



A Stability Indicating Novel UPLC-PDA Method for the Estimation in Bulk and Capsule Dosage form of Isavuconazole Effective for Mucormycosis Caused by COVID-19 Pandemic

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Authors' contributions

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

Article Information

DOI: 10.9734/JPRI/2021/v33i43A32516

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Complete Peer review History: <https://www.sdiarticle4.com/review-history/72997>

Original Research Article

Received 11 August 2021

Accepted 31 August 2021

Published 08 September 2021

ABSTRACT

The present research provides a new proven ultra-high-performance liquid chromatographic technique for isavuconazole in both bulk and capsule dosage forms. DIKMA Waters BEH C18(50 x 2.6mm, 1.7m) column was used for chromatographic separation. With the isocratic elution mode, a mixture phosphate buffer: methanol 40:60 v/v, was used as the mobile phase, and the eluent was measured at 253 nm using a UV detector. The strategy has been maintained and validated in accordance with the International Conference on Harmonization Guidelines. Stressed deterioration has also been investigated in acidic, alkaline, peroxide, thermal, and photolytic conditions. Isavuconazole was eluted with the retention time 0.861 minute in this procedure. The calibration

curve plots for isavuconazole were found to be linear within the concentration ranges of 1-25µg/mL. With a percentage recovery of 100.18 percent, the limit of detection was 0.025g/ml and the value for limit of quantification was 0.50µg/mL. In a force degradation analysis, the current approach was also determined to be stable. Based on the experiential evidence, the present method was found appropriate of isavuconazole estimation in the form of bulk and capsule.

Keywords: Isavuconazole; method development; method validation; UPLC; ICH guidelines.

ABBREVIATIONS

UPLC : Ultra performance liquid chromatography;

PDA : Photo diode array;

LOD : Limit of detection;

LOQ : Limit of quantitation;

SD : Standard deviation;

RSD : Relative standard deviation.

API : Active Pharmaceutical ingredient.

ICH : International conference on harmonization;

1. INTRODUCTION

Mucormycosis, formerly known as zygomycosis and better known as black fungus, refers to a group of diseases caused by fungi belonging to the Mucorales order. The main causative organisms in this group are Rhizopus species. Doctors believe that mucormycosis, which has an overall mortality rate of 50%, may be being triggered by the use of steroids, a life-saving treatment for severe and critically ill Covid-19 patients. [1]. It affects the nose, eye, sinuses and brain most commonly, causing a runny nose, one-sided facial edema and headache, fever, pain, swollen and bulging eye, impaired vision and tissue death. As of May 25, 2021, more than 11,700 people in India were being treated for mucormycosis as a result of the COVID-19 pandemic, according to the Indian government. Because of the black appearance of dead and dying tissue caused by the fungus, it was dubbed "black fungus" by many Indian news sites. Mucormycosis rates in India were predicted to be 70 times greater than the rest of the world even before the COVID-19 pandemic [2]. Some Indian state governments have labelled it an epidemic due to the fast-increasing number of cases [3]. One treatment was a daily injection of amphotericin B, an antifungal intravenous injection that was in short supply, for eight weeks. Isavuconazole is a novel extended-spectrum triazole that can be used to treat yeasts, moulds, and dimorphic fungi. It has been licensed to treat invasive aspergillosis and mucormycosis [4]. 3-(2,5-difluorophenyl)-3-hydroxy-4-(1,2,4-triazol-1-yl)butan-2-yl]-1,3-thiazol-4-yl] benzonitrile (CAS 241479-67-4), is

its chemical name (Fig. 1). It acts by preventing the formation of fungal cell membranes. Patients with invasive fungal infections, especially those who are immunocompromised, have considerable clinical problems [5]. For the estimation of isavuconazole using modern analytical instruments, a meticulous literature review was conducted. It is evident that only one RP-HPLC method for estimating isavuconazole in bulk and formulation was available. HPLC has been used in two bioanalytical procedures [6,7] for the quantification of isavuconazole alone from human plasma. The LCMS approach was also used to determine isavuconazole simultaneously with four additional triazoles [8]. The only one described RP-HPLC method [9] of isavuconazole identified with various deficiencies such as the authors did not depict any assay chromatogram of marketed formulation. The authors did not report any stability study of isavuconazole, whereas the reported retention time was 2.96 minutes. The reported linearity levels are extremely narrow, which limits the method's usefulness. In light of the aforementioned drawbacks, a quick, specific, reliable, and validated stability indicating method for this analyte must be developed and reported. The benefits of ultra-performance liquid chromatography over high-performance liquid chromatography in terms of turnaround time, process reliability, method sensitivity, and drug specificity encourage the employment of LC techniques for a variety of drug active chemical groups [10]. Therefore, the current research task aimed to eliminate all drawbacks and develop a fast, stability-indicating UPLC method for estimating isavuconazole in bulk drugs and capsule dosage forms, and also a validation study, in compliance with the International Conference on Harmonization (ICH) Guidelines Q2 (R1) [11].

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

The active pharmaceutical ingredient Isavuconazole was generously provided by Virupaksha Organic Ltd in Hyderabad,

Telangana, India (99.84 percent purity). Pulse Pharmacy India Pvt Ltd, Hyderabad Telangana, delivered the capsule dose form, cresemba-100mg hard capsule (Isavuconazonium sulphate equivalent to Isavuconazole 100mg). Loba chemicals in Mumbai, India, supplied the methanol, which was of HPLC quality. Analytical grade dipotassium hydrogen phosphate, orthophosphoric acid, and hydrochloric acid were given by SD-fine chemicals in Mumbai. BVK technology services in Hyderabad, India, provided HPLC quality distilled water.

2.2 Instrumentation Conditions

The analysis of Isavuconazole was carried out using a Waters Acquity BEH C18 (2.1 x 50mm, 1.7 μ m) UPLC column. Empower 2 is a software package that includes an Auto Sampler and a PDA detector. 0.1 mg sensitivity AfcoSet ER-200A analytical balance was utilized. In the investigation we also used a pH meter (Adwa – AD 1020), and a Labline India's Ultra sonicator model 1.5L(H). With isocratic elution, the flow rate was maintained at 0.3ml/min. The PDA detector was used to detect the signal at 253 nm.

2.3 Preparation of Volumetric Solutions

2.3.1 Preparation of 0.05 M Phosphate buffer

6.805 grams potassium dihydrogen orthophosphate was weighed and put to a 1000 mL beaker, where it was dissolved and diluted with HPLC water to 1000 mL. Orthophosphoric acid was used to bring the pH down to 4.2.

2.3.2 Preparation of mobile phase

Phosphate buffer 400 mL (40%) and 600 (60%) mL Methanol UPLC grade (95%) were mixed together and degassed in an ultrasonic water

bath for 5 minutes. Under vacuum filtration, filter through a 4.5-micron filter.

2.3.3 Preparation of standard solution

Accurately 50 mg of Isavuconazole was carefully weighted and transferred into a 50 mL clean dry volumetric flask, 5 mL of diluent was used to dissolve the analyte, and sonication was used to completely dissolve the analyte. To get a concentration of 1000 g/mL, the volume was brought up to the mark using the diluent.

2.3.4. Preparation of working solution

From the aforementioned stock solution, 10 mL of isavuconazole standard solution was pipetted into a 100 mL flask. 1 mL aliquot was transferred to a 10 mL volumetric flask and dilute up to mark with diluent to produce 10 μ g/mL.

2.4 Assay of Marketed Formulation

The weight of ten isavuconazole hard capsules was calculated as the average. The capsule cells were removed, and the granules were triturated with a mortar. A quantity of powder equal to 50 mg of pure Isavuconazole was placed in a 50 ml volumetric flask, 5 ml of methanol was added, and the flask was sonicated for 15 minutes to dissolve the contents. To make the volume 50 ml, the mobile phase was added. The final solution with a concentration of 100 μ g/ml was prepared by diluting 10 mL of the above aliquot to 100 mL. One mL aliquot was transferred to a 10 mL volumetric flask, and the mobile phase was used to fill the flask to the desired volume. To remove the gas, the solution was filtered through a membrane filter (0.45 μ m) and sonicated. The prepared solution was injected into the UPLC system in five replicates using the optimum chromatographic state. The amount of isavuconazole in the sample was calculated.

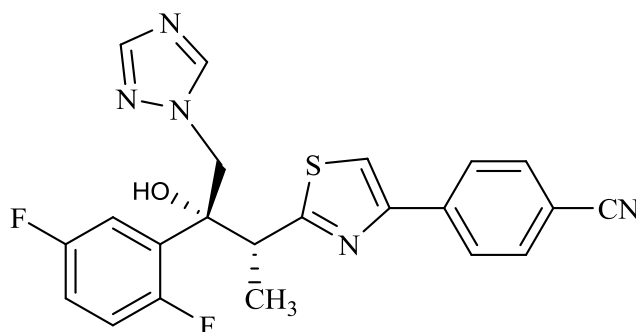


Fig. 1. Chemical structure of the Isavuconazole

2.5 Methods Validation

2.5.1 Specificity

It was accomplished by performing a placebo intervention test on the sample solution, which required dissolving 500 mg of placebo (equal to one pill) in 100 ml of mobile phase and treating the placebo solution as if it were a regular solution. To test if there were any interference peaks, the solution was injected into the UPLC instrument.

2.5.2 System suitability

This study was conducted to determine whether the analytical procedure was working effectively. Six duplicates of a standard isavuconazole solution were injected. The percent RSD of several optimal parameters has been computed, including theoretical plates, peak area, retention period, and asymmetry factor.

2.5.3 Accuracy

The recovery research [11] was carried out with different concentrations of pure isavuconazole (80%, 100%, and 120%) to verify the accuracy of the established procedure. The different levels were made by mixing varying amounts of standard isavuconazole with different concentrations of isavuconazole tablet sample solution. The percentage recovery and percentage mean recovery time were measured three times in this investigation.

2.5.4 Intraday & Inter day precision

The precision of the new approach was tested [12] by determining the working concentration of an isavuconazole sample. Six sample solutions of 10g/mL isavuconazole solution were analyzed in triplicate (n=6) to determine the quality of the sample. The intraday- and inter-day precision were done by examining six times on the same day (intra-day study) and repeat this process on the second and third days (inter-day study). The results were tabulated and the chromatograms were taken down. The relative standard deviation, as well as the peak area and retention time of isavuconazole, were calculated.

2.5.5 Limit of detection

To reach a concentration of 100µg/mL, 10 ml of the isavuconazole solution from the primary stock solution was accurately diluted to 100 ml

with mobile phase. 1 ml of the aforementioned solution was extracted and the volume raised to 10 ml to create a further dilute concentration of 10 g/ml. The 0.25 ml aliquot was removed and the final volume was elevated to 10 ml to get a final concentration of 0.25 µg/ml. The isavuconazole solution was introduced into the UPLC system after filtering. For the computation of the limit of detection, the signal to noise (S/N Ratio) value must be 3. The solution for LOD study were made as fixed, injected three replicates and the injection region was estimated for each injection. This study was performed in six replicates of injections; the percent RSD was calculated.

2.5.6 Limit of quantitation

To attain 100 µg/ml, 10 ml of isavuconazole solution was collected from the primary stock solution and diluted to 100 ml using mobile phase. To obtain dilute concentrations of 10 µg/ml, 1 ml of the aforesaid solution was taken and the volume increased to 10 ml. The 0.5 ml aliquot was removed and transferred to a 10 ml flask, where the liquid was increased to the desired concentration (0.5 µg/ml). After filtering the solution, it was injected into the UPLC system. For the computation of the limit of quantitation, the signal to noise (S/N Ratio) ratio must be 10. Three times, the prepared LOQ solutions were injected. The % RSD was calculated and it should be within the specified range.

2.5.7 Linearity

Standard isavuconazole standard solution was prepared as previously stated, and an aliquot of these solutions was diluted with mobile phase in seven different strengths to provide a solution with isavuconazole concentrations ranging from 1 to 50 µg/ml. After preparing a calibration curve employing concentration versus peak area, the resulting values were subjected to regression analysis. The connection between concentration and peak area in the examined range should be linear, with a correlation coefficient of at least 0.99.

2.5.8 Robustness

The robustness analysis of isavuconazole was conducted to establish the present technique's ability to persist unaffected by minor, but deliberate modifications in the study parameters, and also to provide an indicator of the developed

method's reliability in standard use. The isavuconazole standard solution was injected into the chromatographic system while the detection wavelength (251nm and 255nm), flow rate (0.2 and 0.4mL/min), and organic solvent composition (58 percent and 62 percent methanol) were changed from the typical chromatographic conditions. The USP plate count and tailing factor were employed to investigate the robustness of the recognized procedure.

2.6 Force Degradation Study of Isavuconazole

The force degradation study [13] was conducted under a variety of ICH-recommended stress settings, including acidic, alkaline, oxidative, thermal, and photolytic stresses. In such circumstances, degradation tests were performed in triplicate, with the mean peak area considered into the calculations.

2.6.1 Acid degradation

To do perform a force degradation investigation in acidic media, 1 ml of 1 M HCl was added to 1 ml of isavuconazole working solution and heated at 80°C for around 2 hours before being neutralized with 1 M NaOH and set aside for 24 hours before being injected into the chromatographic apparatus.

2.6.2 Base degradation

Using 1 mL of 1 M NaOH to 1 mL of isavuconazole stock solution resulted in a forced degradation study on the basic media. The solution was heated for 2 hours at 80°C, then the solution was neutralized with HCl (1M) and stored at room temperature for 24 hours. Prepared solution was injected and chromatograms were taken.

2.6.3 Oxidative degradation

An aliquot of isavuconazole stock solution was obtained for this study, and 6 percent H₂O₂ solution (1mL) was added and set aside for 24 hours. Chromatograms were taken after the stressed solution was administered.

2.6.4 Thermal degradation

Isavuconazole aliquot stock solution was kept at 80°C for 2 hours and then kept at room temperature for 24 hours to investigate the

impact of temperature. The resulting solution was injected into the chromatographic system, and analyzed.

2.6.5 Photolytic degradation

An aliquot of isavuconazole stock solution was held at 80°C for 2 hours and then kept for 24 hours to evaluate the influence of temperature. The solution was then injected into a UPLC device and evaluated.

3. RESULTS AND DISCUSSION

3.1 Methods Optimization

Various UPLC chromatographic settings were attempted in order to find the best method for estimating isavuconazole in API and capsule format. During the early trials, several factors such as mobile phase composition, column type, mobile phase pH, and diluents were modified. Various solvent and buffer proportions were investigated in order to get an appropriate mobile phase composition for method optimization. Finally, using a mobile phase of methanol and phosphate buffer (60:40) and a flow rate of 0.4 mL / min, isavuconazole was eluted with excellent peak shape and low retention time. With PDA detection at 253 nm, the isavuconazole had a retention time of 0.86 minute which is sufficiently less therefore, the elution was consider faster. The established technique was validated in accordance with ICH criteria. Fig. 2 depicts the optimized chromatogram.

3.2 Method Validation

The established optimized method for isavuconazole was used in succeeding validation experiments. The present method's applicability for the quantitative investigation of isavuconazole capsule dosage form is demonstrated by the percentage assay of 98.7% in the marketed capsule dosage form. The assay result was found within the acceptance criteria. The chromatogram of the capsule dosage form is illustrated in Fig. 3, and the results are listed in Table 1.

Standard quality control samples were used to determine the accuracy and precision of the established procedure. The accuracy (mean percent recovery) was found to be 100.18, and the percent RSD was determined to be less than 2%. The accuracy of the proposed method was confirmed after an accuracy study was conducted in accordance with the ICH criteria, and the %

recovery was found to be within the permissible level, as discussed in the results and confirms the accuracy of the developed method. The percent RSD of the intraday and interday precision studies was 0.91 and 0.87, respectively, The precision (% RSD) of intraday and interday data was judged to be adequate and within acceptable bounds. The precision study's findings revealed that the suggested methodology was confirmed to be precise. Table 2 also included the results of the accuracy and precision investigation. A system suitability analysis was carried out to ensure the efficient functioning of the analytical measuring equipment by looking at many parameters (retention time, peak area, theoretical plate, tailing factor). The relative standard deviations of peak area, theoretical plates, tailing factors, and retention duration were 0.25 percent, 0.73 percent, 0.87 percent, and 1.21 percent, respectively. It indicates the suitability of the system to carry out the present method. The results of the specificity analysis clearly showed that no excipient peaks were identified at the retention time of isavuconazole, proving the method's specificity for the present developed

method. The linearity analysis was conducted in the concentration range of 1-50 $\mu\text{g/ml}$, and the correlation coefficient for the analyte isavuconazole listed in Table 2 was 0.999. The acceptable correlation coefficient, which is close to 1, is used to create the regression line and find the linear curve in the method's linearity analysis. The results indicate the linearity of the developed method. Isavuconazole has detection and quantitation limits of 0.25 $\mu\text{g/ml}$ and 0.5 $\mu\text{g/ml}$, respectively, The acquired limit of detection and quantitation values demonstrated the proposed method's sensitivity. The method's robustness was tested by changing three parameters from the chromatographic conditions: mobile phase composition (2%), flow rate (0.1ml/min), and detection wavelength (2 nm), and the percent RSD of the tailing factor, which was used as a tool parameter, was found to be 1.44 as shown in the table of validation parameters (Table 2), indicating that the current established method for isavuconazole is robust, as no such significant changes were observed on the deliberate changes in the process parameters.

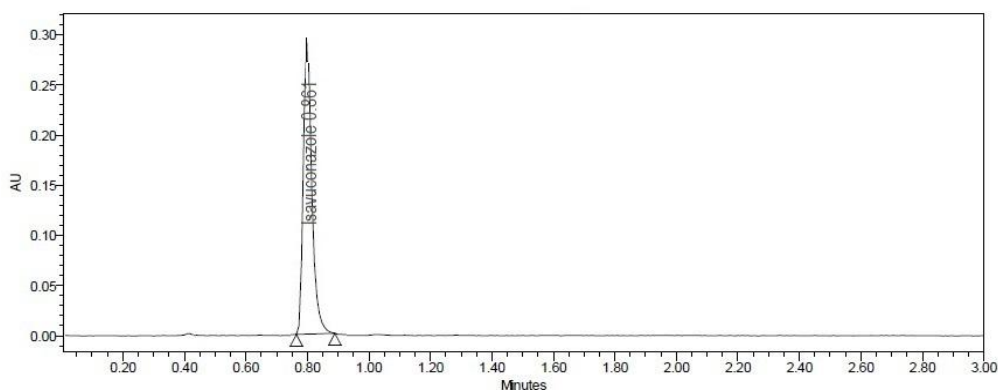


Fig. 2. Optimized chromatogram of the isavuconazole

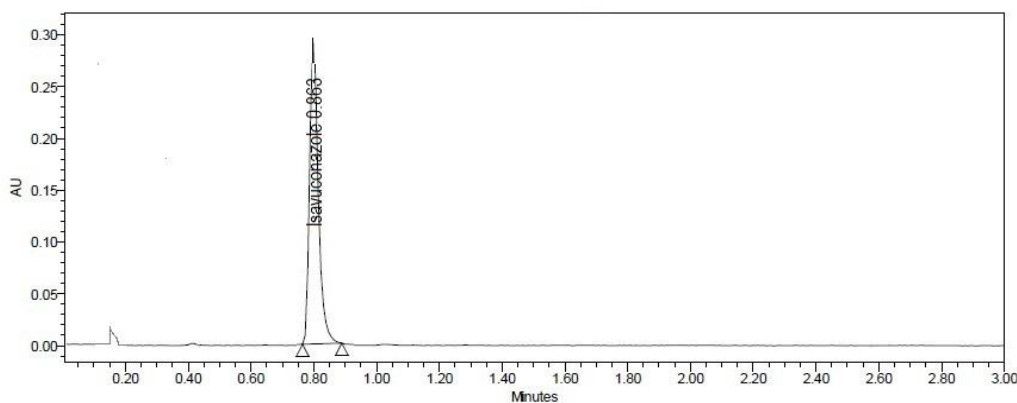


Fig. 3. Chromatogram of the marketed isavuconazole capsule

Table 1. Assay of Isavuconazole marketed formulations

Marketed formulation	Labelled claimed	Amount obtained*	Percentage purity of Isavuconazole*
Cresemba-100mg hard capsules (Isavuconazole 100) manufactured by Pfizer limited, Purchased from pulse pharmacy India Pvt. Ltd.	100 mg	98.72 mg	98.72 %

*Average of three replicates

Table 2. Summary of the validation parameters results

Parameters	Isavuconazole
LOD µg/ml	0.025
LOQ µg/ml	0.5
Linearity range (µg/ml)	1-25
Regression co-efficient	0.999
% *Mean recovery (accuracy)	100.18
Intraday precision** (% RSD)	0.91
Inter-day precision** (% RSD)	0.87
% RSD of tailing factor* (Robustness study)	1.44

*Average of three replicates. ** Average of six replicates

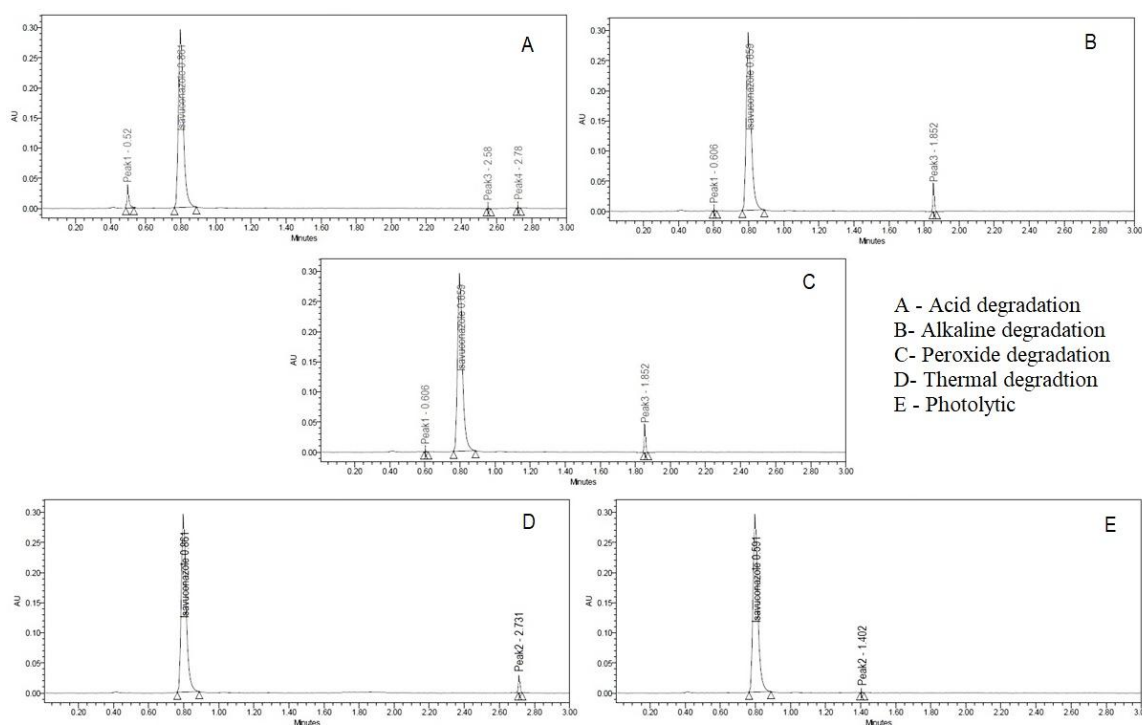


Fig. 4. Chromatograms of the isavuconazole during force degradation studies

Isavuconazole degradation tests were carried out in a variety of stressful circumstances, including acid, alkali, oxidation, thermal, and photolytic environments. Almost all stressed conditions, with the exception of photolytic stressed conditions, showed degradation. Degradation is 4.39 percent

in acidic strained conditions, 2.07 percent in alkaline stressed conditions, and 2.19 percent in peroxide stressed conditions. The heat degradation was found to be 1.03 percent, with the results summarized in Table 3 and chromatograms illustrated in Fig. 4.

Table 3. Force degradation study results for Isavuconazole solution

Sample Name	Isavuconazole				
	Mean Area*	% Degraded	Purity Angle	Purity Threshold	Peak purity
Acid	385778	4.39	1.26	2.49	Passes
Base	406354	2.07	0.72	1.52	Passes
Peroxide	391189	2.19	0.65	1.41	Passes
Photo	526390	1.03	0.26	0.98	Passes
Thermal	536295	0.17	0.05	0.78	Passes

According to the results of isavuconazole force degradation investigations, acidic and peroxide stressed conditions cause slightly higher degradation than other stressed conditions, however photolytic conditions cause no degradation. The results of the force degradation study indicates that in every stressful scenario, the chromatogram of isavuconazole was shown to be exceedingly specific.

4. CONCLUSION

The authors can safely claim that the present developed approach is innovative in comparison to other cases depending on the experimental findings of this developed method. Because the overall analysis time was 0.86 minutes, which is the least time required, the present method is the first reporting stability indicating UPLC method that is termed "rapid." It has shown less degradation in challenged situations and particular separation of isavuconazole from other degraded peaks, the current method is considered "stability promising." All validation parameters yielded findings that were acceptable according to the ICH Q2B criteria. Finally, it can be stated that the newly proposed method must be used for quality control and routine analytical tests of isavuconazole in capsule dosage form, which is an innovative, accurate, and validated approach.

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.

ACKNOWLEDGEMENT

The authors are thankful to Netaji Subhas Chandra Bose Institute of Pharmacy, Chakdaha, West Bengal for providing the necessary facilities to carry out the research work.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:

The peer review history for this paper can be accessed here:
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