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RESEARCH ARTICLE

A Validated High-Pressure Liquid Chromatography (HPLC) Method for Molnupiravir

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ABSTRACT
Molnupiravir is an approved antiviral drug that inhibits RNA replication of viruses. It is approved recently (November 2021) for the treatment of infectious diseases caused by SARS-CoV-2. However, it is reported that Molnupiravir is a nucleoside analogue and shown that nucleoside analogues are reagents that resemble the structure of natural nucleosides. It is widely applied in antiviral and anticancer therapy. Therefore, there is a general need appeared for an efficient, fast, simple, and validated analytical assay method for the quantification of this new product in any samples. We report here that an HPLC method was developed, optimized, and validated for molnupravir. The developed method was found to be easy to apply, cheap, and time-saving. Isocratic elution with 10mM Phosphate Buffer pH:7 / Acetonitrile mixture (80:20) with a Phenomenex C18 column was performed and a photodiode array detector was used for the detection. The total time for the analysis was only 10 minutes. The method was found to be selective, linear, accurate, reproducible, and robust. This proposed method can be easily adapted for molnupravir analyses in pharmaceutical products or in any other biological or non-biological samples.

Key words: Molnupiravir, Raw material, HPLC, Validation

1. Introduction

Molnupiravir is a RNA replication inhibitor and an antiviral drug receiving first approval in UK for the treatment of severe acute respiratory syndrome coronavirus (SARS- CoV-2) by 4th of November, 2021. It acts similar to Remdesivir [1,2]. According to Phase 2 and Phase 3 clinical study results, it was generally well tolerated and less adverse events were reported which were found to be not dose related [1,3]. The drug is administered orally and is more advantageous than other medications used for SARS CoV-2 infections since it is manufactured on a large scale. Although Molnupiravir is known to be effective on viruses, it is very recently reported that it is also a nucleoside analogue [4]. Nucleoside analogues and nucleobases are a pharmacologically diverse family, which includes cytotoxic compounds, antiviral agents, and immunosuppressive molecules. It is also shown that nucleoside analogues are reagents that resemble the structure of natural nucleosides. It is widely applied in antiviral and anticancer therapy [4,5]. Therefore, there is a general need appeared for an efficient, fast, simple, and validated analytical assay methods for the quantification of new products. Patients don't require hospitalization if treated with molnupravir. Molnupiravir is not temperature sensitive and does not need to be transferred with cold chain delivery. This can be accepted as one of the indications for stability of the compound meaning that analysis method can be developed at room temperature. Chromatographic methods are widely used for the purification of analytes or for any analytical purposes. Molnupiravir is a new active ingredient that definitely finds wide use in the world as long as viral infections continue. There is no analytical method available in the literature for the quantification of molnupiravir in pharmaceutical raw materials. Determinations of the drug itself and its impurities or drug degradation products are very important from both pharmacological and toxicological perspectives for scientists and the drug industry [6,7]. The establishment of monitoring methods for stability tests on raw materials, their impurities, and the degradation products during pharmaceutical development is necessary because of their potential toxicity. Simultaneous or direct determinations are more attractive if the application of the method is easy and adoptable [6,7]. An impurity in the drug mass is any chemical component originated from the drug substance or during the synthesis. The safety of the drug is dependent not only on the toxicological properties of the active substance itself but also on its pharmaceutical impurities. It is important to be detected and quantitate them on raw pharmaceutical material and degradation products can be formed during the formulation manufacturing process and/or storage of raw drug substances or formulated products. Pharmaceutical impurities also referred to as 'related substances', could often have pharmacological or toxicological relevance. Therefore, the presence of such impurities and their levels in products are indicators of product quality, which can impose a risk to patient safety [8-10].

There is a Liquid Chromatography with tandem mass spectrometry (LC-MS/MS) method for the analytical determination of molnupiravir in biological samples, and there is a Reverse Phase High-performance liquid chromatography (RP-HPLC) was also method developed for the determination of molnupiravir in transport mediums [11,12]. There is still a need to develop analytical methods for the determination of molnupiravir in samples because molnupiravir is already approved in many countries for the use in SARS Co-V-2 infections.

High-performance liquid chromatography (HPLC) methods are useful for the determination of drugs and their degradation products or impurity products in raw materials or pharmaceutical dosage forms. Since HPLC has been used widely in routine analysis of raw materials or drug during acceptance of materials, pre-formulation, quality controls, and the storage it is important to develop an HPLC method that is thoroughly validated [13-15]. Here we report an easy, cheap HPLC analytical method for quantification of molnupiravir in raw materials which can be completed in a short time. The method was validated according to ICH; Validation of Analytical procedures: Text and Methodology [16]. Specificity, linearity, recovery, precision (system precision, method precision, and intermediate precision), robustness, solution stability, and system suitability parameters were determined. All results obtained were within the limits and can be used for their intended purpose.

2. Materials and methods

2.1. Chemicals

Molnupiravir supplied from Atabay (Kocaeli, Turkey). Analytical grade water, Acetonitrile (Isolab, Istanbul, Turkey), Potassium hydroxide (Isolab, Istanbul, Turkey), and Potassium Dihydrogen Phosphate (Merck, Merck KGaA, Darmstadt, Germany) were used. The internal standard supplied from Atabay (Kocaeli, Turkey). Other chemicals and ingredients were of analytical grade.

2.2. HPLC

Phenomenex C18 column (150 x 4.6 mm, 3µm) (CA, USA) was used for the separation of compounds in HPLC (Shimadzu, Maryland, USA) analysis with photodiode array detector. Run time was 10 min with 1.0 ml/min. flow rate, column temperature was set to 25°C. The analytical detection was achieved with the isocratic elution at the wavelength of 230 nm.

2.3. Assay

Molnupiravir standard and sample solutions were prepared by dissolving molnupiravir in distilled water at a concentration of 0.1 mg/ml and used as a working standard. Samples were diluted in a same manner. 10mM Phosphate Buffer pH:7 / Acetonitrile mixture (80:20) was used as a mobile phase. 10µl of were injected into the chromatographic system several times (6 times). System compatibility was checked with consecutive runs/readings of the standard solution.

2.4. Validation Parameters

Molnupiravir assay on raw materials for validation was carried out according to ICH guidelines; Validation of Analytical Procedures: Text and Methodology. Specificity, linearity, recovery, precision (system precision, method precision and intermediate precision), robustness, solution stability and system suitability parameters were tested.

2.4.1. Specificity

The specificity is the ability to measure the analyte of interest accurately and specifically in the presence of other components that may be expected to be present in the sample matrix [16]. Mobile phase, standard and sample solutions were prepared and analyzed as in the analytical method. In the mobile phase and dilution solution of chromatograms, any peak near to the retention time of the active substance should not significantly affect the analysis result. Retention time of molnupiravir is 4.5 minute. Samples or standard solutions concentration were 0.1 mg/ml.

2.4.2. Linearity

The linearity of the analytical method is results of linear regression of concentration and related absorbances of the active substances in the samples [16]. The molnupiravir standards were prepared as 10%, 25%, 40%, 50%, 80%, 100%, 150% of the standard solution. Three replicates of solutions were subjected to the analyses. Correlation coefficient was then determined. Stock solution concentration was 0.5 mg/ml. Correlation coefficient represented as r2 (it should be > 0.995). RSD% value of the peak areas for each concentration should be ≤ 1.0. The intercept value should be less than 5% of the average areas.

2.4.3. Recovery

The recovery (accuracy) of the analytical method is expressed as the convergence of the prepared concentration of the substance to be analyzed and the concentration of the resultant assay [16]. Recovery studies were carried out in duplicate of solutions containing 50%, 100%, and 150% of the Molnupiravir. % Recovery was then calculated. % Recovery should be 98.0% - 102.0%. RSD% of the calculated results from the recovery studies should be ≤ 1.0.

2.4.4. Precision

The precision of an analytical method is the amount of scattering in the results obtained from multiple analyzes of a homogeneous sample [16]. System precisions, repeatability, intermediate precisions were evaluated.

System Precision

System precision is a measure of system performance, regardless of errors from sample preparation. 6 consecutive injections of standard solutions were performed. The mean of the areas, standard deviation (SD) and percentage relative standard deviation (RSD%) were calculated. RSD% of the areas of standard solutions should be ≤ 1.0.

Intermediate Precision

The intermediate precision study is to analyze the same homogeneous sample on different days, on different devices and by different analysts to see the effect of random changes in the laboratory on the precision of the analytical method. In the same laboratory, six different 100% molnupiravir test solutions were prepared on different days and by a different analyst. Samples were tested same column with different serial numbers and with different equipment. The RSD should be ≤ 2.0.

2.4.5. Repeatability

Evaluation of the system performance to determine sample preparation errors were done [16]. Analyzing of six samples from the same batch of molnupiravir raw material using the developed method described above were performed. Molnupiravir sample concentration was adjusted to 0.1 mg/ml. The RSD of the results should be ≤ 2.0.

2.4.6. Robustness

The robustness of an analytical method determines the parameters of the method remain unaffected by small changes and it is an indicator of the reliability of the analytical method [16]. Some alterations will be made in the method parameters and the effects of these changes on system suitability parameters and test results will be examined.

The Effect of the Temperature

Column/oven temperature is set to ± 5°C and samples were stores at different temperatures (20°C - 25°C - 30°C) and analyzed. 6 subsequent injections into the system from the standard solution were done and alterations were determined.

The Effect of Flow Rate

Flow rate is set to 0.2 ml /min. and minor changes were made (0.8 ml / min. – 1.0 ml / min. – 1.2 ml / min.). Standard solutions were prepared as described in the method. 6 subsequent injections were done and alterations were calculated and compared. RSD of the results should be ≤ 2.0.

2.4.6. Stability of the working solutions

The effect of the storage period on the molnupravir were investigated and determined under various conditions. If the active substance breaks down during this period and storage conditions, these conditions are limited and should be specified in the method. Standard and test solutions were prepared according to the method and kept at room temperature for at least 24 hours and analyzed. RSD of the results should be ≤ 1.0.

3. Results

The retention time of molnupravir was 4.5 minutes. Different columns with different polarities were tried. System for standard solutions was accepted as suitable when the relative standard deviation between readings did not exceed 1%, tailing factor was less than 1.5, and theoretical plate number was higher than 5000.

3.1. Specificity

The method was found to be solely targeted to Molnupiravir. Mobile phase and diluted solutions chromatograms, any peak at or around the retention time of the active substance did not significantly affect the analysis result.

3.2. Linearity

After 3 consecutive analyses of 7 different concentration, the correlation coefficient (r^2) was higher than 0.995. Linearity study results are summarized in Table 1; RSD% values of the areas for all were less than 1.0 and the intercept values were less than 5% of the average area for the 100% molnupravir.

Table 1. Molnupiravir Linearity Table

Theoretical Concentration %	Concentration (mg/ml)	Peak Areas	Average Area	SD	RSD%
10.0	0.010	589756	589477	474.4961	0.08
		589457			
		588969			
		588897			
		589659			
		590125			
25.0	0.025	1484129	1484617	2349.3252	0.16
		1482550			
		1487172			
40.0	0.040	2260841	2261024	204.5711	0.01
		2261245			
		2260987			
50.0	0.050	2959157	2958796	580.8192	0.02
		2959105			
		2958126			
80.0	0.080	4714802	4714698	149.2414	0.00
		4714527			
		4714765			
100.0	0.100	5893630	5918510	13432.8784	0.23

		5918585			
		5931111			
		5916917			
		5921754			
		5929062			
150.0	0.150	8900150	8901341	778.4717	0.01
		8902144			
		8902213			
		8900912			
		8901415			
		8901211			

3.3. Recovery

Recovery studies were carried out in duplicate injections of three different concentrations of standard solutions. Recovery study results are summarized in Table 2. Recovery% values were between 98.0% - 102.0% and RSD values were not more than 1.0.

Table 2. Recovery Results for Molnupiravir

% Molnupiravir Level	Theoritical Concentration of Molnupiravir (mg/ml)	Experimental Concentration of Molnupiravir (mg/ml)	% Recovery Level	Mean Recovery
50%	0.050	0.049985	99.97	99.97
	0.050	0.049985	99.97	
	0.050	0.049990	99.98	
100%	0.100	0.10013	100.13	100.12
	0.100	0.10011	100.11	
	0.100	0.10012	100.12	
150%	0.150	0.14999	99.99	99.98
	0.150	0.14997	99.98	
	0.150	0.14997	99.98	
Mean				100.03
SD				0.07
RSD%				0.07

3.4. Precision

Intermediate precision, System precision and repeatability were determined. Mean areas, standard deviation (SD) and percentage of relative standard deviation of 6 consecutive injections of the standard solution (RSD%) were calculated for the system precision. Intermediate precision was calculated using the same laboratory, the same column with different serial numbers and different equipment's on different days by different analyst. Six replicates of molnupravir test solutions were prepared and analyzed. The repeatability values of six samples from the same batch of molnupravir raw material were analyzed. RSD% for system precision was not more than 1.0 and RSD% for repeatability and intermediate precision was not more than 2.0. Table 3, Table 4 and Table 5 summarizes system precision, repeatability and intermediate precision respectively.

Table 3. System Precision for Molnupiravir

Number of Test Samples	Retension Time	Peak Areas	Tailing Factor	Theoretical Plates
1	4.521	5893630	1,153	66346
2	4.529	5918585	1,153	66915
3	4.527	5931111	1,152	67231
4	4.536	5916917	1,151	67058
5	4.535	5921754	1,152	67220
6	4.524	5929062	1,154	66841
Mean	4.529	5918510	1,152	66935
SD	0.006	13432	0,001	328,6
RSD%	0.14	0.23	0,09	0,49

Table 4. Repeatability Results for Molnupiravir Raw Material

Number of Test Samples	Peak Areas	Assay %	Assay Average %
1	5893280	100.11	100.05
	5885627	99.98	
2	5894457	100.13	100.06
	5886216	99.99	
3	5892691	100.10	100.11
	5893280	100.11	
4	5894541	99.97	99.97
	5891442	99.97	
5	5891179	99.98	100.05
	5891972	100.11	
6	5891617	100.12	100.10
	5892389	100.08	
Mean	5891558	100.05	100.05
SD	2848.59	0.07	0.05
RSD%	0.05	0.07	0.05

Table 5. Intermediate Precision Results for Molnupiravir Raw Materials

Sample	Analyst I % Molnupiravir	Analyst II % Molnupiravir
1	100.05	99.97
2	100.06	99.96
3	100.11	100.05
4	99.97	100.09
5	100.05	99.99
6	100.10	99.98
Average %	100.05	100.01
SD	0.05	0.05
RSD%	0.05	0.05
RSD% (n=12)	0.05	

3.5. Robustness

Column temperature and the flow rate were changed and RSD% of the results were less than 2.0. Table 6-9 summarizes the results.

Table 6. Column Temperature Effect 20 °C Column Oven Temperature

Sample	Assay %
20 °C Column Oven Temperature S-1	99.98
20 °C Column Oven Temperature S-2	99.97
20 °C Column Oven Temperature S-3	99.98
25 °C Column Oven Temperature	100.06
Mean	100.00
SD	0.04
RSD%	0.04

Table 7. Column Temperature Effect 30 °C Column Oven Temperature

Sample	Assay %
30 °C Column Oven Temperature S-1	99.99
30 °C Column Oven Temperature S-2	99.98
30 °C Column Oven Temperature S-3	100.01
25 °C Column Oven Temperature	100.06
Mean	100.01
SD	0.04
RSD%	0.04

Table 8. Flow Rate Change 0.8 mL /min

Sample	Assay %
Flow Rate 0.8 mL /min S-1	99.96
Flow Rate 0.8 mL /min S-2	99.96
Flow Rate 0.8 mL /min S-3	99.97
Flow Rate 1.0 mL /min	100.06
Mean	99.99
SD	0.05
RSD%	0.05

Table 9. Flow Rate Change 1.2 mL /min

Sample	Assay %
Flow Rate 1.2 mL /min S-1	99.95
Flow Rate 1.2 mL /min S-2	99.96
Flow Rate 1.2 mL /min S-3	99.94
Flow Rate 1.0 mL /min	100.06
Mean	99.98
SD	0.06
RSD%	0.06

3.6. Stability of the working solution

Standard and test solutions were prepared and tested t at room temperature for at least 24 hours and analyzed. RSD% of the results was not more than 1.0. Solution stability test results are summarized in Table 10.

Table 10. Solution Stability Results

Time Intervals (hours)	Retention Time	Peak Areas
0	4.52	5886125
2	4.52	5887521
4	4.53	5887432
6	4.52	5886942
8	4.53	5886704
10	4.53	5887469
15	4.52	5887529
20	4.52	5887109
24	4.52	5887395
Mean	4.52	5887136
SD	0.01	476
RSD%	0.11	0.01

4. Discussion

The retention time of molnupravir was found to be fast enough for rapid analysis. (4.5 minutes). Different columns did not give variable results and it was accepted as suitable (relative standard deviation < 1%, tailing factor < 1.5, and theoretical plate number > 5000). The method was found to be specific for Molnupiravir. Mobile phase and other solutions did not give any peak at or around the retention time of the active substance and all these did not significantly affect the analysis. The calibration curve was linear with 7 consecutive concentrations of molnupravir and the correlation coefficient r^2 was higher than 0.995. The RSD% values of the areas were less than 1.0. Recovery values were between 98.0% - 102.0% and RSD values were not more than 1.0. Intermediate precision and repeatability values found to be suitable. The proposed method was determined to be robust and stable for more than 24 hours.

Molnupiravir can be analyzed using this simple "inverse gradient" HPLC method in the literature [17]. This is offered as an orthogonal approach for a Reversed Phased Method and will show various polar impurities not detected by Reversed Phase HPLC. Cogent Diamond Hydride™, 4µm, 100Å column with a dimensions of 4.6 x 75mm was used and the mobile phase was consisted of (95:5) Acetonitrile / DI Water having 0.1% formic acid. Detection was carried out at 254nm. Molnupiravir is dissolved at a concentration of 0.5mg / mL and used as a sample. Although the method was proposed to be easily adaptable it was still rather expensive than our method. Another method similar to proposed method was published recently [18] showing rapid analysis and precise determination. It proposes a rapid analysis but the column was heated up to 30°C and it is not solely for raw material.

5. Conclusion

The developed method was found to be specific for Molnupiravir. The mobile phase and diluted solutions were not representing any significant interfering peak. Correlation coefficient was r^2 =0.9998, and recovery was 100.03%. RSD% was 0.23, and 0.05 for system precision, repeatability and reproducibility respectively. A little change in parameters such as oven temperature and flow rate did not affect the results. Standard solution was found to be stable at 25 °C for 24 hours. RSD% was 0.01. RSD% for system suitability was 0.23. Finally, the developed method is fully validated and can be used as a cheap, easy, and time saving method for analysis of Molnupiravir raw material. This proposed method can be easily adapted for molnupravir analyses in pharmaceutical products or in solutions or in biological samples.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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