 0.198
	R2	1.013	20.25	101.25	
	R3	1.011	20.21	101.05	

Precision: The method demonstrated excellent precision, both intra-day and inter-day, with % RSD values for recovery ranging from 0.61% to 0.90%, as shown in table 4. This consistency confirms the method's compatibility with routine analysis.

Sensitivity: The limits of detection (LOD) and limits of quantification (LOQ) were found to be 2.42 µg/ml and 7.34 µg/ml (Table 5), respectively.

Ruggedness: The recovery values obtained by analyst 1 and analyst 2 were 104.38% and 102.89%, respectively, with % RSD values well within acceptable limits (Table 6), demonstrating the method's ruggedness across different operators.

Robustness: Robustness was assessed by varying the detection wavelength (236 nm and 240 nm). The results, summarized in table 7, show that the method remains consistent under these conditions, with % RSD values within acceptable limits, confirming the method's robustness.

Drug content analysis: The percentage recovery of molnupiravir was determined to be 96.77%, as presented in table 8. This value is in accordance with the specifications outlined in pharmaceutical monographs, validating the method for content analysis.

Equivalency studies: ANOVA (table 9), paired t-test (table 10) and paired equivalence test (table 11) were performed to verify the statistical equivalency of the developed UV method with an established RP-UHPLC method in terms of % recovery (Faruk *et al.*, 2023). A one-way ANOVA yielded a p-value of 0.818, indicating that the means of both methods are statistically equivalent, with an R^2 value of 0.39% showing minimal variance explained by the model. A paired t-test further confirmed this, with a mean difference of 0.31 between the methods and a p-value of 0.803, suggesting no significant difference in recovery data. The equivalence test reinforced these findings, as the mean difference of -0.31 fell within the equivalence interval (-5, 5), and the confidence interval (-2.60, 1.98) supported equivalency. Both null hypotheses for the equivalence test were rejected with p-values below 0.05, confirming that the HPLC and UV methods are statistically equivalent for % recovery.

Table 4. Results of precision study.

Types of precision		Replicate	Absorbance	Amount recovered (µg/ml)	% Recovery	Average % recovery (Mean ± % RSD)
Intra day		R1	0.475	10.32	103.2	102.4 ± 0.61
		R2	0.472	10.27	102.7	
		R3	0.470	10.23	102.3	
		R4	0.469	10.21	102.1	
		R5	0.465	10.14	101.4	
		R6	0.472	10.27	102.7	
Inter day	Day 1	R1	0.470	10.23	102.3	101.95 ± 0.52
		R2	0.467	10.18	101.8	
		R3	0.464	10.12	101.2	
		R4	0.466	10.16	101.6	
		R5	0.472	10.27	102.7	
		R6	0.469	10.21	102.1	
	Day 2	R1	0.462	10.08	100.8	101.73 ± 0.66
		R2	0.465	10.14	101.4	
		R3	0.466	10.16	101.6	
		R4	0.470	10.23	102.3	101.32 ± 0.90
		R5	0.466	10.16	101.6	
		R6	0.472	10.27	102.7	
	Day 3	R1	0.460	10.05	100.5	100.28 ± 0.29
		R2	0.458	10.01	100.1	
		R3	0.461	10.06	100.6	
		R4	0.460	10.05	100.5	
		R5	0.457	9.99	99.9	
		R6	0.458	10.01	100.1	

Table 5. LOD and LOQ of molnupiravir.

Parameters	Value
Standard error of y-intercept (SE)	0.01259
Number of samples (N)	10
Standard deviation of y-intercept (SD)	0.0398
LOD	2.42 µg/ml
LOQ	7.34 µg/ml

Table 6. Results of ruggedness study.

Type of ruggedness	Replicate	Absorbance	Amount recovered (µg/ml)	% Recovery	% Recovery (Mean ± % RSD)
Analyst 1	R1	0.479	10.39	103.9	104.38 ± 0.34
	R2	0.480	10.42	104.2	
	R3	0.483	10.47	104.7	
	R4	0.480	10.42	104.2	
	R5	0.481	10.43	104.3	
	R6	0.484	10.49	104.9	
Analyst 2	R1	0.477	10.36	103.6	102.89 ± 0.76
	R2	0.472	10.27	102.7	
	R3	0.473	10.29	102.9	
	R4	0.479	10.39	103.9	
	R5	0.470	10.23	102.3	
	R6	0.468	10.19	101.9	

Table 7. Results of robustness study.

Parameters	Variations	Replicate	Absorbance	Amount recovered (µg/ml)	% Recovery	% Recovery (Mean ± % RSD)
Detection wavelength	236 nm	R1	0.455	9.95	99.5	99.73 ± 0.53
		R2	0.454	9.94	99.4	
		R3	0.455	9.95	99.5	
		R4	0.453	9.92	99.2	
		R5	0.460	10.05	100.5	
		R6	0.459	10.03	100.3	
	240 nm	R1	0.450	9.86	98.6	98.13 ± 0.37
		R2	0.449	9.84	98.4	
		R3	0.445	9.77	97.7	
		R4	0.448	9.82	98.2	
		R5	0.445	9.77	97.7	
		R6	0.448	9.82	98.2	

Table 8. Assay of molnupiravir.

Amount added (µg/ml)	Absorbance	Amount recovered (µg/ml)	% Recovery	% Recovery (Mean ± % RSD)
10	0.438	9.64	96.4	96.77 ± 0.33
	0.442	9.71	97.1	
	0.440	9.68	96.8	
	0.438	9.64	96.4	
	0.440	9.68	96.8	
	0.442	9.71	97.1	

Table 9. ANOVA for statistical analysis of the % recovery data.

One-way ANOVA method					
Null hypothesis			All means are equal		
Alternative hypothesis			Not all means are equal		
Significance level			$\alpha = 0.05$		
Analysis of variance					
Source	DF	Adj SS	Adj MS	F-value	P-value
Factor	1	0.391	0.3906	0.05	0.818
Error	14	99.870	7.1335		
Total	15	100.260			
Model summary					
S	R-sq		R-sq(adj)	R-sq(pred)	
2.67087	0.39%		0.00%	0.00%	
Means					
Factor	N	Mean	St Dev	95%CI	
HPLC	8	100.259	1.812	(98.223,102.284)	
UV	8	99.95	3.31	(97.92, 101.97)	
Pooled St Dev	2.67087				

Table 10. Paired t-test for statistical analysis of the % recovery data.

Descriptive statistics				
Sample	N	Mean	StDev	SE Mean
HPLC	8	100.26	1.81	0.64
UV	8	99.95	3.31	1.17
Estimation for paired difference				
Mean	StDev	SE Mean	95% CI for µ_difference	
0.31	3.42	1.21	(-2.54, 3.17)	
Test				
Null hypothesis		H_0:µ_difference=0		
Alternative hypothesis		H_1:µ_difference≠0		
T value	P-value		Remarks	
0.26	0.803		Claimed equivalent	

Table 11. Paired equivalence test for statistical analysis of the % recovery data.

Equivalence test with paired data: HPLC, UV method				
Test mean = mean of UV				
Reference mean = mean of HPLC				
Descriptive statistics				
Variable	N	Mean	StDev	SE Mean
UV	8	99.946	3.3143	1.1717
HPLC	8	100.26	1.8119	0.64059
Difference: Mean (UV) – Mean (HPLC)				
Difference	StDev	SE	95% CI for equivalence	Equivalence interval
-0.31250	3.41634	1.2079	(-2.60088, 1.97588)	(-5,5)
CI is within the equivalence interval. can claim equivalence				
Test				
Null hypothesis:			Difference ≤ -5 or Difference ≥ 5	
Alternative hypothesis:			-5 < Difference < 5	
α level:			0.05	
Null hypothesis	DF		T-value	P-value
Difference ≤ -5	7		3.8808	0.003
Difference ≥ 5	7		-4.3983	0.002
The p-values for both null hypotheses are less than 0.05, both of the null hypotheses are rejected. Can claim equivalence.				

Conclusion

The developed UV spectrophotometric method for the analysis of molnupiravir was thoroughly validated and found to be specific, linear, accurate, precise, sensitive, rugged and robust. The method is suitable for routine quality control of molnupiravir in pharmaceutical formulations.

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