Asian Journal of Chemistry; Vol. 35, No. 11 (2023), 2749-2753

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2023.28420

A Rapid, Simplified and Validated Reverse Phase Liquid Chromatography Method for Quantitation of Molnupiravir and Its Generic Versions

SABIHA KAMAL, MOHAMMAD ABDULLAH TAHER, MD. MONIRUZZAMAN, RICKTA RANI DAS and MALA KHAN*

Bangladesh Reference Institute for Chemical Measurements (BRiCM), Dr. Qudrat-e-Khuda Road, Dhanmondi, Dhaka-1205, Bangladesh

*Corresponding author: E-mail: bricmdg@yahoo.com

Received: 9 August 2023; *Accepted*: 26 September 2023; *Published online*: 31 October 2023; AJC-21431

Molnupiravir, an orally active RdRp inhibitor, though originally developed to use against influenza, has become a promising therapeutic candidate for COVID-19 infection by acting as prodrug of the nucleoside analog β-*d*-N4-hydroxycytidine (NHC or EIDD-1931) in the isopropyl ester form. Accordingly, it has been approved by MHRA, UK in November 2021 to use against COVID-19. This work presents the development and validation of a high-performance liquid chromatography (HPLC) method for the rapid and accurate assessment of molnupiravir. The objective of this approach is to ensure the quality control of Lagevrio® capsules and their generic versions available in Bangladesh. The separation was carried out by isocratic elution through C18 column (4.6 mm × 250 mm i.d., 5 µ particle size) at 40 °C temperature at a constant flow rate of 1.0 mL/min using acetonitrile and 1% orthophosphoric acid as buffer (20:80, v/v). A 10 µL sample was injected and elution was monitored at 210 nm wavelength. The calibration curve over the concentration range of 80-120% were found to be linear (r^2 = 0.9998), the %RSD for intra-day (0.31%) and inter-day (0.73%) precision indicate good precision where %recovery was found in the acceptable range of 99.9-100.4% as per ICH guidelines. The method was successfully applied to commercial pharmaceuticals, Lagevrio® and its generic versions and and the results were found to be consistent with the label claims (95-105%).

Keywords: Molnupiravir, Prodrug, SARS-CoV-2, HPLC, MHRA.

INTRODUCTION

Human coronaviruses have several variations, some of which cause minor upper respiratory tract disease. In late 2019, the unexpected inception of COVID-19 commenced due to novel coronavirus, SARS-CoV-2 first identified in Wuhan, China and has been declared as a pandemic by WHO because of its nature and severity. SARS-CoV-2 infection can proceed to severe COVID-19 infection in people of any age, although the risk rises with age. According to the Centers for Disease Control and Prevention (CDC), people age 65 and older are affected by more than 80% of COVID-19 fatalities, whereas adults age 45 and older are affected by more than 95% of COVID-19 deaths [1]. To treat mild to moderate COVID-19, three antiviral monoclonal antibodies (mAbs) have been approved. Those products are administered in the human body either intravenous (IV) or subcutaneous (SC) injection. The UK's (MHRA) authorized molnupiravir (Fig. 1), an antiviral medication, as the world's first oral medicine for symptomatic treatment of COVID-19 for emergency purposes on November 4, 2021 [2]. The US-FDA (Food and Drug Administration) then authorized molnupiravir (MPV) to use to treat COVID-19 patients, who are at the biggest risk of developing severe COVID-19 by a committee of advisers on November 30, 2021 [3].

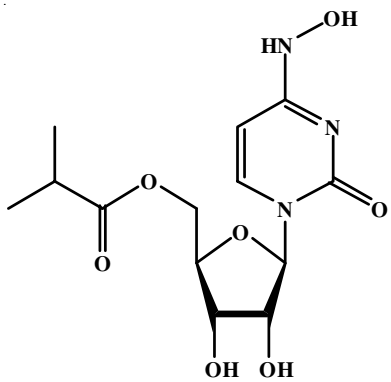

Fig. 1. Chemical structure of molnupiravir (MVP)

Molnupiravir (MPV) is an antiviral oral prodrug active against the coronavirus-2 that causes critical respiratory syndrome (SARS-CoV-2). It is metabolized to N4-hydroxycytidine (NHC), a cytidine nucleoside analogue that enters cells and is phosphorylated to generate ribonucleoside triphosphate (NHC-TP) which is pharmacologically active. NHC-TP works by a process known as viral error catastrophe or viral fatal mutagenesis. The viral RNA-dependent RNA polymerase incorporates NHC-TP into viral SARS-CoV-2 RNA, as a result of a build-up of mistakes in the viral genome and replication inhibition [4,5]. Adult patients should take 800 mg (four 200 mg capsules) orally once every 12 h for 5 days, with or without meals. Once the symptom is identified, the treatment must begin within 5 days.

Molnupiravir (MPV) was developed by Merck & Co. and is currently marketed by Merck Sharp & Dohme (MSD) under the trade name Legevrio. The manufacturer has permitted arrangements for a number of companies to produce Legevrio under license to meet the fast-growing world demand for this drug [6]. Thus in Bangladesh, from 11 November 2021, DGDA has authorized a few pharmaceutical companies to produce molnupiravir.

A limited number of validated analytical techniques for these drugs have been found in the literature. Liquid chromatography/tandem mass spectrometry (LC-MS/MS) has been used to measure plasma levels of certain drugs in animal models [7-9]. The goal of this research is to establish and validate a simple, fast and reliable isocratic reverse-phase high performance liquid chromatography method with photodiode array detection for the determination of molnupiravir in Legevrio® and its commercially available generics in Bangladesh. Emorivir® & Monuvir® manufactured by two reputed Pharmaceutical Company of Bangladesh. The suggested technique's key advantages and uniqueness comprised a simple sample extraction procedure at room temperature, a quick analysis time, high precision, accuracy and excellent recovery. Confirmation of the applicability of the developed method was validated according to the International Conference on Harmonization (ICH), a determination of MPV in Legevrio® capsules and its generics has been also performed.

EXPERIMENTAL

Acetonitrile (HPLC grade), orthophosphoric acid (A.R. grade) and hydrochloric acid (A.R. grade) were collected from Alfa Aesar, England; Sigma-Aldrich Co., USA and PT Smartlab, Indonesia respectively. High quality water, applied throughout the study, was obtained by (Water Pro PS, Lab Conco, USA). Molnupiravir analytical standard was provided by a renowned and leading pharmaceutical company in Bangladesh. The innovator of MVP Legevrio® & two different brands of MVP, commercially available in Bangladesh, were purchased from a local pharmacy. All of the commercial samples were collected as hard capsules label claimed as 200 mg molnupiravir.

Instrumentation and analytical conditions: The HPLC system (Shimadzu, Japan) consisted of a pump (LC-20AD) equipped with a SIL-20 AC HT model Autosampler, a Photo diode array detector (SPD M-20A) set at 210 nm wavelength. The analytical column, C18 (250 mm × 4.6 mm i.d., 5 µ particle size) Phenomenex, USA was used at temperature 40 ºC. Isocratic elution with acetonitrile and 1% orthophosphoric acid HPLC grade at a ratio of 80:20 was used at a constant flow rate of 1.0 mL/min. The mobile phase was freshly prepared and filtered through a 0.45 µm polyamide-membrane filter (Sartorius, Goettingen, Germany) using a glass vacuum filtration apparatus (Rocker 300, Taiwan) and degassed by sonicating for 15 min before use (Powersonic 510, Hwashin Technology, Korea).

Standard preparation: Stock solution for the standard of MVP at a concentration of 0.18 mg/mL was prepared freshly by accurately weighing 18 mg of MVP standard into a volumetric flask of 100 mL and deionized water was used to fill up the volume. The system suitability standard was prepared by accurately weighing about 18.00 mg of molnupiravir standard and made up to 100 mL by diluent (mixture of water and acetonitrile in the ratio of 50:50). Before injecting solutions to the HPLC system, all the solution was passed through a 0.45 µm syringe filter and the mobile phase was allowed to equilibrate the column for a minimum of 30 min.

Sample preparation for assay: Twenty capsules from two commercial brands (label claim 200 mg Molnupiravir) were each weighed and their average weight was determined. The shells of the capsules were then removed and placed all the capsules content in a mortar pestle. The powder was ground in a glass mortar until it was homogeneous, and then an exact 18 mg of molnupiravir was weighed out and transferred to a 100 mL volumetric flask containing around 60 mL of diluent. After 15 min of sonication, the solution was diluted to its final volume with the diluent and thoroughly mixed. An aliquot of this solution was passed through a 0.45 µm syringe filter prior to injection.

Validation procedure: The proposed HPLC method was validated as per the International Conference on Harmonization (ICH) guidelines for the Registration of Pharmaceuticals for human consumption [10]. The linearity, accuracy, precision, specificity, system suitability and filter paper compatibility were done to validate this method.

Linearity: Linearity of MVP was tested at five different concentrations ranging from 80- 120% of the standard solution. To get the peak area that corresponded to each concentration, each concentration was injected three times. A calibration curve was established by plotting AUC *versus* analyte concentration (%) [11].

Precision: As a major parameter for validation of an analytical method, the intra-day and inter-day precisions were studied. Intra-day precision was conducted by six replicate injections at a concentration of 100% of freshly prepared MVP standard at exactly the same equipment and the environmental condition. Inter-day precision was achieved by administering the same concentration with different analyst for two consecutive days.

Accuracy: Method accuracy was tested for % recovery and %RSD of individual measurements by adding a known amount of standard to that of placebo below and above the working level (80, 100 and 120%) [12]. The outcomes were

expressed as the percentage of molnupiravir recovered from the samples.

Specificity: The specificity test was evaluated by comparing the chromatogram obtained from the placebo used in the formulation of the pharmaceutical product and the standard solution with the same diluent and mobile phase and confirmed for the absence of interference [12].

System suitability: Five replicate injections of 0.18 mg/mL standard solution were injected to perform system suitability testing and determine the % RSD of the peak area and retention time (RT).

Filter paper compatibility: The filter paper compatibility was carried out to evaluate the impact of filtration on the analyte. Both standard solution and sample solution were passed through different types of filters (Whatman# 41 filter, 0.22 µm and 0.45 µm syringe filter) and recovery (%) was calculated against the unfiltered standard solution. For the sample solution, the assay value of the filtered solution was compared against centrifuged (unfiltered) sample solution.

RESULTS AND DISCUSSION

Development and optimization of method: For method development of molnupiravir quantitation, various conditions such as different columns (C8 and C18) and mobile phase mixtures were tried. The Phenomenex C18 (250 mm × 4.6 mm i.d., 5 µ particle size) at 40 ºC was found to be appropriate for the separation of the target analyte. A 1% orthophosphoric acid and acetonitrile at different ratios and also at different flow rates were tried as mobile phase. The ratio of 80:20 (1% orthophosphoric acid: acetonitrile) at the flow rate of 1.0 mL/min showed a better peak shape with a small retention time of about 6.8 min (Fig. 2). The optimum detection wavelength was found to be 210 nm by using the Shimadzu PDA detector at that optimum condition. The isocratic method was chosen over the gradient mode due to its simplicity and reduced number of variables in the sample formulation, as the gradient mode is associated with disturbances such as baseline noise, dwell volume change, prolonged analysis time and increased cost [13].

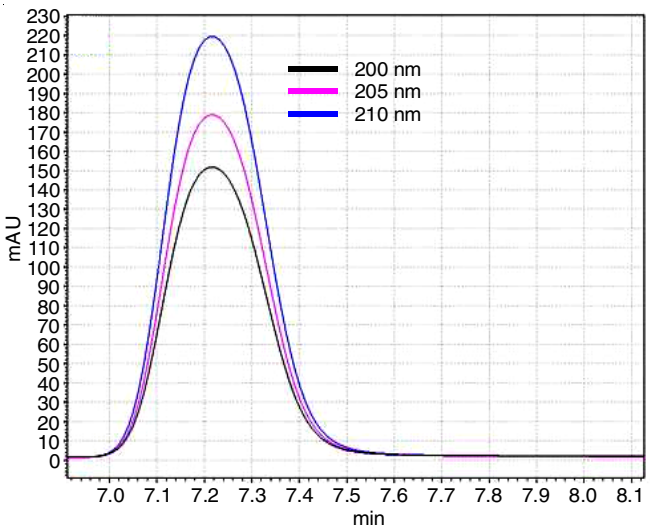

Fig. 2. Optimization of the wavelength

Validation of method

Linearity: Five point calibration graphs were constructed covering a concentration 80%, 90%, 100%, 110% and 120% of standard solution. Three independent determinations were performed at each concentration. The regression coefficient (r^2) value was determined which was 0.9998 (Fig. 3) indicating a good correlation. The relative standard deviation of slope is found to be zero, whereas the y-intercept is 0.62%.

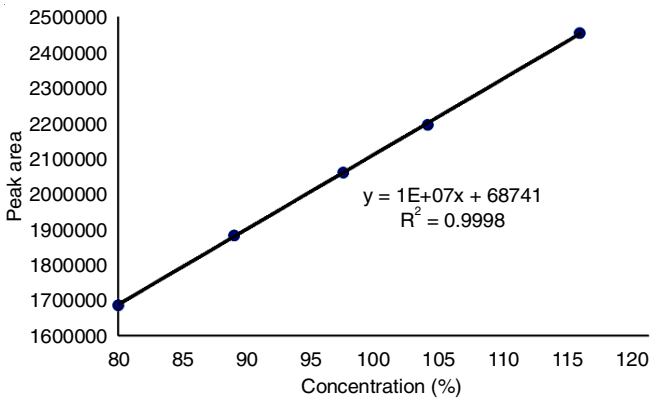

Fig. 3. Calibration curve for linearity of molnupiravir

Precision: In the creation and validation of novel methods, precision is crucially important as it indicates the repeatability of the data obtained on the same day (intra-day precision) or other days (inter-day precision). Six different independent MVP solutions were used to test intra-day precision at 100% level concentrations. Inter-day precision was achieved by administering the same concentration different analyst on a different day. Table-1 shows inter- and intra-day precision of the developed method where %RSD of intra-day and inter-day precision of MVP 100% level concentration was found to be 0.31% and 0.73%, respectively conforming ICH guidelines [14] and thus indicating a good precise method.

TABLE-1
% RSD DETERMINED FOR
INTRA-DAY & INTER-DAY PRECISION

S/N	Level (%)	% Recovered Day-1	% Recovered Day-2
1	100	99.42	98.77
2		100.01	99.61
3		100.04	99.94
4		99.67	99.72
5		99.52	100.91
6		99.28	101.53
Mean (n = 6)		99.66	100.08
% RSD (n = 6)		0.31	0.99
Mean assay (n = 12)			99.87
Mean %RSD (n = 12)			0.73%

Accuracy: The accuracy of molnupiravir concentration of three levels at 80%, 100% and 120% from the calibration curve was determined in three replicates. The data for accuracy was expressed in terms of percentage recoveries of molnupiravir. These results are summarized in Table-2. The mean recovery data of molnupiravir were within the range of 99.90

TABLE-2
ACCURACIES (%) DETERMINED FOR REFERENCE STANDARDS AT GIVEN CONCENTRATIONS

Level (%)	Recovered (%)	Mean value (%)	RSD (%)
80	100.78 99.57 99.36	99.90	0.77
100	100.28 100.86 101.43	100.86	0.57
120	100.92 100.25 100.04	100.40	0.46
Mean %RSD			0.6%

to 100.86% and the mean %RSD was found to be 0.6%, hence satisfying the acceptance criteria for the study.

Specificity: No peak within the retention time range of 6.2-6.8 min was revealed by examining the HPLC chromatogram (Fig. 4) for the mixture of the drug and excipients. The results exhibited that the developed method was specific for the analyte of interest as none of the excipients interfered with it.

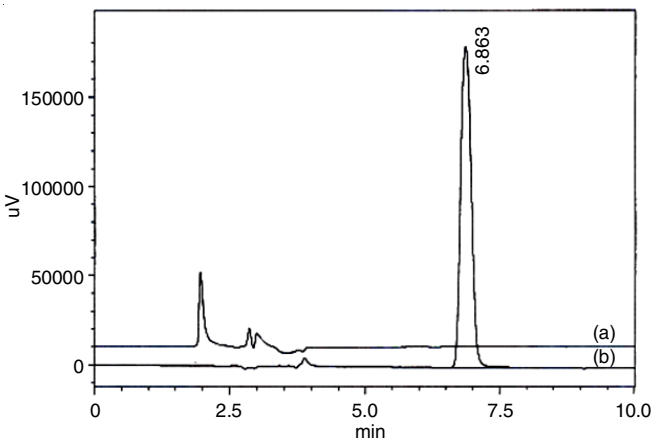

Fig. 4. HPLC chromatograph of (a) placebo and (b) molnupiravir standard

System suitability: The tailing factor as well as the theoretical plate of system suitability standard was found to be 1.18 and 5832, respectively and indicating the performance of column and well-shaped peak since the tailing factor is under 2 and theoretical plate number is greater than 2000. The % RSD of peak area and retention time of system suitability standard was calculated (Table-3), which shows the suitability of the system. Based on statistical analysis, it was determined that the suggested method has good linearity and accuracy and that it has been validated for a wide range of attributes, suggesting that it might be used to quickly and accurately estimate MVP.

Filter paper compatibility: There was no significant difference between filtered and unfiltered content for standard and test sample solutions (Tables 4 and 5). Any filter can be used however, in regular analysis, it is recommended to use a 0.45 µm disc filter and discard the first 5 mL of the solution.

Assay of capsules: The validated method was applied for the assay determination of three commercial capsules containing 200 mg of MVP. Each sample was analyzed in triplicate after extracting the drug as mentioned in assay sample preparation

TABLE-3
SYSTEM SUITABILITY STUDY

Level (%)	Area	RT (min)	Tailing factor (Mean)	Theoretical plate (mean)
100	2449324 2453111 2437534 2432253 2413547	6.8 6.8 6.8 6.7 6.7	1.18	5832
Mean	2437154	6.76		
%RSD	0.64	0.81		

TABLE-4
FILTER EFFECTS ON MOLNUPIRAVIR IN STANDARD SOLUTIONS

Filtration types	Content (%)	Absolute difference
Unfiltered	100.00	Reference
0.22 µ disc filtered	99.36	0.64
0.45 µ disc filtered	99.38	0.62
Whatman#41 filtered	99.98	0.02

TABLE-5
FILTER EFFECTS ON MOLNUPIRAVIR IN SAMPLE SOLUTIONS

Filtration types	Content (%)	Absolute difference
Centrifuged & unfiltered	99.01	Reference
0.22 µ disc filtered	99.53	0.52
0.45 µ disc filtered	98.72	0.29
Whatman#41 filtered	99.18	0.17

of the experimental section and injected. Fig. 5 shows the HPLC chromatograms of MVP standard and as the active ingredients in the pharmaceutical capsules. The analyte peak was not affected by any of the capsule ingredients. The chromatogram is in good agreement with the labeled content. Assay results of Lagevrio®, Emorivir and Monuvir expressed as the percentage of the label claim, were identified to be 100.5%, 98.3% and 96.5%, respectively showing that the content of MVP in the capsule formulations confirmed to the content requirements (95-105%) of the label claim. A low standard deviation indicated a high degree of precision in the measurement. Based on these findings, it appears that the established HPLC method for the rapid and accurate determination of MVP may be applied to the selection of MVP in pharmaceutical formulations and active pharmaceutical ingredients.

Conclusion

As molnupiravir (MVP) is recently approved drug used as a medication for SARS-COV-2, there is a necessity to establish a simple and quick method for analysis of molnupiravir and quality check of its commercial batches. For the accurate quantification of Lagevrio® and generic molnupiravir, a validated isocratic HPLC-UV technique has been developed. The proposed method is straightforward and quick while also being reliable and precise. Due to its rapid chromatographic run time, it can handle a large number of samples in a short amount of time. Subsequently, it is appropriate for analyzing molnupiravir in the pharmaceutical dosage forms on a regular basis. Due to the straightforward nature of the proposed method, there is less of a need for sophisticated analytical instruments

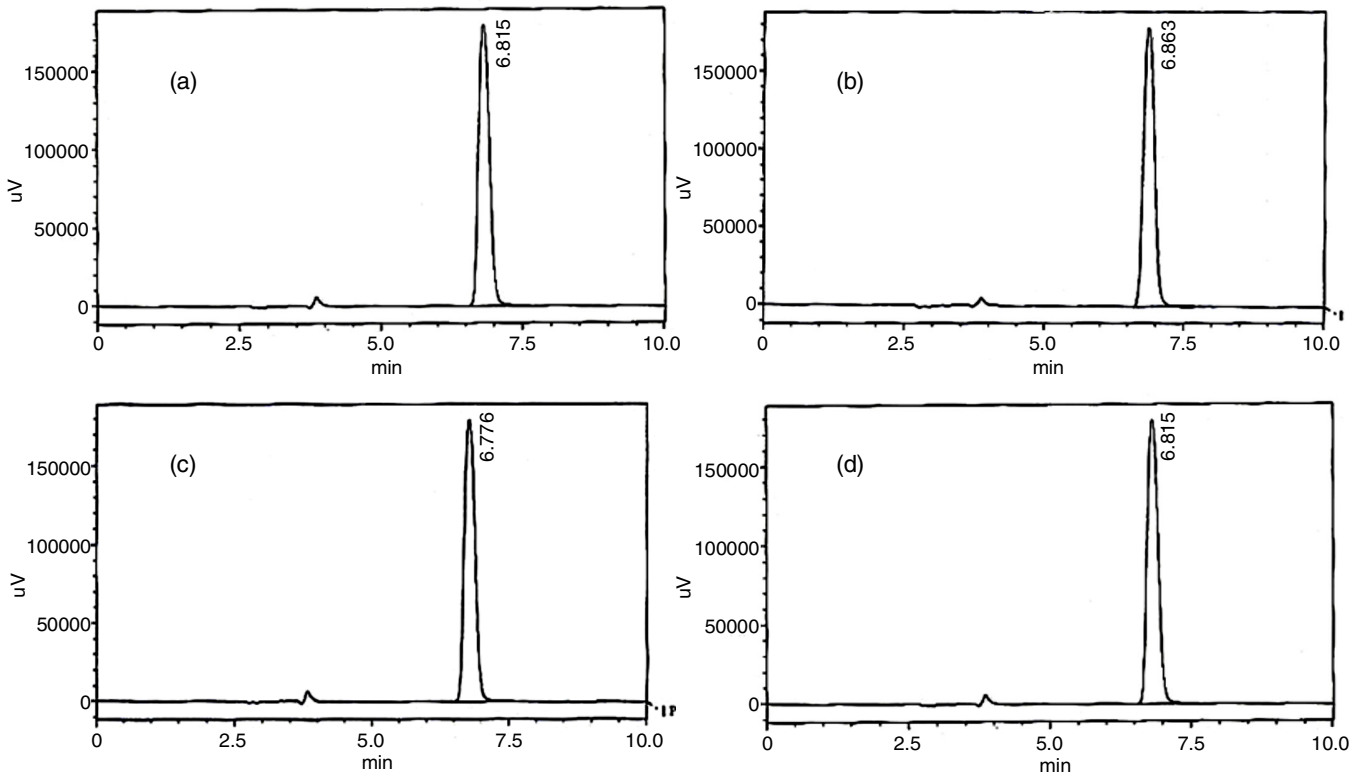

Fig. 5. Chromatogram of (a) molnupiravir standard, (b) lagevrio, (c) emorivir and (d) monuvir

that are tedious expensive and time consuming. Given the prevailing global pandemic and the imperative need for effective SARS-COV-2 treatment on a global scale, the suggested approach is both time-intensive and potentially beneficial for the national quality control laboratories and pharmaceutical enterprises, particularly in developing nations.

ACKNOWLEDGEMENTS

This study was funded and supported by the Bangladesh Reference Institute for Chemical Measurements (BRiCM), Ministry of Science and Technology, Bangladesh.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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