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ORIGINAL ARTICLE

Molnupiravir detection by tandem mass spectrometry

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ABSTRACT

Background: After the COVID-19 epidemic 2019, studies on antiviral drugs accelerated. In clinical studies with both re-purposed and newly discovered drugs, the need for reliable methods that measure drug levels in the blood has increased. Molnupiravir is one of the drugs considered under the treatment of COVID-19 and is on the agenda with conflicting findings. However, limited validated methods report the measurement of molnupiravir levels. Therefore, our aim in this study was to develop a practical, robust, validated tandem mass spectrometric method that allows measuring molnupiravir levels.

Methods: Method development studies for the measurement of molnupiravir levels were performed with a liquid chromatography-tandem mass spectrometry (LC-MS / MS) device, and the method was validated according to CLSI (The Clinical & Laboratory Standards Institute) and FDA (Food and Drug Administration) protocols. Linearity, recovery, precision, stability, matrix effect, carry-over, and lower limit determination studies were performed.

Results: The method was linear with a correlation coefficient value of 0.993 in the 20 ng/mL-20 μg/mL range. The sensitivity of the method was 20 ng/mL. The CV% obtained from the intra- and inter-assay studies was below 6.2%, and the mean recovery was over 95%. The total analysis time was 5 minutes for each sample.

Conclusion: A simple, cost-effective, reliable tandem mass spectrometric method with high sensitivity and accuracy based on protein precipitation alone has been developed to measure molnupiravir levels.

Keywords: COVID-19, molnupiravir; pandemic; tandem mass spectrometry; drug monitoring.

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INTRODUCTION

COVID-19 disease, caused by the SARS-CoV-2 virus, has been associated with a significant increase in mortality and morbidity worldwide since 2019 and was declared a pandemic by the World Health Organization in 2020 [1]. The course of COVID-19 ranges from asymptomatic to life-threatening events. Early intervention is crucial, especially for asymptomatic and mild patients, while effective oral antiviral drugs are needed to reduce serious morbidities, hospitalizations, and mortality in severe clinical courses [2, 3]. During the COVID-19 pandemic, approved antivirals and candidates with broad-spectrum antiviral activity have been re-purposed, and studies for developing new molecules have accelerated [4]. Although some broad-spectrum antiviral drugs such as remdesivir, chloroquine, and favipiravir played an essential role at the beginning of the pandemic, studies on the development of small molecule, oral antiviral drugs targeting SARS-CoV-2 have accelerated with the understanding of COVID-19 [5]. Molnupiravir is a small-molecule oral antiviral prodrug effective against Sars-Cov-2 and other RNA viruses. Molnupiravir was developed by Merck and Ridgeback Biotherapeutics for the prevention and treatment of COVID-19 [6].

Molnupiravir is converted to the ribonucleoside analog N-hydroxycytidine (NHC) by host esterases in the plasma. NHC enters the systemic circulation and is intracellularly phosphorylated to NHC triphosphate. NHC triphosphate is incorporated into viral RNA by viral RNA polymerase and then misdirects viral polymerase to incorporate guanosine or adenosine during viral replication. Thus, it causes a series of lethal mutations in the viral genome that render the virus non-infectious. Results of the MOVE-OUT Phase 3 trial, published in 2021, reported a significant reduction of hospitalization and mortality in unvaccinated COVID-19 outpatients administered molnupiravir [7]. However, a recent Oxford PANORAMIC trial showed no decrease in hospitalization and mortality rates in vaccinated outpatients treated with molnupiravir [8]. Although it is stated that molnupiravir has mild side effects such as nausea, vomiting, and headache and is well tolerated by patients, safety concerns were expressed in an animal reproduction study due to its cytotoxic, mutagenic potential, and teratogenic effects. These concerns have been raised that molnupiravir may cause mutations similar to Sars-Cov-2, particularly in rapidly dividing human tissues. However, current health authorities, including the FDA, have approved the clinical use of molnupiravir, stating that it has low mutagenic and cytotoxic potential [9, 10].

Considering all these data, it is clear that further clinical trials on molnupiravir are needed. However, it has been observed that studies evaluating clinical findings of molnupiravir with the molnupiravir blood concentrations are lacking. Therefore, there is a need to develop reliable methods to measure molnupiravir blood levels and to interpret clinical findings together with blood levels in these clinical studies. Various High-Performance Liquid Chromatography-Ultraviolet (HPLC-UV) [11], UV-spectroscopic [12], Highly Sensitive High-Performance Thin-Layer Chromatography (HP-TLC) [13], Liquid Chromatography with tandem mass spectrometry (LC-MS-MS) [14, 15] methods have been developed for the measurement of molnupiravir levels until today. However, these methods had disadvantages such as laborious pre-treatment procedures, extended analysis time, and large sample volume requirements [11-17]. Our aim in this study is to develop a reliable and robust tandem mass spectrometric method for quantifying molnupiravir levels.

MATERIALS AND METHODS

Tandem mass spectrometric analysis

Chemicals and reagents

Molnupiravir capsule (200 mg) was obtained from Clinical Services. Acetonitrile (CAS Number: 75-05-8, HPLC grade, ≥99.9%), HPLC grade water (CAS Number: 7732-18-5, HPLC grade, ≥99.9%), formic acid (CAS Number: 64-18-6, reagent grade, ≥95%), methanol (CAS Number: 67-56-1, HPLC grade, ≥99.9%), carbamazepine (CAS Number 298-46-4, analytical standard, ≥99.9%), bovine serum albumin (CAS Number 9048-46-8, ≥98.5%), potassium chloride (CAS Number: 7447-40-7, ACS reagent, 99.0-100.5%), sodium chloride (CAS Number: 7647-14-5, ACS reagent, ≥99.0%), disodium hydrogen phosphate (CAS Number: 7558-79-4, ACS reagent, ≥99.0%), potassium dihydrogen phosphate (CAS Number: 7778-77-0, ACS reagent, ≥99.0%) were obtained from Sigma Aldrich (St. Louis, MO, USA).

To eliminate the problems associated with matrix effect, molnupiravir was dissolved in a surrogate matrix similar to the human matrix (serum or plasma). For this purpose, different matrices such as methanol, ethanol, acetonitrile, HPLC-grade water, phosphate-buffered saline (PBS)

solution (0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4 at 25 °C) were evaluated. However, considering the factors such as protein content, ionic strength, and pH similar to plasma, it was seen that the most suitable surrogate matrix was PBS solution containing 1% BSA. 2 mg/mL stock solution was prepared by dissolving the molnupiravir capsule in PBS solution containing 1% BSA. Then, standard solutions were prepared in the concentration range of 20 ng/mL-20 μg/mL by serial dilution from the stock solution. 100 000 ng/mL stock carbamazepine solution was prepared by dissolving 100 mg carbamazepine standard in 1000 mL methanol. Then, the internal standard working solution of 100 ng/mL was prepared by diluting this stock solution in methanol at a rate of 1/1000. All working and standard solutions were freshly prepared and stored at +4 °C.

The ethical approval was obtained from the Selcuk University local Ethics Committee (Number: 2023/19, Date: 24/10/2023).

Equipment conditions

The analytes were detected using the API 3200 (Applied Biosystems/MDS Sciex) tandem mass spectrometer coupled with the Shimadzu HPLC system. Shimadzu HPLC system (Kyoto, Japan) consisted of a pump (LC-20 AD), an automatic sampler (SIL-20 AC HT), and a unit for online degasser (DGU-20A3). Mass spectrometric analyses were performed using an API 3200 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Canada) with an electrospray ion source (ESI) operating in positive mode. As the mobile phase, a mixture of mobile phase A (HPLC grade water containing 0.1% formic acid) and B (acetonitrile containing 0.1% formic acid) was applied by gradient elution. A Phenomenex Luna C18 reverse phase column (50 × 4.6 mm, 5 μm; part no: 00B-4041-E0) was used to separate the analytes. The column oven temperature was 35 ◦C, and the flow rate was 0.6 mL/min. The precursor/product ion transitions for molnupiravir and internal standard carbamazepine were 328.1/126.0 and 237.0/194.0, respectively. The method optimization parameters were as follows: ion spray voltage, 4500 V; ion source temperature, 500 ◦C; gas1, 60 psi; gas2, 60 psi; curtain gas, 30 psi; collision gas, 6 psi. Declustering potential (DP), collision cell exit potential (CXP), collision energy (CE), and entrance potential (EP) parameters were set to 50, 7, 30, 11 V and 30, 4, 48, 10 V for molnupiravir and carbamazepine, respectively.

Sample Preparation

200 μl working or standard solution, 100 μl internal standard (100 ng / mL carbamazepine), and 500 μl acetonitrile were added to eppendorf tubes and vortexed for 30 seconds. Afterward, the mixture was centrifugated at 3500 rpm for 10 minutes. Then, 25 μl supernatant was injected into the LC-MS/MS system.

Method Validation

The developed method was validated according to the CLSI (Clinical and Laboratory Standards Institute) [18] and FDA (Food and Drug Administration) [19] protocols. The validation process includes linearity, precision, matrix effect, recovery, carry-over, and stability studies.

Statistical analysis

The method validation performance was evaluated using the Ep-Evaluator Release 8.0 version (Data Innovations, South Burlington, VT) and Excel (2010) programs.

RESULTS

Linearity study

Linearity studies were performed according to the CLSI EP6-A protocol [18], and the linearity study findings were evaluated with the Ep Evaluator Release 8 program. The standard solutions were prepared in the concentration range of 20 ng/mL-20 μg/mL by serial dilution from the stock solution. Method linearity was evaluated by linear regression analysis, and the correlation coefficient was calculated as 0.993. The limit of detection (LOD) and limit of quantitation (LOQ) values were determined by signal/noise ratio according to CLSI EP17-A protocols [18]. A signal/noise ratio of approximately 3 was considered LOD, and a value of 10 was considered LOQ. Accordingly, the LOD value was determined as 5 ng/mL and the LOQ as 20 ng/mL. The The calibration curve was presented in Figure 1. Chromatograms of LOD (5 ng/mL) and LOQ (20 ng/mL) values were presented in Figure 2 and Figure 3, respectively.

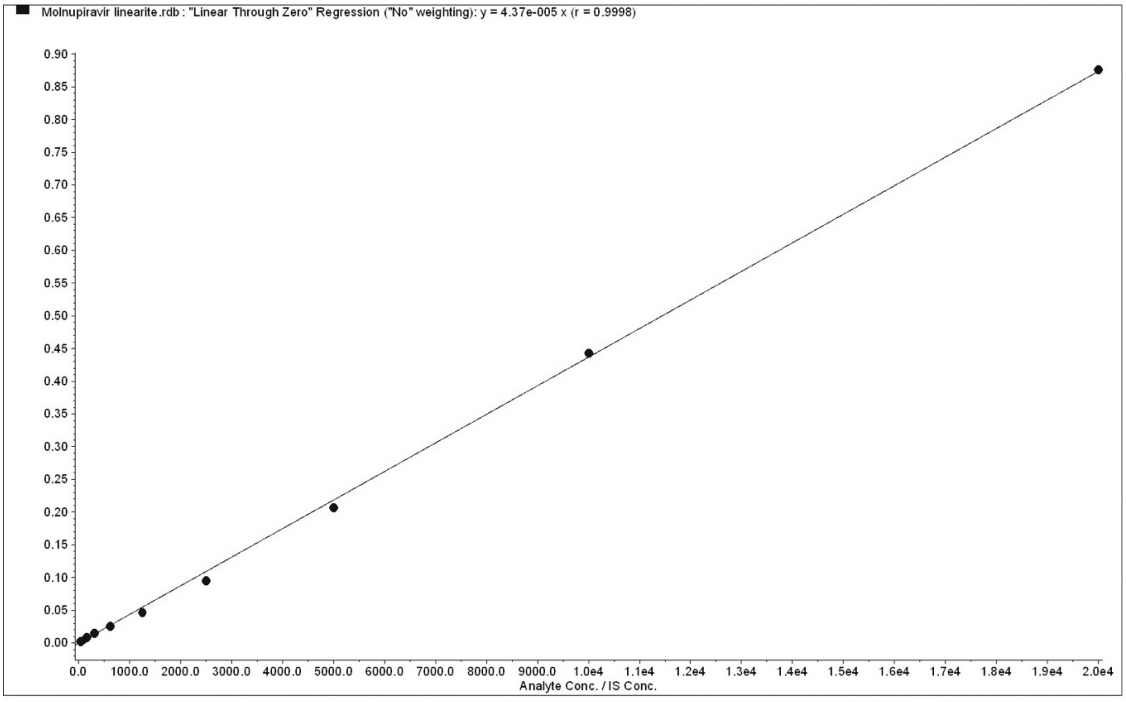

Figure 1. The calibration curve of molnupiravir.

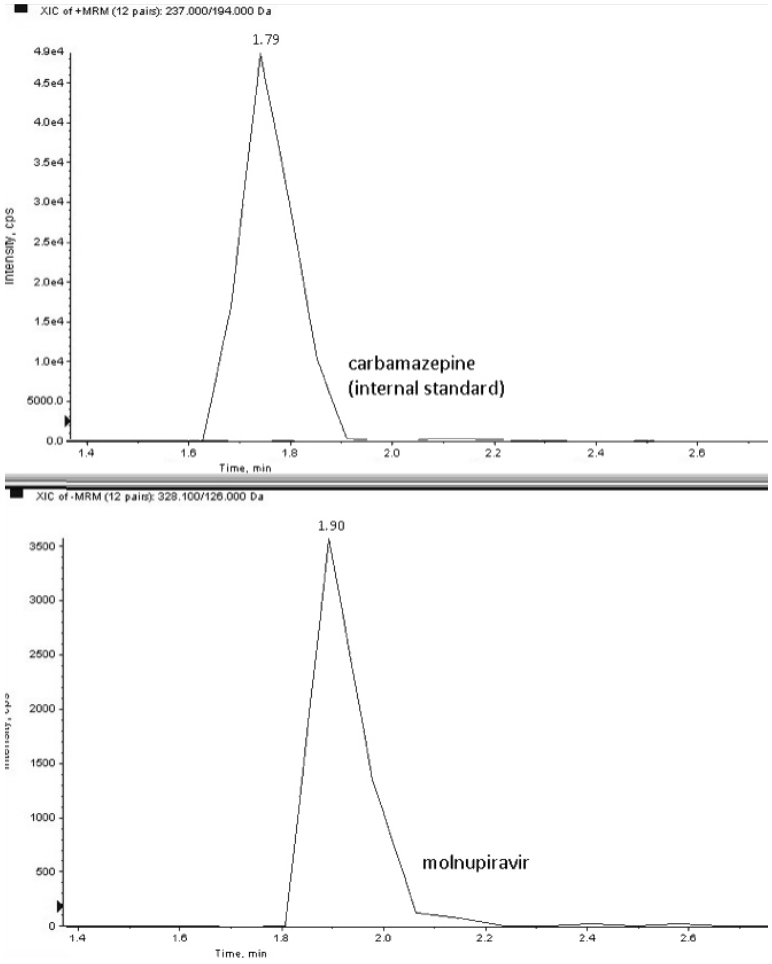

Figure 2. An example chromatogram of the molnupiravir standard at a concentration (LOD) of 5 ng/mL.

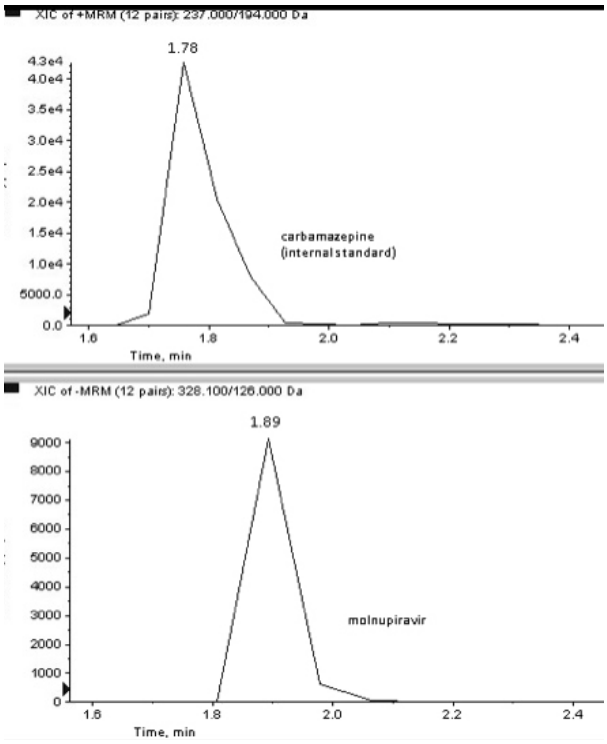

Figure 3. An example chromatogram of the molnupiravir standard at a concentration (LOQ) of 20 ng/mL.

Precision and accuracy study

The precision study was performed according to FDA protocols [19], including intra- and inter-assay precision. The precision study was performed with five different QC samples (LLOQ, low, medium 1, medium 2, and high QC). For the preparation of QC samples in the precision study, a 2 mg/mL stock solution of molnupiravir in PBS solution containing 1% BSA was used. During method validation, the QCs for accuracy and precision runs should be prepared at a minimum of 4 concentration levels within the calibration curve range: the LLOQ, within 3 times of the LLOQ (low QC), around 30% to 50% of the calibration curve range (medium QC) and at least 75% of the ULOQ (high QC) [19]. Accordingly, five different QC samples (LLOQ, low, medium 1, medium 2, and high QC) were prepared using the solution from the stock. LLOQ level was 20 ng/mL. A low QC sample was prepared at a concentration of 3 times the LLOQ. Medium 1 level corresponds to the middle level of the linear range. Medium 2 level was 75% of the high QC sample. For inter-assay precision, 4 replicates of each level were run for 5 days. To calculate the intra-assay CV%, 40 replicates were run at each level, 20 in the morning and 20 in the afternoon. The method's intra- and inter-assay CV% values ranged between 4.3% and 6.2%. The precision (CV%) of the concentrations determined at each level should not exceed 15%, except at the LLOQ, which should not exceed 20% according to FDA guidelines[19]. The results were expressed in Table 1.

Table 1. The results of molnupiravir precision study.

	Intra-assay				Inter-assay			
Concentration(ng/mL)	Mean	SD	CV%	Accuracy%	Mean	SD	CV%	Accuracy%
20	20.62	1.06	5.14	103.1	20.75	1.28	6.17	103.7
60	59.91	2.60	4.35	99.8	58.91	2.96	5.03	98.2
10000	10313	492	4.78	103.1	10221	613	6.01	102.2
15000	15184	688	4.53	101.2	15063	907	6.02	100.4
20000	20018	1017	5.08	100.1	19965	1112	5.57	100.1

An accuracy study was performed by analyzing 4 replicates per level at the LLOQ, low (LQC), medium 1 (MQC1), medium 2 (MQC2), and high-quality control (HQC) samples for five consecutive days. Accuracy was calculated as a percentage of the measured value to the expected value. The acceptability criteria for accuracy studies, according to the FDA guidelines, are that the bias value for LLOQ should be <20% and for other quality control (QC) values <15% [19]. The accuracy ranged between 98.2 and 103.7% for molnupiravir.

Recovery and matrix effect study

The recovery study was conducted with QC samples at 3 different concentration levels. The recovery study results were calculated as the average "measured value/expected value" ratio (%). The matrix effect study was performed according to the procedure specified by Chambers et al. [20]. The results were expressed in Table 2. In the matrix effect study, the response of the analyte in a neat solution was compared to the response of the spiked analyte in the pretreated surrogate matrix. Accordingly, 3 different levels of QC samples were prepared in the mobile phase mixture (water and acetonitrile, 1:1), and the response of the analyte in these QC samples was compared with the response of the analyte in the pretreated surrogate matrix. The matrix effect was calculated using the formula: (ME% = (mean post-extracted peak area / mean un-extracted peak area) × 100). The recovery value of the method was between 98% and 102%, and the matrix effect was below 7%.

Table 2. Recovery and matrix effect results of molnupiravir measurements.

Analyte	Concentration(ng/mL)	Recovery	Matrix effect
Molnupiravir	50	98.5%	-6.9
	5000	101.1%	4.5
	15000	97.4%	-4.9

Stability study

The stability study was performed according to the CLSI EP25-A protocol. For stability studies, 3 QC levels were prepared in the surrogate matrix, including the low, medium, and high QC samples. These samples were kept at -20 °C for freeze-thaw stability and then freeze-thawed 4 times with an interval of 5 days. For the long-term stability study, 4 aliquots were prepared for each QC level. The first replicate was run before freezing, and the other replicates were run on days 15, 30, and 45, respectively. The bias% values were calculated compared to the measured analyte levels on the collection day (expected value) via the following formula: Bias%=((measured value-expected value) / expected value) ×100

The results were expressed in Table 3

Table 3. The stability of molnupiravir at different temperatures (bias%).

Analyte	Concentration(ng/mL)	Frozen -(20C) for 45 day			Freeze-thaw stability		
		15. Day (%)	30. Day(%)	45. Day(%)	2.	3.	4.
Molnupiravir	50	3.66	5.12	7.53	4.15	6.63	8.65
	5000	2.98	4.97	5.88	3.94	5.36	7.39
	15000	4.15	5.56	6.52	2.63	4.58	6.36

The processed sample stability was also investigated by maintaining the QCs samples in an auto-sampler at 4 °C for 20 h, followed by analysis. The bias% values changed between 4.5% and 7.8%.

Carry-over study

This study has been performed according to CLSI EP10-A [18]. The high and low-level QC samples were analyzed using the order specified in the CLSI EP10-A protocol. The mean and standard deviations of the groups were calculated using the EP Evaluator Release 8 program. The carry-over study was conducted individually for each analyte. The orders of samples were expressed as follows: L1-L2-L3-H1-H2-L4-H3-H4-L5-L6-L7-L8-H5-H6-L9-H7-H8-L10-H9-H10-L11. The carry-over value was calculated as 1.22 ng / mL for molnupiravir. The EP Evaluator program determined the acceptability criteria based on CLSI protocol EP10-A3 guidelines, and according to EP Evaluator software, evaluation carry-over value was acceptable for molnupiravir. This study was approved by the clinical research ethics committee of the Selçuk University Faculty of Medicine (Date: 24.10.2023, Number: 2023/19).

DISCUSSION

With the declaration of COVID-19 as a pandemic, many therapeutic compounds have been re-purposed, and studies on developing new agents have accelerated. Molnupiravir is one of the antiviral drugs considered for the treatment of COVID-19. However, findings from clinical studies regarding the efficacy and safety of molnupiravir in the treatment of COVID-19 are contradictory. One of the most important limitations of these studies is the lack of co-evaluation of drug blood levels and clinical data. Therefore, there is a need for methods that allow reliable and practical measurement of molnupiravir levels [21].

Various HPLC methods have been reported for the measurement of molnupiravir levels. However, these methods were disadvantageous due to their low sensitivity, incomplete peak separation, long retention time, laborious pre-treatment procedures, and low recovery and precision [11,12,17, 22]. LC-MS/MS is accepted as the gold standard for drug-level measurement due to its high accuracy, sensitivity, precision, and low risk of interference [23].

For this reason, we developed a new method for quantitating molnupiravir levels in our study. However, limited studies report the development of a validated tandem mass spectrometric method for measuring molnupiravir levels. For example, Gouda et al. reported a tandem mass spectrometric method that allows measurement of molnupiravir levels in plasma. The method indicated was linear for molnupiravir in the 20 to 10000 ng/mL range. The CV% calculated from the precision study ranged from 0.7% to 9.4%. The extraction recovery % results ranged from 78.2 to 80.1%. The CV% calculated from the precision study was below 15%, and the recovery% ranged from 95% to 100%. However, the pre-treatment procedures consisted of a laborious procedure involving the concentration of samples under nitrogen [14]. Amara et al. reported a tandem mass spectrometric method based on measuring molnupiravir and its metabolite in plasma and saliva. The method's intra- and inter-assay CV% values ranged from 1.25% to 9.05%. The mean recovery was over 90% in plasma samples. In addition, the long and laborious pre-treatment steps based on the concentration of the samples under nitrogen were another disadvantage of the method [15]. Parsons et al. reported a validated tandem mass spectrometric method that allows the measurement of molnupiravir metabolites in plasma and peripheral blood mononuclear cell lysates. The CV% value of this method ranged from 1.42% to 11.8%. The average recovery was 74.2%. However, this method did not include validation of parent drug levels [16].

Considering the scarcity of reported methods for measuring molnupiravir levels, it is clear that new, practical, and reliable methods are needed. For this purpose, we developed a validated tandem mass spectrometric method for quantitating molnupiravir levels in our study. Our method was advantageous because it required a minimal sample volume, good sensitivity, expanded measurement range, high precision, low matrix effect, simple and economical pre-treatment procedures, and short analysis time.

Compared to other LC-MS/MS methods [14, 15], the method we developed relied only on a simple pre-treatment step involving protein precipitation followed by centrifugation of the samples. So, its simple, economical pre-treatment steps, short analysis time (3.5 min), and relatively low sample volume (200 μl) were significant advantages of our method.

The method we developed also had a wide measurement range (20 ng/mL-20 μg/mL). It had adequate sensitivity with LOD of 5 ng/mL and LOQ of 20 ng/mL. For example, Gouda et al., the measurement range of the tandem mass spectrometric method specified was 20-10000 ng/mL,

and the LOQ value was 20 ng/mL [14]. In various HPLC methods, the LOQ values for molnupiravir varied between 100 and 5000 ng/mL [24-26].

In the matrix effect study, molnupiravir was dissolved in a surrogate matrix similar to the human matrix (serum or plasma) to eliminate the problems associated with the matrix effect. Considering factors such as protein content, ionic strength, and pH similar to those of plasma, it was seen that the most suitable surrogate matrix was a PBS solution containing 1% BSA. As a result of our study, the matrix effect value was less than 7%. According to CLSI protocols, the matrix effect should be less than 15%. Therefore, the matrix effect value of the method was low and at an acceptable level [18].

The accuracy ranged between 98.2 and 103.7% for molnupiravir. The method's intra- and inter-assay CV% values ranged between 4.3% and 6.2%. According to the FDA guidelines, the acceptability criteria for accuracy studies are that the bias value for LLOQ should be <20% and for other quality control (QC) values <15%. The precision (CV%) of the concentrations determined at each level should not exceed 15%, except at the LLOQ, which should not exceed 20% [19]. Therefore, our method had acceptable accuracy and precision.

In this study, a cost-effective, simple, robust, and reliable measurement method was developed to measure molnupiravir levels. However, our study has limitations regarding the lack of measurement of molnupiravir metabolite levels and drug and metabolite levels in real patient samples or biological matrices. The decrease in COVID-19 patients and the reduction in molnupiravir administration made working with the real patient population difficult. In this study, only the method for measuring parent drug levels was developed with molnupiravir commercial capsules. Further studies, including real patient samples, are needed. However, our study is important considering the limited studies in this area and the importance of measuring antiviral drug levels. There is a need for new studies that allow the measurement of molnupiravir and metabolite levels in the real patient population or various pharmacokinetic models.

Declarations

The authors received no financial support for the research and/or authorship of this article. There is no conflict of interest.

This study was approved by the clinical research ethics committee of the Selçuk University Faculty of Medicine (Date: 24.10.2023, Number: 2023/19).The authors contributed equally to the study.

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