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Reversed-Phase High Performance Liquid Chromatography Procedure for the Determination of Isavuconazole in Human Plasma

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Abstract

Introduction: Isavuconazole is an antifungal drug used for treating patients with invasive fungal infections. Efficacy and safety of isavuconazole is monitored by measuring plasma isavuconazole concentration using LCMS which is a non-affordable method. We used the HPLC system with a UV detector to measure plasma Isavuconazole concentration.

Objective: Improved Reversed-phase high performance liquid chromatography procedure with UV detection is described which is cost effective, simple, precise and easily processed for the measurement of Isavuconazole, a drug used to treat the patients with invasive fungal infections, in blood plasma. **Method:** The method involves protein precipitation, addition of ammonium dihydrogen phosphate and chromatographic separation on a Hypurity C18 Column using an isocratic mobile phase of acetonitrile and ammonium acetate buffer (pH 8.0, 10 mM) (55:45, v/v). The UV detection was performed at 285 nm. The method provides rapid resolution of Isavuconazole in a 50 uL injection.

Result: Lower limit of Quantification (LLOQ) is 0.25 µg/ml in a 50 uL injection volume for Isavuconazole with a recovery consistently > 100 %. The assay is validated over linear range of 0.25 to 10 µg/ml. The intra-assay precision is < 3.53 % and inter-assay is <6.38% relative standard deviation of Isavuconazole. The method demonstrated clean separation, clinically acceptable detection limit and a linear range upto 25 ug/mL.

Conclusion: The assay demonstrated applicability in quantifying the drug level and monitoring the therapeutic dose for maintaining effective biological level to have better response in fungal infected patients. The method is cheaper as compared to LC-MS/MS and Tandem Mass spectrometry and the results are reportable on the same day of blood collection.

Keywords: Isavuconazole, Therapeutic drug monitoring, HPLC

Introduction

Isavuconazole is an antifungal drug used for the therapy of patients with both invasive aspergillosis and mucormycosis, approved by the Food and Drug Administration and European Medicine Agency in 2015 [1]. It is administered as a prodrug Isavuconazonium sulfate in the form of oral capsules or a water soluble Intravenous (iv) formulation and is hydrolyzed to its active moiety BAL4815 by plasma esterases [2]. The explanation of wide antifungal activity of Isavuconazole when compared to other triazoles is the side arm of Isavuconazole which orients the triazole ring of molecule to the binding pocket in fungal CYP51 protein [3].

The recommended loading dose of Isavuconazole is 200 mg every 8 hours for 48 hours by both oral and IV formulation. This is followed by maintenance dose of 200 mg of Isavuconazole once daily [1]. The pharmacokinetics (PK) of Isavuconazole are very predictable, with high oral bioavailability (98%), is rapidly absorbed after oral administration with a C_{max} of 2-3 h which undergoes hepatic metabolism and has a long half-life (100-130 h). The efficacy of isavuconazole is similar to amphotericin B in treatment of mucormycosis, but Isavuconazole is well tolerated.

To reduce drug-related adverse effects and optimize clinical outcome for improving efficacy, therapeutic drug monitoring (TDM) of Isavuconazole is required in certain clinical cases; e.g. severe gut disease where oral absorption may be problematic, treatment of central nervous system disease, treatment of a non-wild-type fungal pathogen. TDM may also be indicated in circumstances where there is currently little information e.g. dosing in children or adolescents. TDM of Isavuconazole is indicated in unresponsive infections, in the treatment of pathogens with reduced susceptibility, or in the case of drug-drug interactions .TDM of Isavuconazole is also recommended in subjects with severe hepatic impairment, as little is known about the drug pharmacokinetics properties in this group of patients [3,4].

So far few analytical methods have been reported for Isavuconazole measurement in human plasma by using mass spectrometry or fluorescence detection [5]. These specialized equipments are not easily available in clinical laboratories in Indian settings. The LC-MS/MS is very sensitive but it is an expensive instrument. Some have reported Isavuconazole estimation by tandem mass spectrometry method [6]. and by UPLC with UV [7]. The HPLC-UV is simple and economical instrument for measuring drug concentration. A method was developed using HPLC-UV by Nannetti G et al, where they have used deproteinization and Solid Phase Extraction (SPE) as well. To support extensive analysis of Isavuconazole, a HPLC-UV method was validated, which is cost effective, easier and simpler to setup in a clinical laboratory in India with limited financial resources using existing standard equipments of HPLC. Validation and development of method for Isavuconazole in human plasma using HPLC-UV is done for the first time in India, to the best of our knowledge till date. The objective of the study was to develop a modified HPLC-UV method which is cost effective, simple, and precise and can be performed easily in laboratories in Indian Settings for the patients being treated with Isavuconazole. We report here an improved HPLC method using C18 reverse phase column and ultra violet detection.

Materials and Methods

Equipment and Accessories

UHPLC System was of Model-Ultimate 3000 from Thermo Fisher Scientific India Pvt Ltd and UV Detector (VWD-3400RS) was used and linked to the Software Chromeleon 7.2.10. Chromatographic separation was carried out using Isocratic column, Hypurity C18 Column (250x4.6) mm, particle size-5µ and Guard Column (ClinRep).

Chemicals and Reagents

Isavuconazole powder was obtained from Sigma Aldrich with chemical purity of ≥98%. Acetonitrile (HPLC Grade) and Methanol (HPLC Grade) was obtained from Merck Life science Pvt. Ltd. Ammonium acetate (HPLC Grade) was obtained from Sisco Research Laboratories Pvt. Ltd. Distilled water (Type-II grade) was used in the experiment was from Rions lab water purification system (RO-

DI ULTRA). Blank human plasma was prepared from pooled plasma of healthy persons who were not taking any antifungal drugs and was used for the preparation of quality controls and calibrators. Premix solution was prepared for sample preparation; 2.3 g of Ammonium dihydrogen phosphate (HPLC Grade, Fisher Scientific) was dissolved in 100 ml of distilled water and the volume was adjusted to 500 ml. (pH 7.0) and filtered through a 0.45 µm membrane filter.

Standards

Stock solution of Isavuconazole, at the concentration of 1 mg/mL, was prepared in methanol. Working solutions, at the concentration of 1000, 800, 400, 200, 100, 50 µg/mL, were then obtained through serial dilutions in methanol. Both the stock and working solutions were stored at –20°C. Plasma calibration standards at 10, 8, 4, 2, 1, 0.5, 0.25 µg/mL were freshly prepared by 1:100 dilution of the respective working solution in pooled human plasma and analyzed on the same day. 50 uL was injected into HPLC

Quality Control (QC) Samples Preparation

Quality control (QC) samples; low (2 µg/mL), medium (4 µg/mL), high (8 µg/mL) concentrations were prepared by diluting the working solutions in pooled plasma and stored at –20°C, in several aliquots. Thawed aliquots were used as and when required.

Chromatographic Conditions

Chromatographic separation was done using Mobile phase of acetonitrile and ammonium acetate buffer (pH 8.0, 10 mM) (55:45, v/v), previously filtered through a 0.45 µm membrane filter (Millipore). Prior to injecting solutions, the column was equilibrated by flowing the mobile phase through the system at least for 15 min. Autosampler temperature was 8°C and Column temperature was 40°C. 60: 40 :: methanol: water was used for washing the column.

Flow rate was adjusted to1.0 mL/min, and assay run time was 15 min to remove all plasma components from the column. UV detection was set at 285 nm where Isavuconazole exhibits peak. The injection Volume for each sample was 50 µL. These conditions allowed elution of Isavuconazole with good resolution at retention time of 9.2 min (Figure 1).

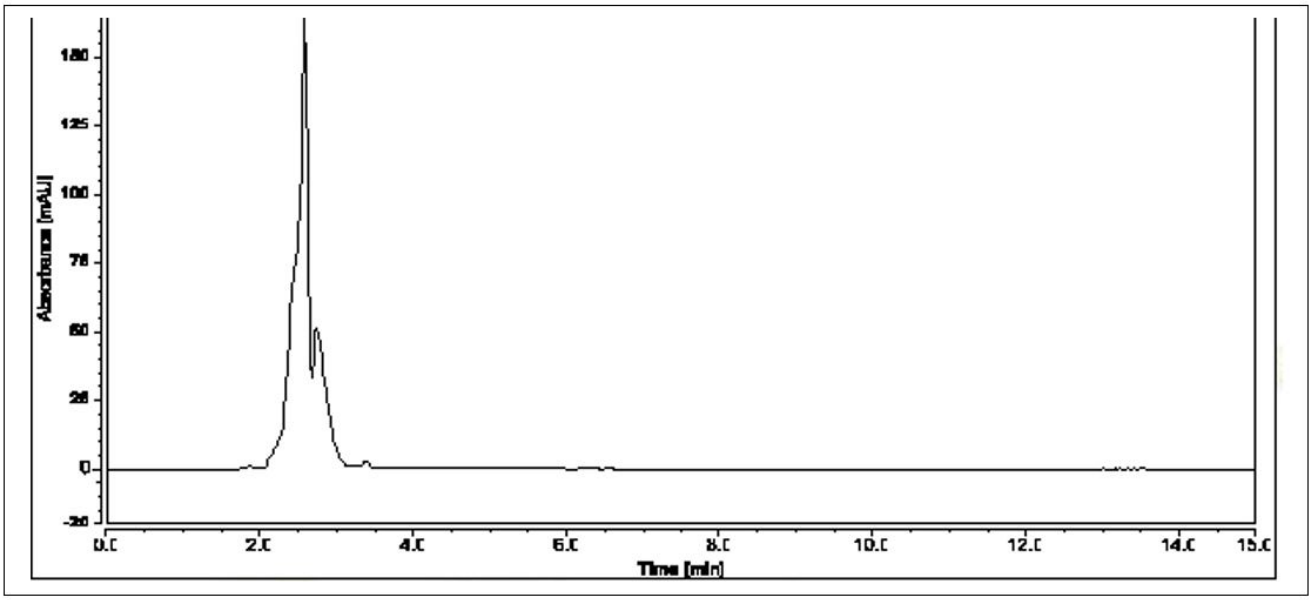


Figure 1: Chromatogram of blank plasma sample

Sample Preparation

Blood samples were withdrawn from patients and collected in K3EDTA vacutainers. Plasma samples were obtained after centrifugation of blood samples at 3500 rpm for 15 min at 4°C (Eppendorf, Model 5804 R). Plasma samples were deproteinized by addition of 400 µL of acetonitrile to 250 µL of plasma, vortexed thoroughly, and then centrifuged at 10000 rpm for 5 min at 4°C. 200 µL of supernatant was mixed with 200 µL of premix solution, vortexed and then centrifuged at 10000 rpm for 5 min at 4°C. The supernatants were transferred to HPLC vials for analysis. The injection volume was 50 µL.

Stability

The test is not being done anywhere in India, we expect samples to reach DMH within 3-5 days from all over. Therefore we studied the stability for 7 days under cold conditions. The stability of Isavuconazole was investigated in plasma samples spiked with the drug at two different concentration levels and subjected to four different storage and treatment conditions which clinical samples can commonly experience during the whole TDM process. Three replicates of low and high QC were used for each storage conditions. The spiked plasma samples were exposed to the following different conditions: (a) storage at -20°C for 7 days (b) storage at RT for 48 h in the dark; (c) storage at cold condition for 3-5 days under ambient light and (d) after three freeze-thaw cycles. The Isavuconazole peak areas measured in treated QC samples were compared with those of immediately analyzed QCs at the respective concentration.

Statistical Analysis

All statistical analyses were performed using SPSS 20.0 (IBM) and Microsoft Excel. In stability studies, comparisons of assay values were made by one-way analysis of variance (ANOVA).

Validation

Linearity was checked for 50 uL injection volume by using calibration curve three times**.** A statistical analysis of the regression line was performed, evaluating the resulting correlation coefficient, Y intercept, slope of the regression line, and residual sum of squares. A seven point calibration standard curve of ISC in plasma, ranging from 0.25 to 10 µg/mL, was prepared.

The recovery assessment was done by the standard addition method. Known amounts of Isavuconazole standards were added to the aliquots of plasma. Plasma samples spiked with three different concentrations of standard (low, medium & high) of Isavuconazole and unspiked plasma were used. The samples were then treated and chromatographed as described. Percentage of recovery was assessed by subtracting the value obtained from patient plasma from that of plasma with added standards, dividing by concentration of standard added and mean of percent recovery was obtained Three replicates of patient plasma and of patient plasma added with standards were measured.

The method was validated on the basis of intra and inter assay variations. Five replicates of each QC sample (spiked with six different concentrations of Isavuconalzole) were analyzed to determine intra- day precision and accuracy and three replicates were used for inter-day variation. Inter-assay values were obtained by analyses of the QC samples repeated on three different days. Precision was calculated as the percentage of the coefficient of variation (CV) of the measured values, whereas the accuracy (% bias) represents the percentage of deviation between expected and measured concentration.

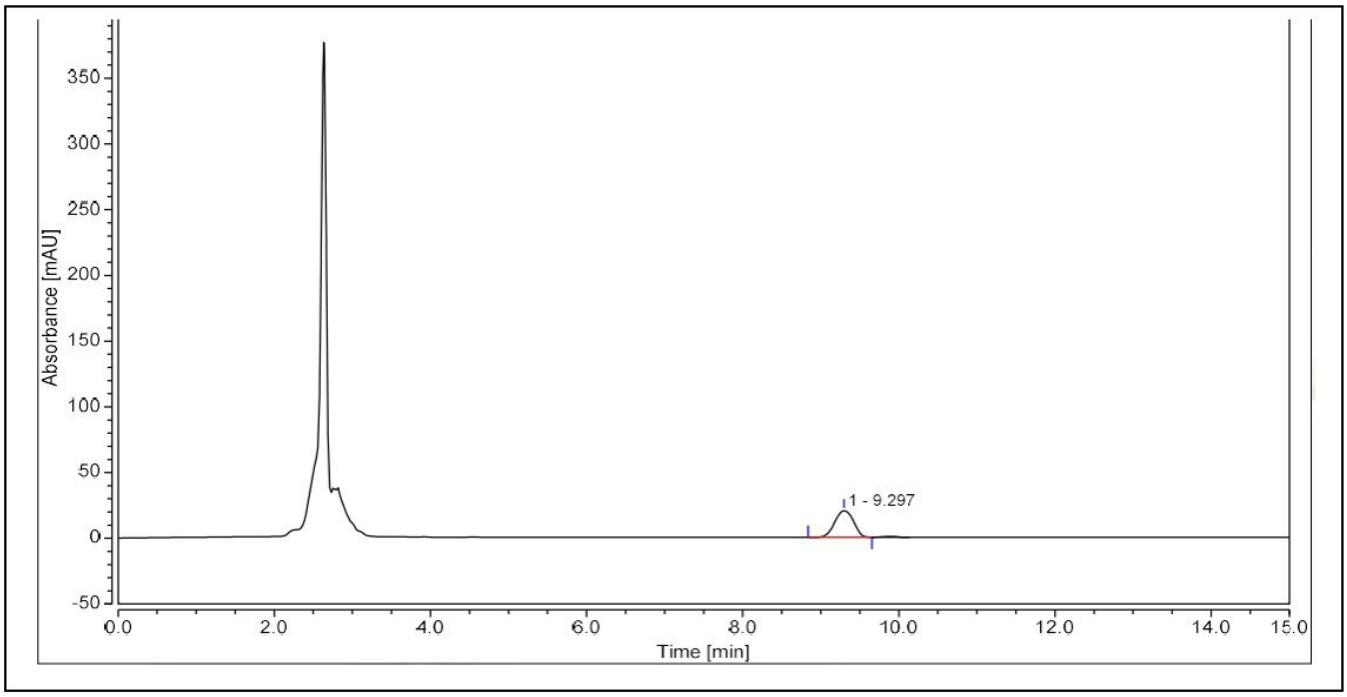
Results and Discussion

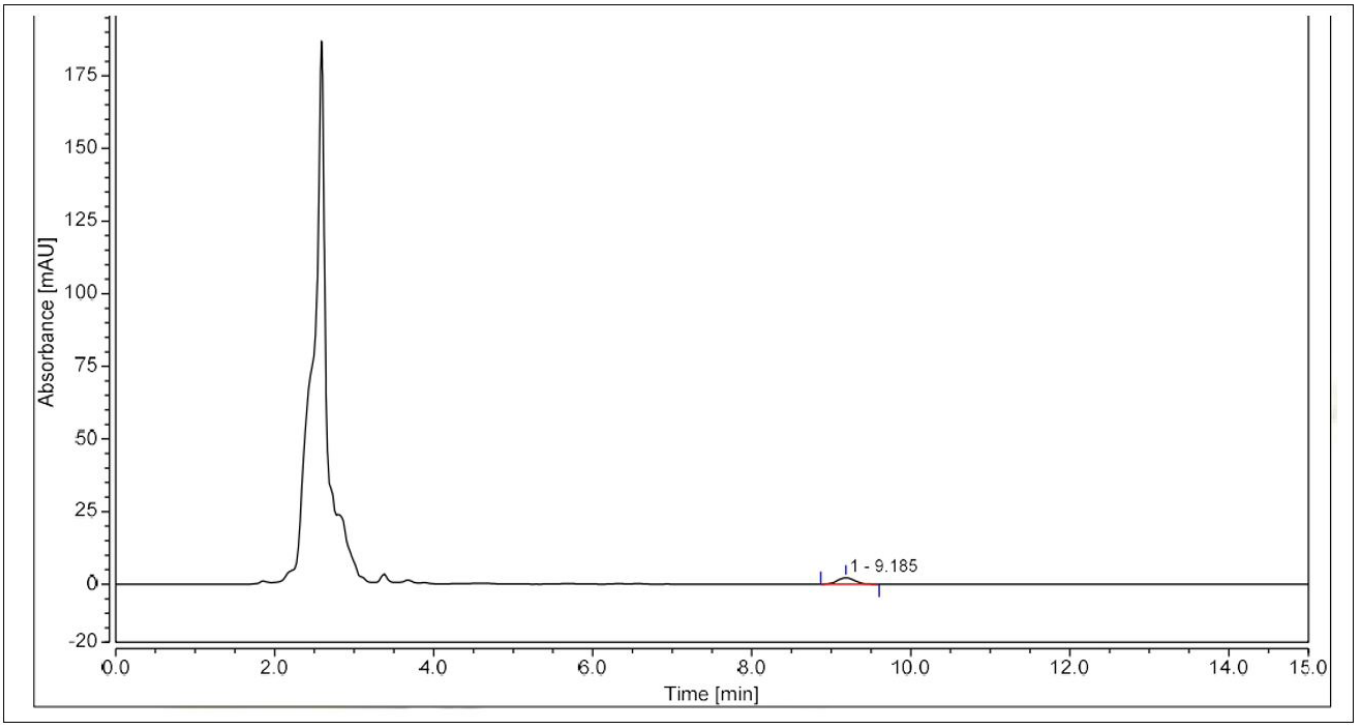
Assay characteristics

Chromatogram of Isavuconazole standards is presented in Fig 1A. Intra assay and inter-assay chromatograms are presented in Figure 1B. The retention time for Isavuconazole was 9.23 minutes. The chromatograms of plasma extracts do not have any interfering

peaks. The assay of Isavuconazole has been reported[19]. The mobile phase we used is able to separate Isavuconazole with consistency. Previously published methods for the determination of Isavuconazole levels in plasma involved the use of LC-MS/MS, fluorescence detectors, reverse phase UPLC which are not always available in analytical laboratories.[6,14,15,16] To the best of our knowledge, this is the first modified UHPLC method with UV detection developed in India for the determination of Isavuconazole in patient's plasma, which uses commonly available equipment and offers a sensitivity required for therapeutic drug monitoring of Isavuconazole. This method exhibits great robustness and reproducibility, having acceptable intra-day and inter-day bias and CV values.

A. Chromatogram of blank plasma sample spiked with ISA at 10 µg/ml





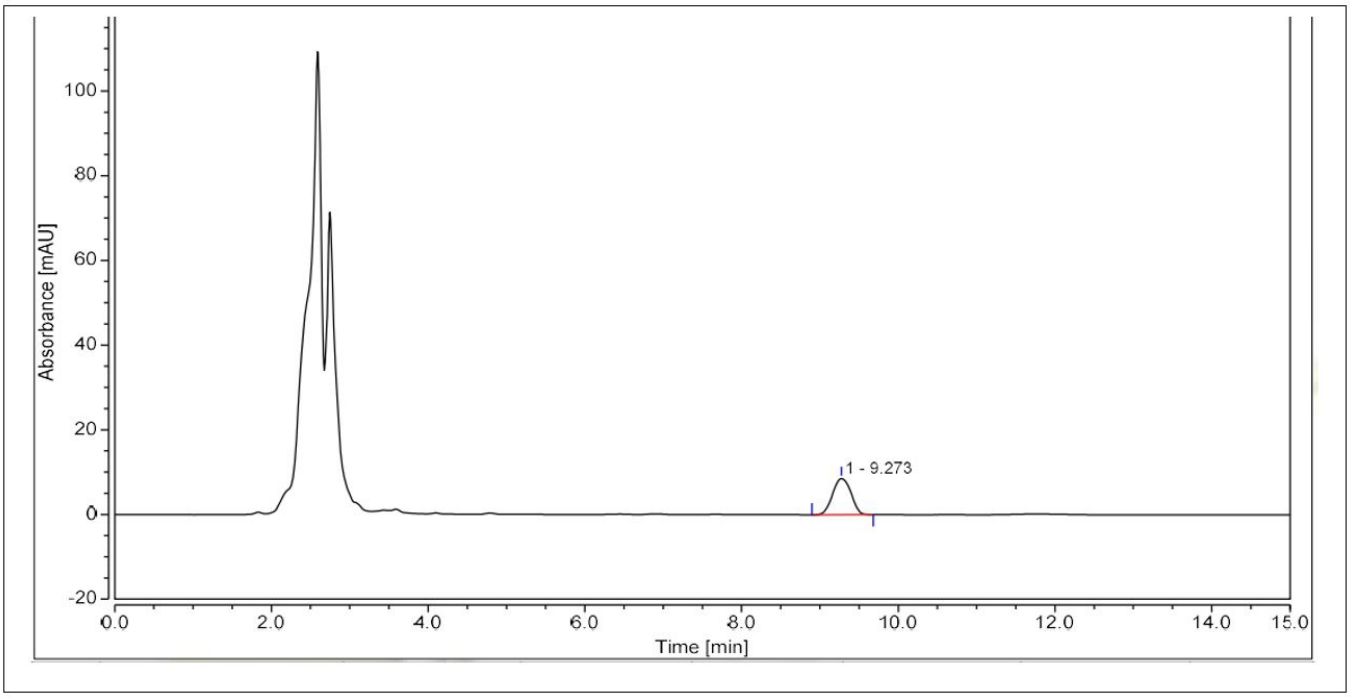


Figure 2: A) Chromatogram of patient treated with Isavuconazole in combination with Amphotericin and Posaconazole. Concentration of Isavuconazole was 1.04 µg/ml

B) Chromatogram of patient Isavuconazole in combination with Amphotericin and posaconazole. Concentration of Isavuconazole was 4.39 µg/ml

Limits of quantification, calibration curves and recoveries

A seven-point calibration standard curve of Isavuconazole was prepared. The calibration curve was linear over the range of 0.25 to 10.0 ug/mL concentration per 50 uL injection. The equation of calibration curve was y=0.568 x (r^2= 0.9977) where y represents the corresponding peak area and x represents the analyte concentration (Figure-3).

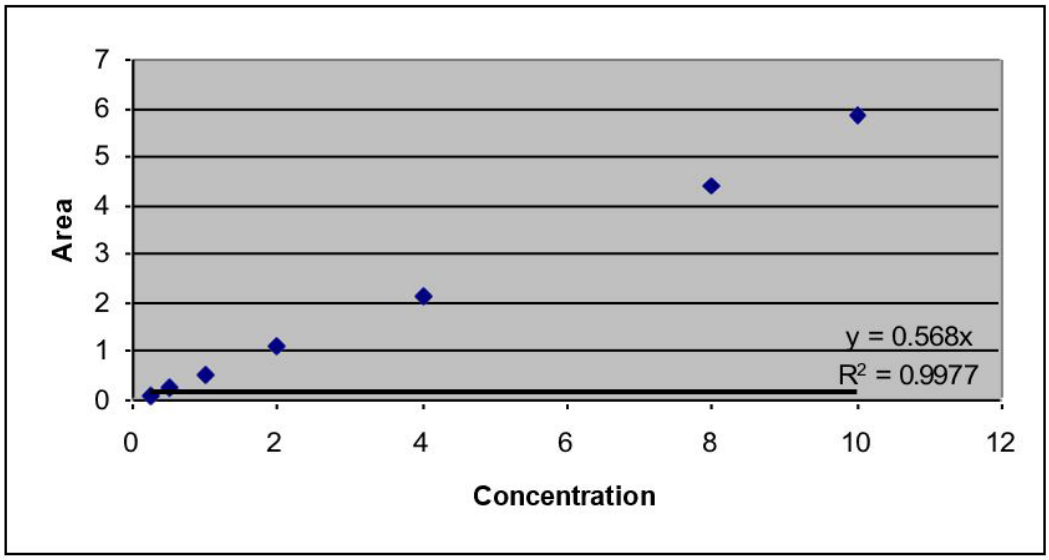


Figure 3: Linearity Check

Lower limit of quantitation was calculated based on precision and accuracy (bias) data and found to be 0.25 µg/ml with percent CV and bias less than 15%. Limit of detection was found to be 0.125 ug/ml with percent CV and Bias > 20 % and upper limit of detection was found to be 10 ug/ml with percent CV and Bias of 0.31 & 6.3 respectively.

We have plotted concentrations against responses and the graph was in the range of 0.25-10.0 µg/mL. The mean regression equation was $Y = 568x$. The correlation coefficient (R2) was 0.9977 for the calibration curve. Injecting the calibrator with more than 10 ug/mL i.e. 15 ug/mL was not able to define the linear curve.

The recovery of Isavuconazole was determined by adding known amount of standards. The spiked plasma was then extracted and chromatographed. For the spike and recovery assessment pooled plasma was used to evaluate the effect of sample matrix on plasma Isavuconazole. Plasma samples spiked with three different concentrations of standard (low, medium & high) of Isavuconazole with unspiked plasma were used. The absolute recovery of Isavuconazole from human plasma ranged from 94 % to 110 % with CV values varying from 1.05 % to 4.64 % (Table-2). This indicated that the recovery of Isavuconazole from the plasma was almost complete. It also indicated that Isavuconazole was stable throughout the sample preparation and chromatography. Hence, an internal standard and correction for recovery would not be needed when measuring concentration of Isavuconazole in human plasma.

Intra- and Inter- assay variations

Intra-assay and inter-assay CVs were equal or lower than 6.12% and 10.34%, respectively, in the tested concentration range with 50 uL injection.

The intra-day and inter-day % bias of the means of the measured concentrations from their known true concentrations varied from -12.4 % to 0 % and from -10.6% to 3.0%, respectively.(Table-1)

Precision and accuracy were evaluated in plasma spiked with Isavuconazole at 6 concentrations which were back calculated from Isavuconazole calibration curve (Table-1). Intra-assay and inter-assay CVs were equal or lower than 6.12% and 10.34%, respectively, in the tested concentration range with 50uL injectionThe data indicates that this HPLC method very consistent, accurate and reliable. Furthermore, Isavuconazole was found to be stable atleast for 7 days when stored at cold temperature.

Nominal Value	**Intra-day (n=5)**			**Inter-day (n=3)**		
	Mean Value (µg/ml)	**Accuracy (%bias)**	**Precision (%CV)**	**Mean Value** (µg/ml)	**Accuracy (%bias)**	**Precision (%CV)**
0.25 (LLOQ)	0.25	0.0	0.0	0.24	-1.33	10.20
2.0 (low QC)	1.83	-8.3	3.46	1.88	-6.0	5.55
4.0 (Medium QC)	3.59	-10.2	3.53	4.12	3.0	6.57
8.0 (High QC)	7.72	-3.47	1.15	7.73	-3.29	3.03
10.0 (ULOQ)	10.63	6.38	0.31	9.84	-1.56	0.10

Table 1: Intra-day and inter-day accuracy and precision results in QC plasma samples

Isavuconazole				
µg/ml **added**	µg/ml **found**	**Area of spikedsample**	**% Recovery**	**% Mean Recovery**
0.5	0.47	3.90	94	99.3
0.5	0.51	3.87	102	
0.5	0.51	3.85	102	
2	2.14	5.57	107	108
2	2.14	5.50	107	
2	2.2	5.54	110	
5	5.16	5.03	103.2	104.3
5	5022	5.02	104.4	
5	5.27	5.04	105.4	

Table 2: Sample and Recovery Assessment

Stability

Isavuconazole was found to be stable in human plasma under all tested stability conditions (Table-3). Stability was confirmed for Isavuconazole in plasma samples stored at RT for 48 h in ambient light and dark, after three freeze-thaw cycles, as reported in previous studies.[14 15 5 16] and for plasma stored at – 20°C for 7 days. No significant difference in Isavuconazole levels between plasma samples stored at RT for 48 h either in the dark or under ambient light was observed in this study.

	Concentration	
	2 µg/ml	**8** µg/ml
storage at –20°C for 7 days		
% Stability	100.3	98.98
% CV	1.41	1.25
% deviation	0.35	-1.01
storage at RT for 48 h in the dark		
% Stability	100	100
% CV	1.88	2.18
% deviation	0	0
storage at RT for 48 h under ambient light		
% Stability	98.74	100.34
% CV	0.94	1.65
% deviation	-1.25	0.33
after three freeze-thaw cycles		
% Stability	98.21	98.64
% CV	4.85	1.49
% deviation	-1.78	-1.35

Table 3: Isavuconazole stability in plasma samples under different treatment/storage conditions

Utilities

The HPLC method was used to measure the Isavuconazole concentrations in plasma of patients treated for invasive fungal disease. Invasive Fungal Disease patients were administered oral or intravenous preparations of Isavuconazole and monitored for circulating levels for after therapy. The levels were considered for efficacy of the administered drug and accordingly the dosage was fixed.

The applicability of the method in the clinical setting was evaluated by analyzing plasma samples obtained from 11 patients treated with Isavuconazole. The measured Isavuconazole plasma level ranged from 0.53 μg/mL to 5.76 μg/mL (median of 3.02 μg/mL), which were within the range reported in the literature (Maertens et al., 2016). Figure-2 shows representative chromatograms of plasma samples of patients at steady-state receiving the recommended maintenance oral dose of 200 mg daily of Isavuconazole. No potentially interfering peaks at the retention time of ISC were observed in all analyzed clinical samples, thus confirming the clinical applicability of the present analytical method. This assay is currently employed successfully for the routine TDM of Isavuconazole in our diagnostic Unit

Interlaboratory Comparison

Participation in EQAS is a common practice but Isavuconazole has not been available so far in EQAS programs like BioRad, Randox, WeQAS, etc. Though interlaboratory quality control is important to ensure the quality of the method interlaboratory comparison was not possible as this test was not available any where in India. However, there was acceptable clinical opinion from the treating clinicians regarding measured Isavuconazole plasma level.

HPLC method for Isavucanozole

The present paper describes the development and validation of a simple, accurate, selective, and sensitive HPLC assay with UV detection for the quantification of Isavuconazole in human plasma. The determination of drug exposure is required in selected clinical cases to adjust the drug dosage, in order to monitor the effectiveness of therapy and to minimize the toxicity.[17] Previously published methods for the determination of Isavuconazole levels in plasma involved the use of LC-MS/MS, fluorescence detectors, reverse phase UPLC which are not always available in analytical laboratories.[6,14,15,16] To the best of our knowledge, this is the first modified UHPLC method with UV detection developed in India for the determination of Isavuconazole in patient's plasma, which uses commonly available equipment and offers a sensitivity required for therapeutic drug monitoring of Isavuconazole. This method exhibits great robustness and reproducibility, having acceptable intra-day and inter-day bias and CV values. Also the run time of this assay is similar to assay similar to reported by others.[5,7,14,18] However, an Indian study has validated Isavuconazole estimation in pharmaceutical formulations by using RP-HPLC with a very narrow range of calibrators/standards ranging from 1.25 to 3.75 μg/ml[19] .

The stability of Isavuconazole was investigated in plasma samples spiked with the drug at different concentration levels which were subjected to different storage and treatment conditions which clinical samples can commonly experience during the whole TDM process. It showed that Isavuconazole concentration remain stable in plasma including in plasma exposed to ambient light and dark. Finally clinical applicability of the method and the appropriateness of validated concentration range have been demonstrated in the analysis of plasma sample of patient receiving Isavuconazole. Clinical feedback towards the turnaround time, reproducibility is satisfactory. In order to cater near by area/state the method was validated for seven days at cold conditions and found to be useful for providing results.

Limitation of this study is not being able to compare with EQAS or ILC.

Conclusion

Using HPLC method with Acetonitrile as protein precipitating solvent we could measure as low as 0.25 ug/L of Isavuconazole in human plasma samples in 50 uL injection volumes with good recovery. The therapeutic range for effective Isavuconazole treatment is 2 to 10 ug/ml and this method is able to fulfill the monitoring protocol for clinicians. The result can be delivered on the same day of blood collection in order to manage the patients with invasive fungal infections. The method is suitable for pharmacokinetic investigations.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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