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Development And Validation of a Robust HPTLC Assay Method to Assess the Stability of Molnupiravir Under Various Stress Conditions

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KEYWORDS

Molnupiravir, HPTLC, Method development, Forced degradation, Assay, Validation.

ABSTRACT:

This research focuses on designing and validating a precise, sensitive, a stability-indicating HPTLC test technique for quantification of molnupiravir medicament formulation. Validation process adhered to the ICH guidelines. Developed High-Performance Thin-Layer Chromatography technique employed Silica Gel G60F254 as stationary phase for effective separation and analysis. The optimized mobile phase consisted of ethanol, ethyl acetate, and formic acid in a 3:6.5:0.5(v/v/v) ratio. Molnupiravir exhibited an Rf value of 0.76 ± 0.01, with a maximum absorbance at 282 nm for detailed analysis. The method exhibited strong linearity with an R^2 value of 0.9937 over range of concentration 100-300 ng/spot and was thoroughly authenticated for parameters including accuracy, precision, specificity, robustness and linearity. Research was carried out in many circumstances, such as acidic, alkaline, neutral, oxidative, thermal, and photolytic environments. with % of degradation calculated. Significant Degradation was noted. under oxidative and thermal condition. No degradation occurred observed under acidic conditions. The method proved to be selective and reproducible for molnupiravir in both bulk and capsule formulations. The developed method effectively separated drug degradants from the non-degraded drug, confirming its status as a stability-indicating assay method.

1. INTRODUCTION:

Molnupiravir, the first oral antiviral medication for COVID-19 treatment, was originally identified and cultivated in 2013.It works by focusing on RNA-dependent RNA polymerase (RdRp) of SARS-CoV-2, inhibiting virus's ability to replicate. By introducing errors inside the viral RNA, molnupiravir disrupts the generation of new viruses, thereby reducing the severity of the disease and the need for hospitalization[1]. Molnupiravir, scientifically identified as [(2R,3S,4R,5R)-3,4-dihydroxy-5-[4-(hydroxyamino)-2-oxopyrimidine-1-yl] methyl 2-methylpropanoate, was initially created as potential treatment for Venezuelan equine encephalitis virus (VEEV) infections. However, During the COVID-19 pandemic, researchers repurposed it as an antiviral therapy that hinders Replication of SARS-CoV-2 in human pulmonary cells. molnupiravir has been shown to reduce the risk of hospitalization by 50%. It is also referred to as β-d-N4-hydroxycytidine isopropyl-5′- ester (NHC). it induces RNA mutations, disrupting viral replication. Molnupiravir is rapidly hydrolyzed after administration, with a half-life of 3.3 hours. It is considered safe, with minimal side effects compared to other antiviral drugs like nirmatrelvir and ritonavir[2,3].

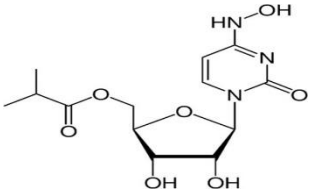

Fig 1 Structure of MLP

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Several documented analytical methods exist for the analysis of molnupiravir, including HPLC-MS-MS for quantifying metabolites, RP-HPLC, HPLC-UV, and stability analysis using HPLC[4-8]. These methods focus on HPLC development and validation, with some addressing stability studies. There is no HPTLC method reported and none of the HPLC methods provide detailed degradation studies. Hence this study aims to validate a method with forced degradation studies of molnupiravir using HPTLC-MS.

2. EXPERIMENTAL

2.1. Materials and Methods:

The HPTLC instrument used was a CAMAG system with Vision CATS software, equipped with a 100 µL syringe and a TLC scanner 3 with a Deuterium lamp. A Camag 15 µL sample syringe (Hamilton, Banaduz) was used for sample application. The UV spectrophotometer employed was a Jasco V-730 with a 1 mm quartz cell, managed by Spectra Manager software, and the wavelength used was 282 nm (Jasco International Co Ltd, Japan). Additional instruments included a pH meter (model Eutech 2700, Eutech Thermo), an analytical balance (model XPE26, Mettler Toledo), and an ultrasonicator (model Ucb-40 D, Spectra Lab).

A standard sample of Molnupiravir was generously provided by Divi's Laboratories Limited (Hyderabad, India). All solvents used in analysis were analytical reagent AR grade, sourced from Loba Chemie Pvt. Ltd. Formic acid, ethyl acetate, and ethanol were procured from Merck Private Limited (Mumbai, India). The analysis was carried out using pre-coated TLC aluminum plates which is having a 0.2 mm thick stratum of silica gel G60F254, measuring 200 × 100 mm. Molnupiravir was found to be soluble in DMSO (Dimethyl Sulfoxide) and ethanol.

2.2. Chromatographic Conditions:

Various mobile phases were tested, including:

- Ethanol: Ethyl acetate: Acetic acid in a ratio 3:6:1 (v/v/v), volumetric flow rate of 1.0 mL/min was used, with executed at 282 nm, resulting in irregular peak shape and hence not optimized.
- Ethanol: Ethyl acetate: Formic acid in ratio of 3:6:1 (v/v/v), volumetric flow rate of 1.0 mL/min was utilized, with executed at 282 nm. resulting in irregular peak shape and hence not optimized.
- Ethanol: Ethyl acetate: Formic acid in a ratio of 3:6.5:0.5 (v/v/v), volumetric flow rate of 1.0 mL/min was utilized, with executed at 282 nm. resulting in regular peak shape was optimized having the chamber saturation time set to 20 minutes, the development distance was 70 mm, and the Rf value was found to be 0.52.

2.3. Preparation of Stock solution

Weigh Accurately 100 mg Molnupiravir API and transfer it in 100ml volumetric flask. Add ethanol to get solubilize it in and then volume fabricated up to 100ml with ethanol to make ultimate concentration 1000 µg/ml. Further dilutions are made with ethanol by pipetting 1ml of stock solution and dissolve it in 100ml to make final concentration 100 µg/ml.

2.4. Stress Studies

Stress studies on molnupiravir API were conducted under various conditions, including acidic, basic, oxidative, thermal, photolytic, neutral.

2.4.1 Acidic environment

To assess the degradation of molnupiravir under acidic conditions, weighed precisely 10 milligrams of the molnupiravir API placed into a 10 ml volumetric flask for solubility add 2ml ethanol to it. For hydrolytic breakdown, transfer 2 mL of 0.1M HCl and then solution was exposed to 40ºC for 30 min. The acid-treated samples were then neutralized with an equivalent strength of base. Finally, HPTLC analysis was performed to identify any potential degradation products.

2.4.2 Basic condition

To assess the degradation of Molnupiravir under basic conditions, precisely weighed 10 mg of the API and placed in 10ml volumetric flask. Add 2ml ethanol to get soluble. Then add 2 mL of NaOH (0.1N). solution was exposed to 40ºC for 30 min. Solution was subsequently neutralized with 0.1N HCl diluted with ethanol to volume makeup of 10 ml.

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2.4.3 Oxidative condition

To evaluate the degradation of Molnupiravir under oxidative conditions, 10 mg of the API was precisely weighed, and 2 ml of ethanol to get solubilize. Add 2 mL of 6% H_2O_2 was transferred. The amalgamation was sustained at ambient temperature for 24 hours subsequently diluted to a volume of 10 mL using ethanol.

2.4.4 Thermal condition

For thermal stress testing, precisely weighed 10 mg of Molnupiravir API and exposed to 40°C in an oven for 30 Min. After the exposure, add ethanol to dissolve the drug and after that made up the final volume of 10 ml.

2.4.5 Photolytic condition

In UV chamber molnupiravir solution was exposed to UV light for 30 minutes. After exposure, TLC plates were used to apply 1 µL of the solution (100 ng per spot), and the chromatogram was then developed.

2.4.6 Neutral condition

To assess the degradation of Molnupiravir under neutral conditions, precisely weighed 10 mg of the API and dissolved in 20ml ethanol. Solution was stored at ambient temperature for 30min. After exposure period, 1 µL of solution (100 ng/spot) was deposited onto Thin Layer Chromatography plates and chromatogram was developed.

2.5 SIAM development

All degradation samples were prepared by pipetting 1ml and diluted to 10ml with ethanol (concentration 100µg/ml). 1µL sample is applied on precoated HPTLC plates for final acquisition final concentration of 100ng/band. Mobile phase, consisting of Ethanol: Ethyl Acetate: Formic Acid (3:6.5:0.5 v/v/v), was used for chamber saturation for 20 minutes. Specimens were spotted on HPTLC plate as bands with an 8 mm width. Bands were affixed at a 25 mm position, maintaining a 12.5 mm distance. The plate was created using a CAMAG HPTLC twin-trough chamber, densitogram was recorded using an HPTLC Scanner 3. Chosen detection wavelength the analysis was 282 nm. Peak regions of medication and degradation products were analyzed to determine the percentage of degradation.

2.6 Validation Method

2.6.1 Linearity

Initially prepared stock solution of 1000µg/ml transferred in to six separate volumetric flasks. Further dilutions were done to make final concentrations from 100 to 300 µg/ml. from that Subsequently, 3.0, 4.5,6.0,7.5 and 9.0 µL of samples were utilized for TLC plates. Calculated average area and the graph was plotted area versus concentration (ng/band). Then documented and calculated incline, captured, and relapse coefficient of the calibration curve.

2.6.2 Precision

Molnupiravir was analyzed on HPTLC at concentration levels of 100, 300, and 500 ng/band, with each concentration being tested six times.

2.6.3 Recovery

A research on recovery was conducted conducted by spiking the sample with a standard drug at three different levels. A research was conducted by using tablet formulation which having strength was 200mg, to which a known amount of Molnupiravir API was included at 50%, 100%, and 150% levels. Selected sample's base concentration 200µg/ml. Solutions of 100,200 and 300µg/ml were prepared. Take 1µl sample from each concentration and apply on TLC Plate corresponding to 100, 200 and 300 ng/band.

2.6.4 Robustness

To assess impact of mobile phase composition minor vatiation, ethyl acetate was adjusted by ±10%, resulting in two modified ratios: (Ethanol: Ethyl acetate: Formic acid) (2.7: 7: 0.3 v/v/v) and (3:5.8:0.7 v/v/v). Effects of these changes on the results were evaluated. Additionally, the time intervals between sample spotting and chromatography, as well as between chromatography and scanning, were varied by ±5 minutes to observe any potential influence on the analysis.

2.6.5 Specificity

Blank, standard and test solutions were examined to assess and evaluate the method's specificity. The purity of the Molnupiravir peak purity existed assessed and was determined to be 99.80% at 3 distinct tiers, based on a comparison with the graph position of the band.

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2.7 Marketed formulation assay

Accurately weighed 20 MOLNUTOR tablets each tablet contains 200mg of molnupiravir to determine its average weight. Then tablets were crushed and make a fine powder. Weighed about 100mg and transferred in 100ml volumetric flask which was added 70 ml ethanol. For 20 min solution was sonicated and then dilted the solution with ethanol for final concentration of 1000µg/ml. The excipients were permitted to sediment. To obtain final concentration 100µg/ml further dilutions were performed. On pre-coated TLC plate, A 1-µL aliquot was applied. after being developed in optimum mobile phase, plates were analyzed.

2.8 Characterization of degradation by products

100µg/ml Molnupiravir solution was prepared for acid base degradation study and applied 1µL spot to thin-layer chromatography plate. Plates were created using refined mobile phase. Electrospray ionization mass spectrometry was performed to analyze the degradation products providing fragment ions of degraded compounds. MS analysis aided in identifying the individual fragments present in the degraded samples.

3. RESULTS AND DISCUSSIONS

3.1 Wavelength selection for analysis

A standard solution of Molnupiravir was prepared in ethanol, and its λ max was observed at 282 nm.

3.2 Choice of chromatographic system

Molnupiravir in bulk dosage forms and degradation samples were quantified and optimized using HPTLC method. The sample was put to the TLC plate and saturated chamber for 20 minutes using mobile phase of ethanol: ethyl acetate: formic acid (3:6.5:0.5, v/v/v). The plates were developed in optimized mobile phase. Then densitogram were recorded and showed an Rf value of 0.76 ± 0.01, as illustrated in Fig. No. 2(a).

3.3 SIAM advancement

Standard molnupiravir was treated with various conditions including Acidic, alkaline, oxidative, photolytic, thermal, hydrolytic conditions. Molnupiravir standard and its degradation products well separated bands were observed each with distinct Rf values. The results are presented in Table 1, with photo-documentation of degradation shown in Fig. No. 2(b). Degradation was observed under oxidative and thermal conditions, while minor degradation occurred under basic, neutral, and photolytic conditions. % degradation found high in oxidative conditions.

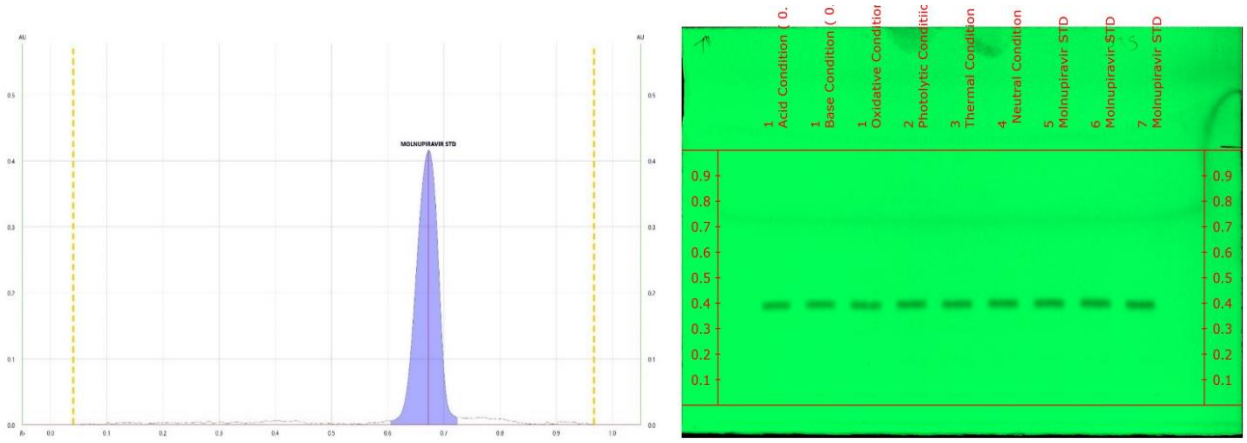

Fig 2 Densitogramof molnupiravir (100ng/band) **a** photodocumentation for degradation **b**

Table 1. Percentage stress degradation of Molnupiravir (%)

Conditions	Degrading agents	Exposure Period	No of peak	% Area	% Degradation
Acidic Decomposition	0.1N HCl	40°C For 30 min	MOL STD	100.54	No Degradation

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			Digredant 1		
Base Degradation	0.1N NaOH	40°C For 30 min	MOL STD	98.45	1.55
			Digredant 1		
Oxidative Degradation	6% H2O2	Room Temp	MOL STD	88.02	11.98
			Digredant 1		
			Digredant 2		
Thermal Decomposition	40°C	40°C For 30 min	MOL STD	91.59	8.41
			Digredant 1		
Neutral Decomposition	Room Temp	For 30 min	MOL STD	97.71	2.29
			Diluent		
Photolytic Degrdation	UV Chamber (UV Light)	For 30 min	MOL STD	98.05	1.95
			Digredant 1		

3.4 Validation

The validation study results for stability-indicating HPTLC method devised for Molnupiravir, using mobile phase ethanol: ethyl acetate:formic acid (3: 6.5: 0.5, v/v/v), are presented below. Summary of validation parameters is provided in Table 2.

Table 2. The summary of validation parameters is as follows:

Specifications	Acceptance criteria	Results
Linearity (ng/band)	≥0.99	R^2= 0.99
Precision	≤2%	precise
Accuracy/ Recovery	98-102%	100.80%
Specificity	No interference	specific
Robustness	≤2%	Robust

3.5 Assay of Marketed Formulation

In the formulation research Molnupiravir Drug content was detected in range of 99-102%. The method was found reliable by analyzing marketed molupiravir formulation which found a low relative standard deviation (%RSD) of ±0.96. Therefore, for the systematic assessment of the pharmaceutical this method can be effectively used.

4 CONCLUSIONS

The HPTLC method for estimating molnupiravir has been successfully developed and validated. Key attributes of this method include speed, precision, and

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accuracy. The the method's specificity, repeatability, and accuracy have been confirmed through statistical analysis. Forced degradation investigations were performed on the API, further supporting the method's reliability. Therefore, It may be inferred that stability is indicated assay a technique was devised for routine examination.

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DECLARATIONS

The author declares that no conflict of interest exists.

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