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Method Development and Validation of a Specific Stability Indicating RP-HPLC Method for Molnupiravir API

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Original Research Article

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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 suggested approach was validated according to ICH (International Council on Harmonisation) principles, and all of the validation parameters' findings were within acceptable limits. The method was proven to be appropriate for quality control of Molnupiravir in bulk and pharmaceutical dose forms, as well as stability testing.

Keywords: Assay; stress studies; validation; molnupiravir.

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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].

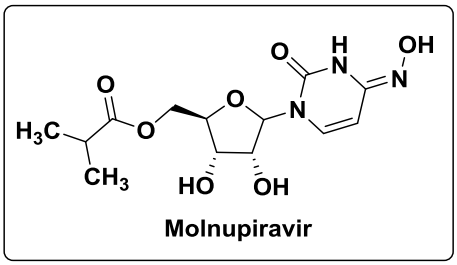

Fig. 1. Molnupiravir structure

Molnupiravir is an antiviral medicine used to treat COVID-19, and it has shown good antiviral effectiveness against SARS-CoV-2 in mice, resulting in reduced virus transmission [1]. Molnupiravir prevent the SARS-CoV-2 transmission in ferrets within 24 hr [2]. When comparatively remdesivir, it has structurally simple, no considerable complicity in manufacturing process and orally available in the market. Now clinical triles are going on related to safety of the drug [3-5]. As part of release, all the drugs must be analysed with a stability indicating assay method in current GMP practice. Based on the stress results we can estimate and identified the degradation products in drug substance and this can help to establishment of the degradation trend and the drug substance stability. The performance of the force degradation testing will be depending on the type of drug product and the individual drug substance. In this view the analysis of molnupiravir done in under variety of conditions, develop the LC method for separation the drug substance from the degradation products formed during forced degradation studies under ICH suggested conditions (thermal stress, hydrolysis, photolysis, and oxidation) [6-17]. These stress study experiments to provide good information about drug substance stability and which can help to establishment of method validation of drug substance [18]. A literature reveals that one method was reported for the determination of molnupiravir in biological fluids and marketed formulations [19]. The earlier literature reported that to developed liquid chromatographic method for estimation and separation of molnupiravir from process impurities and degradation impurities formed during stress studies. Any one not studied retention time of the method, in this present method the analysis of molnupiravir was achieved before 15 min. So, the aim of present work was to develop economic, selective, simple and stability-indicating liquid chromatographic method that can be used to determine the assay of molnupiravir and its related substances in bulk drug sample. This work separated the degradation impurities from drug substance generated from stress studies.

2. MATERIALS AND METHODS

2.1 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.

2.2 Instrumentation

This work was achieved on waters 2695 series from water technologies, USA with PDA detector. The resulted signals were processed and monitored by Empower software from waters technologies on HP computers.

2.3 Chromatographic Conditions

Agilent zobax eclipse C18 column with dimensions 250 mm X4.6 mm X5 µm and 30 mM ammonium phosphate monobasic and Methanol (47:53%V/V) mixture as a mobile phase were used for this analysis. Pumped the mobile phase to the column from solvent reservoir at flow rate of 1.5ml/min for 22min. The injection volume was 2µl and the eluate was monitored at 260 nm with column temperature at 35°C. For the preparation of standard and test samples, mobile phase was used as a diluent.

2.4 Blank Preparation

Mobile phase used as blank refer Fig. 2.

2.5 Preparation of Standard Solution

25 mg of molnupiravir standard and 10 ml of diluent were taken into 25ml of flask. Further

sonicated the solution for 5 min and then make up to volume with diluent. 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).

2.6 Preparation of Sample Solution

Molnupiravir sample solution was prepared by dissolving 25mg sample and 10ml of diluent into 25ml of flask. Sonicated the solution for 5 min and then make up to volume with diluent. The solution was filtered on 0.45µm filter paper for removal of high micron particles in solution. Further aliquot of the solution diluted 0.1 mg/ml concentration with diluent (Fig. 4).

3. RESULTS AND DISCUSSION

The molnupiravir assay method was achieved on Agilent Zorbax eclipsed C18-column (250mm × 4.6mm × 5µ) and it was maintained at 35 °C temperature. The mobile phase flow rate and composition were changed frequently to optimize the separation conditions between main related substances and stressed samples. After several chromatographic runs, 30 mM Ammonium phosphate monobasic buffer and Methanol (47:53, %v/v) were selected as a mobile phase components. All peaks were well separated from the primary peak under the given experimental conditions. In the resilience parameter, the effects of deliberate modifications in optimum technique circumstances were assessed.

4. METHOD VALIDATION

Validated the proposed analytical method by following parameters such as accuracy, precision, recovery, selectivity, robustness, LOD, LOQ and Linearity.

4.1 Linearity

The linearity was evaluated by plotting a graph in between peak area and sample concentration. A Linear dilution of molnupiravir sample solutions having the concentrations of 25 µg/mL to 150 µg/mL was analysed in the proposed method conditions. Correlation coefficient (n=6) was found to be more than 0.9992 with %RSD values being less than 2% across the concentration ranges studied. The calibration curve was showed in Fig. 5.

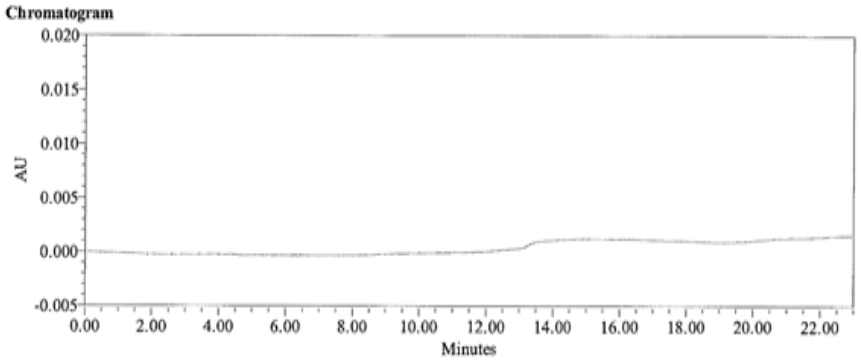

Fig. 2. Blank chromatogram

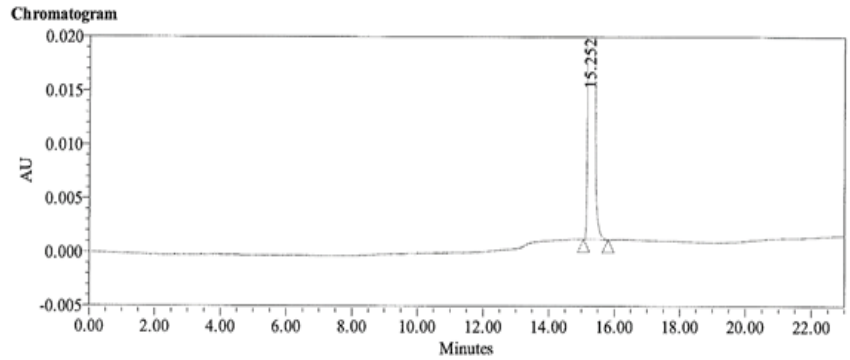

Fig. 3. Standard solution chromatogram of molnupiravir

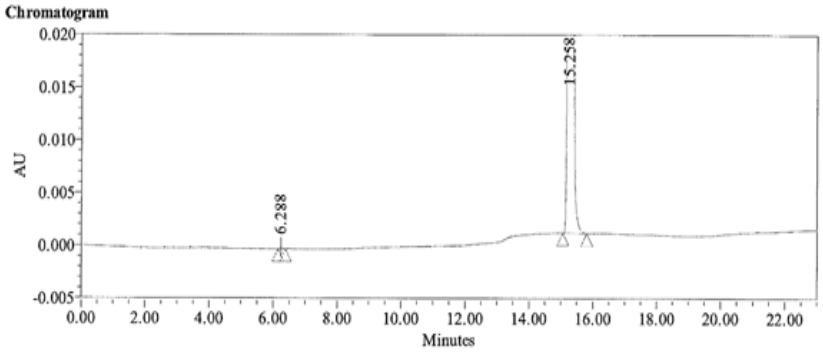

Fig. 4. Sample solution chromatogram of molnupiravir

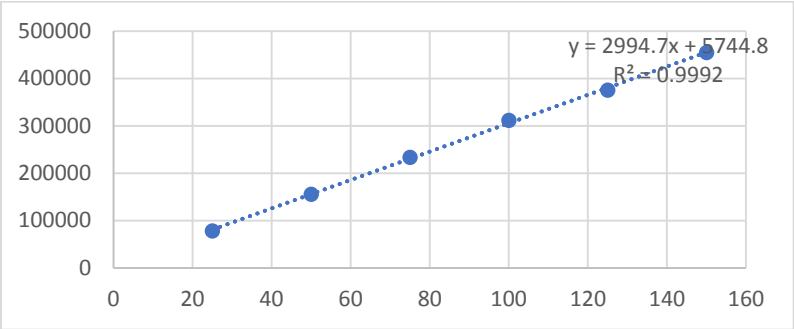

Fig. 5. Linearity Curve for molnupiravir

4.2 Limit of Detection and Limit of Quantitation

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

4.3 Precision

The method precision was determined by investigation of reproducibility and repeatability. By injecting nine replicate samples, repeatability of method was investigated. Each of the sample conc 25, 50 and 75 µg/ml and mean concertation found to be 25.5, 50.2 and 74.8 µg/ml with associated %RSDs of 0.23, 1.24, and 0.42, respectively. By injecting three same concentration samples over the three consecutive days inter day precision was established, 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 method of ruggedness was established by assays results of molnupiravir comparison between inter and intra day under taken by two analysts, resulted %RSD value did not exceed more than 2%.

4.4 Accuracy

For estimation of accuracy, to analysed the recovery of test sample by using known amounts of molnupiravir reference standard. The molnupiravir standard solutions (50 µg/ml) 0.8 mL, 1.0 mL, and 1.2 mL were added to three sample solutions containing a fixed amount of Molnupiravir (50 µg) in diluent, respectively. Therefore, this recovery study was performed with 40.0, 50.0, and 60.0 µg/ml of molnupiravir solutions. 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.

4.5 Specificity

The method specificity was determined to ensure that to distinguish between the drug substance from other components in sample matrix. We found that there is no interference observed between peaks in resultant chromatogram and all

the degradation components formed in stress studies were well separated from the drug substance peak. It indicated that developed method was specific and stability-indicating.

4.6 Robustness

The method robustness was determined by deliberated changes into the chromatography conditions such as column temperature, buffer composition and flow rate. The column temperature changed from 35 °C to 33 °C at lower side and changed from 35 to 40 °C at higher side. Flow rate changed between 1.4 to 1.6 mL/min from 1.5 mL/min and buffer concentration also changed between 20 to 40 mM from 30 mM. The small changes were observed in obtained results and tableted below Table 2.

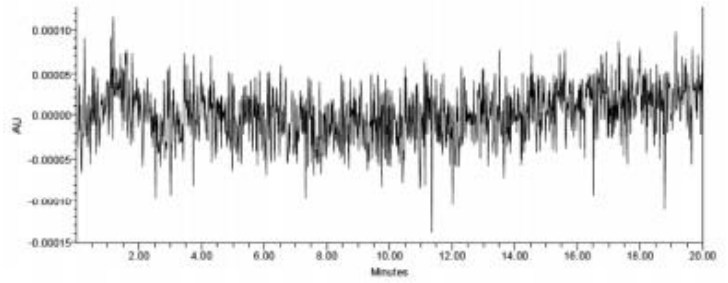

Fig. 6. A representative chromatogram of molnupiravir at LOD

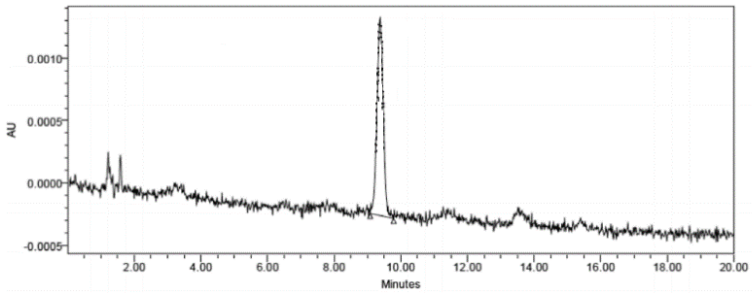

Fig. 7. A representative chromatogram of molnupiravir at LOQ

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

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

4.7 System Suitability Parameters

System suitability is defined that to ensure whether the validation parameters can generate acceptable results or not and usually it is need after completion of method development and validation. The regulatory agencies also recommended that it can be used prior to analysis. The system suitability parameters like tailing factor, resolution and theoretical plates were calculated and compared to the standard molnupiravir values to assess whether developed method given truthful results or not. The results are shown in Table 3.

4.8 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

As per regulatory guidelines ICH Q3B, Q2B, Q2A and 21 CFR, need to be study the forced degradation parameters for developed drug substance and drug product. In present development, molnupiravir stressed under different conditions such as acidic (using 0.2N, 1.0N HCl), basic (using 0.2 N, 1.0 N NaOH), oxidative (using 2.0, 5% hydrogen peroxide), thermal (heated at 50 °C, 150 °C for 5 h) and photolytic (UV cabinet) conditions. To determine proposed method ability for separate molnupiravir from its degradation products and stability of the drug substance. For all degradation studies 0.1 µg/ml conc of molnupiravir was used. After completion of degradation, the solution allowed to room temperature and neutralized with corresponding reagents.

5.1 Acid and Alkaline Degradation

25 mg of drug substance and 2 ml of 0.2 N HCl solution were taken into a 25 ml volumetric flak and kept a side at room temperature (26±2 °C) for 24 hr. after completion of degradation dilute to volume with diluent then pipette out aliquot into flask and diluted to 0.1 mg/ml concentration with diluent. The same procedure applicable to 1.0 N HCl. Similarly, alkaline stress conditions experiment was performed using 0.2 N, 1.0 N NaOH. The resultant chromatograms were shown in Figs. 8 and 9 respectively.

5.2 Oxidative Degradation

25 mg of sample and 2 ml of 2.0% H_2O_2 solutions were taken into a 25ml flask. The flask was kept aside at room temperature for 24 h. further it was diluted to 0.1 mg/ml concentration with diluent and it was analysed. The representative chromatogram is shown in Fig. 10.

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

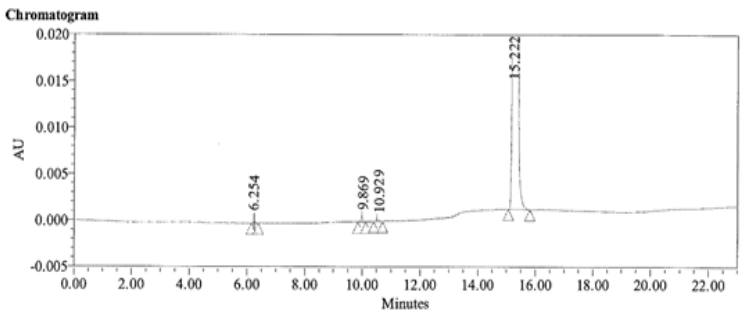

Fig. 8. Typical chromatogram representing the degradation behaviour of molnupiravir in acid hydrolysis

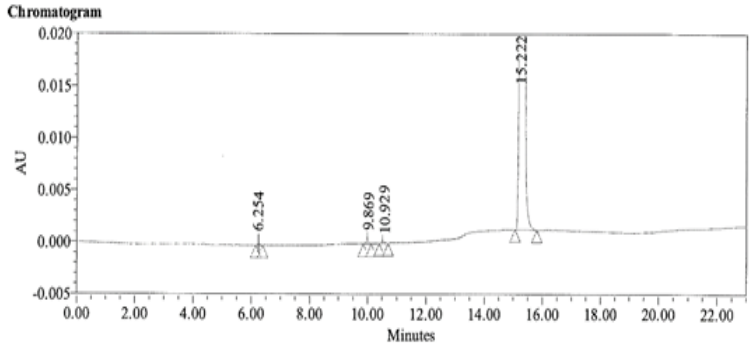

Fig. 9. Typical chromatogram representing the degradation behaviour of molnupiravir in alkaline hydrolysis

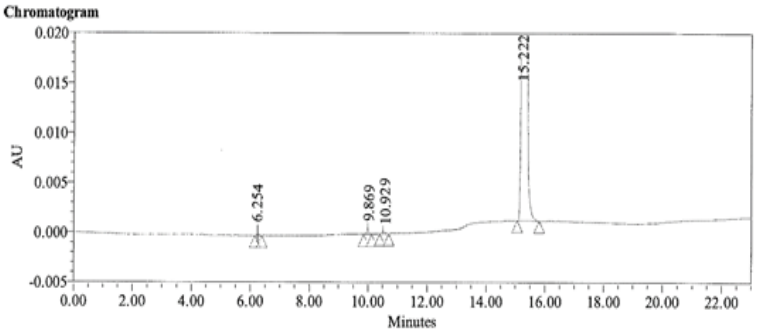

Fig. 10. Typical chromatogram representing the degradation behaviour of molnupiravir in oxidation

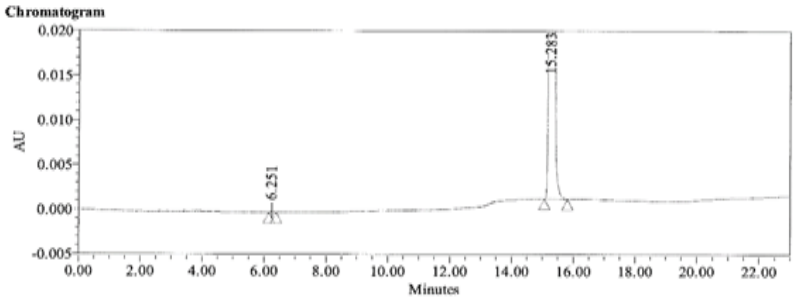

Fig. 11. Typical chromatogram representing the degradation behaviour of molnupiravir in liquid state photo-stability

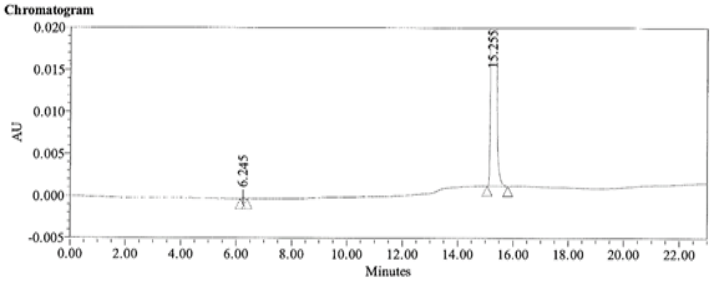

Fig. 12. Typical chromatogram representing the degradation behaviour of molnupiravir in temperature stress studies

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% H2O2 at RT)	24	99.80	15.252	pass
Oxidation (5% H2O2 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

C-mean peak area is the average of three determinations

5.3 Photo Degradation

~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 72 h resulting in an overall illumination of ≥210 Wh/m^2 at 25 °C with UV radiation at 320-400 nm. The representative chromatogram is shown in Fig. 11.

5.4 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 Fig. 12.

5.5 Results of Forced Degradation Studies

Forced degradation studies were performed for examination of the stability of drug substance and specificity of proposed method. Molnupiravir drug substance does not degraded under stress conditions like UV-light, hydrogen peroxide and thermal exposure. When it is exposed to acidic and alkaline hydrolysis mild degradation was observed. 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. Figs 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.

6. CONCLUSION

Simple and accurate stability-indicating HPLC method was developed for the routine analysis of molnupiravir API. The forced degradation results indicate that the method is robust and stability-indicating. The developed method was ability to separate the degradation products, related substances formed during preparation of drug substance and excipients from drug substance. Therefore, this method can be recommended for routine analysis and for checking quality during stability studies of the cited drug.

DISCLAIMER

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.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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