Original Research Article

Method development and Validation of a specific stability indicating RP-HPLC method for Molnupiravir API

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ABSTRACT

A simple, accurate, isocratic stability indicating RP-HPLC method was developed for the determination of Molnupiravir in bulk drug. This RP-HPLC was achieved on "Waters 2695 using an Agilent Zorbax Eclipse C18 (250 mm × 4.6 mm × 5 µm)" column with the mobile phase consisting of 30 mM, ammonium phosphate monobasic and Methanol in the ratio of 47:53 %v/v. The stress testing of Molnupiravir was carried out under acidic, alkaline, oxidative, thermal, and photolytic conditions and formed degradation products were well resolved from Molnupiravir API (active pharmaceutical ingredient). The proposed method was validated as per ICH (International Council on Harmonisation) guidelines and the results of all the validation parameters were well within their acceptance values. The method was found to be suitable for the quality control of Molnupiravir in bulk and pharmaceutical dosage forms as well as for stability-indicating studies.

Keywords: Assay, Stress Studies, Validation, Molnupiravir

1. INTRODUCTION

Molnupiravir is chemically described as ((2*R*,3*S*,4*R*,5*R*)-3,4-dihydroxy-5-(4-(hydroxyimino)-2-oxo-3,4-dihydropyrimidin-1(2*H*)-yl) tetrahydrofuran-2-yl) methyl isobutyrate and Its empirical formula is $C_{13}H_{19}N_3O_7$, its molecular weight is 329.31. [Fig 1].

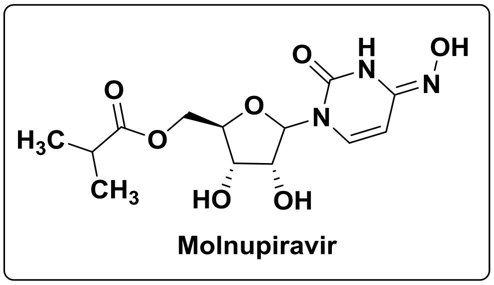

Fig. 1. Molnupiravir structure

Molnupiravir is emerging as a drug candidate of increasing interest based on its potential to treat COVID-19. Molnupiravir showed broad spectrum antiviral activity against SARS-CoV-2 with reduced virus titer in mice[1], and completely blocked SARS-CoV-2 transmission in ferrets within 24 hr of administering the medication[2]. From a pragmatic standpoint, molnupiravir is orally available and is structurally simple compared to remdesivir, making future manufacture considerably less complex. There are safety concerns related to the drug, and these should be answered by the pending clinical trials[3,4,5]. According to current good manufacturing practices, all drugs must be tested with a stability-indicating assay method before release. Stress testing of the drug substance can help identify the likely degradation products, which can in turn help establish the degradation pathways and the intrinsic stability of the molecule and validate the stability-indicating power of the analytical procedures used. The nature of the stress testing will depend on the individual drug substance and the type of drug product involved. Keeping into the view of susceptibility of molnupiravir under variety of conditions, it was felt that a HPLC method of analysis that separates the drug from the degradation products formed under ICH (International Council on Harmonisation) suggested conditions (hydrolysis, oxidation, photolysis, and thermal stress)[6-17] would be of general interest. These studies provide valuable information on drug's inherent stability and help in the validation of analytical methods to be used in stability studies[18]. A survey on literature reveals that a very few methods were developed for the estimation of molnupiravir in biological fluids and marketed formulations[19]. The literature has demonstrated that a LC method for determination of molnupiravir in the presence of its impurities and degradation products generated from forced decomposition studies was developed. Nevertheless, in that reported study, the retention time of molnupiravir was obtained at 28 min. However, in our present method the retention time of molnupiravir was achieved at 15 min. Thus, the aim of our study was to develop a simple, selective, economic, specific stability indicating the LC method that can be used to determine the related substances and also the assay of bulk samples of Molnupiravir. This paper deals with the development of stability indicating the analytical method using the samples generated from forced degradation studies.

2. MATERIAL AND METHODS

Materials

Analytically pure Molnupiravir was received as gift sample from Research and Development laboratory, Andhra University, Visakhapatnam, India. HPLC grade Methanol was purchased from Merck, India. Analytical reagent grade ammonium phosphate monobasic was purchased from Sigma Aldrich, India. High pure water was prepared by using Millipore Milli Q plus purification system.

Instrumentation

The LC system, used for method development, forced degradation studies and method validation was Waters 2695 series (manufactured by Waters Technologies, USA) LC system with an PDA detector. The output signal was monitored and processed using Empower software (designed by Waters Technologies, USA) on Lenovo computer (Digital Equipment Co.).

Chromatographic conditions

The chromatographic column used was Agilent Zorbax Eclipse C18 (250 mm × 4.6 mm × 5 µm). The mobile phase consists of a mixture of 30 mM ammonium phosphate monobasic and Methanol (47:53 %V/V). The mobile phase was pumped from the solvent reservoir to the column at a flow rate of 1.5 ml/min for 22 min. The column temperature was maintained at 35 °C. The eluate was monitored at 260 nm with injection volume was 2 (µl). Mobile phase was used as diluent during the standard and test samples preparation.

Blank preparation

Mobile phase used as blank refer fig-2

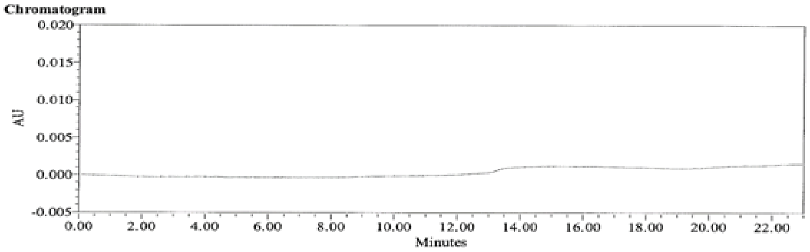

Fig. 2. Blank chromatogram

Preparation of standard solution

Standard solution of molnupiravir was prepared by dissolving 25 mg standard into 10 ml of diluent in 25 ml of volumetric flask. The solution was sonicated for about 5 min and then made up to volume with diluent. This solution was filtered through 0.45 µm filter paper. Suitable aliquot of the filtered solution was added to a volumetric flask and made up to volume with diluent to yield a starting concentration of 0.1 mg/ml (fig-3).

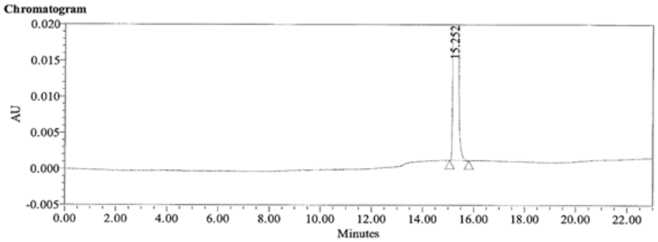

Fig. 3. Standard solution chromatogram of molnupiravir

Preparation of sample solution

Sample solution of molnupiravir was prepared by dissolving 25 mg sample into 10 ml of diluent in 25 ml of volumetric flask. The solution was sonicated for about 5 min and then made up to volume with diluent. This solution was filtered through 0.45 µm filter paper. Suitable aliquot of the filtered solution was added to a volumetric flask and made up to volume with diluent to yield a starting concentration of 0.1 mg/ml (fig-4).

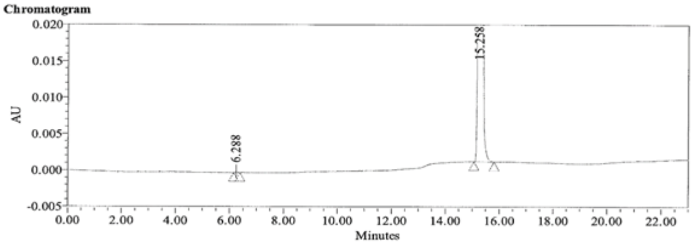

Fig. 4. Sample solution chromatogram of molnupiravir

3. RESULTS AND DISCUSSION

The chromatographic column used was Agilent Zorbax Eclipse C18 (250 mm × 4.6 mm × 5 µm) and it was maintained at 35°C temperature for the separation and the method validated for the determination of Molnupiravir API. The composition and the flow rate of the mobile phase were changed to optimize the separation conditions using stressed samples and the main related substances. A mobile phase consisting of 30 mM Ammonium phosphate monobasic buffer and Methanol (47:53, %v/v) is selected at a flow rate of 1.5 ml/min, after several preliminary investigatory chromatographic runs. Under the described experimental conditions, all peaks were well defined and free from tailing. The effects of small deliberate changes in the mobile phase composition and flow rate were evaluated as a part of testing for method robustness.

4. METHOD VALIDATION

The analytical method was validated with respect to parameters such as linearity, limit of quantitation (LOQ), limit of detection (LOD), precision, accuracy, selectivity, recovery, and robustness/ruggedness.

Linearity

Linearity was established by least squares linear regression analysis of the calibration curve. The constructed calibration curves were linear over the concentration range of 1–200 µg/ml. Peak areas of molnupiravir was plotted against their respective concentrations and linear regression analysis performed on the resultant curve. Correlation coefficient (n=6) was found to be more than 0.9992 with %RSD values being less than 2% across the concentration ranges studied. Typically, the regression equation was y = 2994.7x + 5744.8 (R = 0.9992) (fig-5).

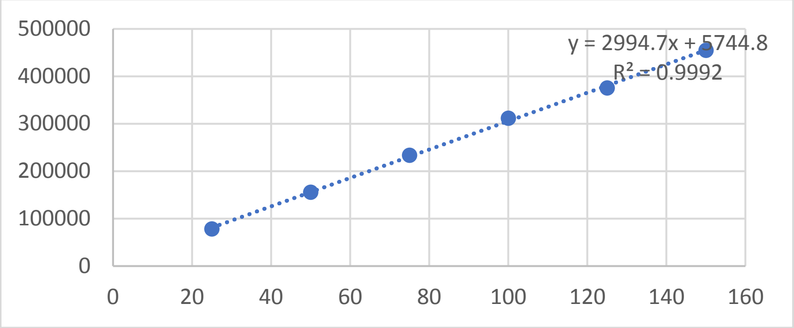

Fig. 5. Linearity Curve for molnupiravir

Limit of Detection and Limit of Quantitation

The limit of quantitation (LOQ) of the present method was found to be 0.993 µg/ml with a resultant %RSD of 0.4% (n = 5). The limit of detection (LOD) was found to be 0.3 µg/ml. The representative chromatograms for blank run and chromatogram at LOQ were shown in Figures 6 and 7, respectively.

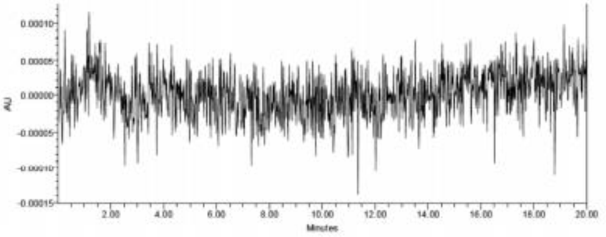

Fig. 6. A representative chromatogram of molnupiravir at LOD

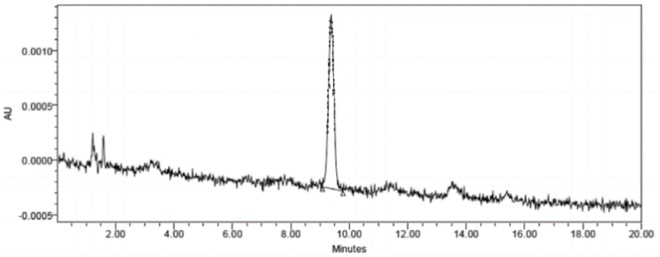

Fig. 7. A representative chromatogram of molnupiravir at LOQ

Precision

Precision of the assay was investigated with respect to both repeatability and reproducibility. Repeatability was investigated by injecting nine replicate samples of each of the 25, 50, and 75 µg/ml standards where the mean concentrations were found to be 25.5, 50.2, and 74.8 µg/ml with associated %RSDs of 0.23, 1.24, and 0.42, respectively. Inter-day precision was assessed by injecting the same three concentrations over three consecutive days, resulting in mean concentrations of molnupiravir of 24.0, 50.3, and 74.1 µg/ml and associated %RSDs of 1.33, 1.59, and 1.7 %, respectively. The ruggedness of the method was assessed by comparison of the intra- and inter-day assay results for Molnupiravir undertaken by two analysts. The % RSD values for intra- and inter-day assays of molnupiravir in the cited formulations performed in the same laboratory by the two analysts did not exceed more than 2%, thus indicating the ruggedness of the method. The mean retention time of molnupiravir was 15.2 min with associated %RSD of 0.1%.

Accuracy

The accuracy of the method was determined through the recovery test of the samples, using known amounts of molnupiravir reference standard. For the LC method, aliquots of 0.8, 1.0, and 1.2 ml of a molnupiravir standard solution (50 µg/ml) were added to three sample solutions containing a fixed amount of Molnupiravir (50 µg) in diluent, respectively. Therefore, this recovery study was performed at a final concentration solution of 40.0, 50.0, and 60.0 µg/ml of molnupiravir. All solutions were prepared in triplicate and analysed. Accuracy data for the assay following the determination of the compound of interest are summarized in Table 1.

Table 1. Accuracy data (n=3)

Amount of drug added	Mean amount of drug recovered (mg)	% Drug recovery	% RSD
40	40.4±0.2	101.10	0.22
50	50.3±0.3	100.82	0.23
60	59.9±0.4	99.72	0.51

Specificity

The specificity of the LC method was evaluated to ensure that there was no interference from the excipients contained in pharmaceutical product or from products resulting from forced degradation. The results of stress testing studies in addition to that of monitoring standard solutions of the drug in the presence of their impurities indicated a high degree of specificity of this method. The degradation product(s) of the parent compound was found to be similar for both the tablets and API powder. All the degradation products formed during forced decomposition studies were well separated from the analyte peak, demonstrating that the developed method was specific and stability-indicating.

Robustness

Test as recommended in the ICH (International Council on Harmonisation) Guidelines, a robustness assessment was performed during the development of the analytical procedure. The robustness of the method was investigated under a variety of conditions including changes of pH of the eluent, flow rate, and buffer composition. The degree of reproducibility of the results obtained as a result of small deliberate variations in the method parameters and by changing analytical operators have proven that the method is robust and the data are summarized in Table 2.

Table 2. Robustness testing of the method

Parameter	Modification	% Drug recovery
Column temperature (°C)	33	101.1
	35	100.1
	37	100.8
Buffer concentration (mM)	20	99.2
	30	100.1
	40	98.4
Flow rate (ml/min)	1.4	103.2
	1.5	100.1
	1.6	98.6

System suitability parameters

System suitability parameters can be defined as tests to ensure that the method can generate results of acceptable accuracy and precision. The requirements for system suitability are usually developed after the completion of method development and validation. (Or) The USP (2000) defines parameters that can be used to determine system suitability prior to analysis. The system suitability parameters, such as Theoretical plates (N), Resolution (R), Tailing factor (T), were calculated and compared with the standard values to ascertain whether the proposed RP-HPLC method for the estimation of molnupiravir in pharmaceutical formulations was validated or not. The results are shown in Table 3.

Table 3. System suitability parameters

Parameter	Values obtained	Preferable values

Theoretical Plates	5054	>3000
Tailing factor	1.02	<1.5
%RSD	0.32	<0.70

System suitability parameters

The R.S.D. of assay of molnupiravir during solution stability experiments was within 1.0%. No significant change was observed in the content of molnupiravir during solution stability experiments. The experimental data confirmed that sample solutions used during assay and related substance determinations were stable up to 48 h.

5. FORCED DEGRADATION STUDIES

In order to determine whether the analytical method and assay were stability-indicating, molnupiravir active pharmaceutical ingredient (API) powder were stressed under various conditions to conduct forced degradation studies. Intentional degradation was attempted to stress conditions acid hydrolysis (using 0.2 N, 1.0 N HCl), base hydrolysis (using 0.2 N, 1.0 N NaOH), oxidative degradation (using 2.0 %, 5 % H_2O_2), photolytic degradation (UV cabinet, an overall illumination of ≥210 Wh/m2 at room temperature with UV radiation at 320– 400 nm), and thermal treatment (heated at 50 °C, 150 °C for 5 h) to evaluate the ability of the proposed method stability indicating and separate Molnupiravir from its degradation products. Molnupiravir at a concentration of 0.1 mcg/ml was used in all the degradation studies. After completion of the degradation processes, the solutions were neutralized and diluted with diluent.

Acid and alkaline degradation

25 mg of sample taken into a 25 ml volumetric flask add 2 ml of 0.2 N HCl solution and kept a side at room temperature for 24 hr, after completion of degradation dilute to volume with diluent. Appropriate aliquot was taken from the above solution and diluted with diluent to obtain a final concentration of 0.1 mg/ml. the same procedure applicable to 1.0 N HCl. Similarly, forced degradation in alkaline media was performed using 0.2 N, 1.0 N NaOH. The representative chromatograms for acid and alkaline degradation studies are shown in Figures 8 and 9, respectively.

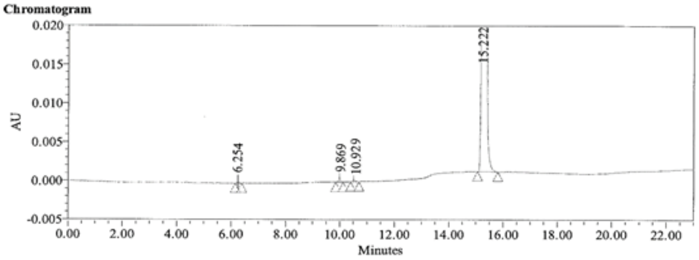

Fig. 8. Typical chromatogram representing the degradation behaviour of molnupiravir in acid hydrolysis.

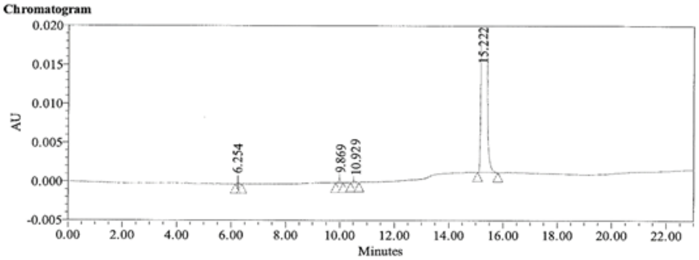

Fig. 9. Typical chromatogram representing the degradation behaviour of molnupiravir in alkaline hydrolysis.

Oxidative degradation

Oxidative degradation was performed by taking 25 mg of sample into a 25 ml volumetric flask and add 2ml of 2.0 % H_2O_2 solution. The flask was kept aside at room temperature for 24 h. further make up the volume with diluent Appropriate aliquot was taken from the above solution and diluted with dilunet to obtain a final concentration of 0.1 mg/ml and the same procedure repeated with 5.0 % H_2O_2. The representative chromatogram is shown in Figure 10.

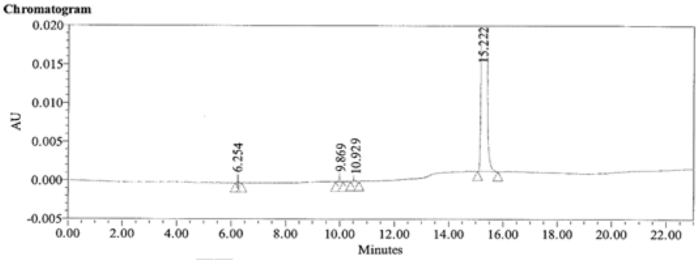

Fig. 10. Typical chromatogram representing the degradation behaviour of molnupiravir in oxidation

Photo degradation

Molnupiravir-API was prepared and exposed to light to determine the effects of light irradiation on the stability of Molnupiravir in solution and in the solid state. Approximately 50 mg of Molnupiravir-API powder was spread on a glass dish in a layer that was less than 2 mm thickness and a solution of API (1 mg/ml) was prepared in mobile phase. All samples for photo-stability testing were placed in a light cabinet and exposed to light for 24 h resulting in an overall illumination of ≥ 210 Wh/m^2 at 25 °C with UV radiation at 320-400 nm. Control samples, which were protected with aluminium foil, were also placed in the light cabinet and exposed concurrently. Following removal from the light cabinet, all samples were prepared for analysis as previously described. The representative chromatogram is shown in Figure 11.

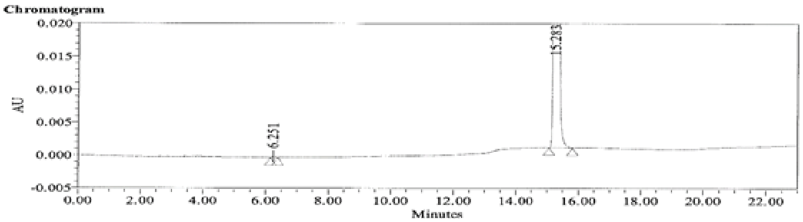

Fig. 11. Typical chromatogram representing the degradation behaviour of molnupiravir in liquid state photo-stability

Photo degradation

For thermal stress, Molnupiravir-API was exposed to a controlled temperature oven at 50 °C and 150 °C for 5 h. The representative chromatogram is shown in Figure 12**.**

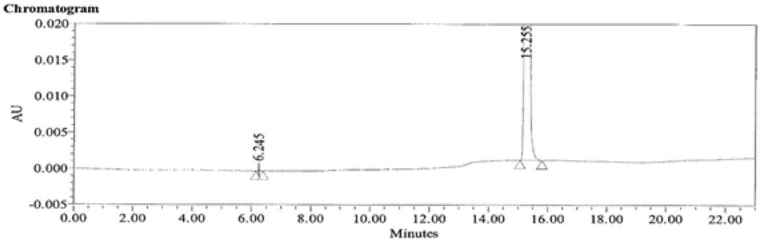

Fig. 12. Typical chromatogram representing the degradation behaviour of molnupiravir in temperature stress studies

Results of forced degradation studies

Forced degradation studies were performed for bulk drug, to provide an indication of the stability indicating property and specificity of the proposed method. Intentional degradation was attempted to under different stress conditions to evaluate the ability of the proposed method to separate Molnupiravir from its degradation products. Degradation was not observed in molnupiravir samples under stress conditions like hydrogen peroxide, UV light, and thermal exposure. However, mild degradation was observed when the drug was exposed to Acidic hydrolysis and alkaline hydrolysis. The concentration of molnupiravir was more slightly decreasing with time in acidic and alkaline hydrolysis. This degradation is mainly observed in terms of loss of assay. Table 4 indicates the extent of degradation of molnupiravir under various stress conditions. Therefore, it may be concluded that Molnupiravir is susceptible to degrade in Acidic and Alkaline conditions. Resolution between the analyte peaks and nearest peak was more than 2.0 in all the chromatograms. Figures 5 to 9 show the chromatograms of forced degraded samples. The degradation products were well resolved from molnupiravir, confirming the stability-indicating power of the method.

Table 4. Summary of forced degradation results

Stress condition	Time (h)	% Recovery	Retention time of the analyte	Peak purity
Acid hydrolysis (0.2 N HCl at RT)	24	96.45	15.250	pass

Acid hydrolysis (1.0 N HCl at RT)	24	94.12	15.222	pass
Base hydrolysis (0.2 N NaOH at RT)	24	94.37	15.216	pass
Base hydrolysis (1.0 N NaOH at RT)	24	92.23	15.224	pass
Oxidation (2% H_2O_2 at RT)	24	99.80	15.252	pass
Oxidation (5% H_2O_2 at RT)	24	99.51	15.253	pass
Photolysis (UV cabinet at 320–400 nm)	24	99.01	15.283	pass
Thermal treatment (50 °C)	5	98.97	15.276	pass
Thermal treatment (150 °C)	5	98.91	15.255	pass

RT-room temperature; API-Active Pharmaceutical Ingredient; cmean peak area is the average of three determinations

6. CONCLUSION

A simple, rapid, accurate, and precise stability-indicating HPLC analytical method has been developed and validated for the routine analysis of molnupiravir in API. The results of stress testing reveal that the method is selective and stability-indicating. The proposed method has the ability to separate the drug from their degradation products, related substances, and excipients found in tablet dosage forms and can be applied to the analysis of samples obtained during accelerated stability experiments

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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