Implementation of a Dual-Column Liquid Chromatography-Tandem Mass-Spectrometry Method for the Quantification of Isavuconazole in Clinical Practice

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Abstract

Objectives   Therapeutic drug monitoring (TDM) of isavuconazole, which is a novel broad-spectrum antimycoticum against invasive fungal infections, ensures an effective exposure of the drug and minimizes the risk of toxicity. This study is aimed at evaluating the analytical performance of a dual-column liquid chromatography-tandem mass-spectrometry (LC-MS/MS) method for isavuconazole quantification.

Materials and Methods   The method was performed on a Voyager TSQ Quantum triple quadrupole instrument equipped with an Ultimate 3000 chromatography system (Thermo Fisher Scientific, San Jose, California, United States). Analytical and preanalytical requirements of the isavuconazole LC-MS/MS method were evaluated. Sample stability measurements were performed at room temperature (RT) and in serum tubes with separator gel.

Results   The isavuconazole LC-MS/MS method was linear over the concentration range of 0.2 to 12.8 mg/L. The coefficient of determination (r^2) always exceeded 0.999. Within- and between-run precision ranged between 1.4 to 2.9% and 1.5 to 3.0%, the recovery between 93.9 and 102.7%. At RT, serum samples were stable for 3 days. Isavuconazole serum concentrations were significantly lower after incubation (18 hours) in serum tubes with separator gel at RT.

Conclusion   The dual-column isavuconazole LC-MS/MS is a reliable tool for the TDM of isavuconazole. Serum samples are stable for at least 3 days and should be collected in tubes without separator gel.

Keywords
► antifungal agents
► isavuconazole
► liquid chromatography-tandem mass-spectrometry
► therapeutic drug monitoring
► triazole

Introduction

Fungal infections are a high-risk factor of morbidity and mortality in patients with immunosuppressive or cancer chemotherapy. Triazole antifungal agents (e.g., voriconazole, itraconazole, posaconazole, isavuconazole) are important drugs for the prophylaxis and treatment of invasive fungal diseases (IFDs). Isavuconazole is a new generation,

broad-spectrum triazole, which is commonly used for IFD caused by a range of rare or multiple fungal species.[1,2] This agent inhibits the 14α-demethylase, which affects the ergosterol biosynthesis and disrupts the fungal cell membrane structure and function.[3]

Serum concentrations of triazoles sometimes require therapeutic drug monitoring (TDM). Since voriconazole, itraconazole, and posaconazole show a high level of intra- and interindividual variability and a narrow therapeutic window, antifungal TDM is generally indicated for these agents.[4,5] In a previous study isavuconazole was shown to have a high bioavailability and dose-proportional pharmacokinetic, which demonstrated no relevant differences between patients with IFD and healthy subjects.[6]

Several methods have been established for the quantification of serum concentrations of antifungal agents, including liquid chromatography-tandem mass-spectrometry (LC-MS/MS), high-performance liquid chromatography (HPLC), and the bioassay.[4] LC-MS/MS is a powerful and valuable tool with the advantages of high specificity, precision, and the potential of simultaneous separation of multiple analytes.[7,8] However, this method is not widely available compared with the HPLC technology and the cheap and simple bioassay.[4]

To date, no Food and Drug Administration (FDA)-approved assay for the quantification of isavuconazole serum concentrations is available.[9] Therefore, a thorough evaluation of the analytical performance by the use of isavuconazole LC-MS/MS instrumentation is recommended by clinical laboratories before this method can be used in clinical routine.

The present study aimed at evaluating a dual-column LC-MS/MS method for the quantification of isavuconazole in serum. Evaluation consisted of the assessment of the linearity, precision, recovery, limit of quantification (LOQ), and limit of detection (LOD). Additionally, we performed sample stability measurements at room temperature (RT) in serum tubes with separator gel.

Materials and Methods

Instrumentation and Conditions

The method was performed on a Voyager TSQ Quantum triple quadrupole instrument equipped with an Ultimate 3000 chromatography system (Thermo Fisher Scientific, San Jose, California, United States). Chromeleon Xpress software for device management and LCQuanTM 2.7 for data processing were used. Isavuconazole and its deuterated isotope were kindly provided by Basilea (Basel, Switzerland). Calibrators and controls were prepared by spiking drug free serum from healthy donors with stock solutions. In brief, 20-μL serum samples (calibrators or controls) were deproteinized by adding 100-μL ice-cold methanol containing the internal standard (IS) (isavuconazole-d4, 1 mg/mL). After vortexing and centrifugation at 24,000 × g (5 minutes), the clear supernatant was tenfold diluted with 5% ACN. Ten microliter were loaded on a trapping column (POROS R1 20, 2.1 × 30 mm, Thermo Fisher Scientific) with mobile phase 1 (5:95 v/v ACN/water). After a short washing period the analytes were

transferred and separated on a Luna 5-μm Phenyl–Hexyl column 100A 50 × 2.1 mm (Phenomenex, Aschaffenburg, Germany) with a linear gradient of mobile phase 2 (0.1% formic acid in MS grade water) and mobile phase 3 (0.1% formic acid in MS grade ACN). Isavuconazole and the IS were monitored in a positive multiple reaction monitoring mode using characteristic precursor–product ion transitions: m/z 438.1→214.9 (438.1→368.9 as a second qualifier) and m/z 442.1→218.9 (442.1→372.9 as a second qualifier), respectively.

Method Evaluation

A calibration curve was prepared using serum samples spiked with six different isavuconazole concentrations ranging from 0.2 to 12.8 (0.2, 0.8, 1.6, 3.2, 6.4, 12.8) mg/L and analyzed in triplicate.[10] The linear calibration curve was constructed by plotting the ratios of peak areas of the analyte divided by the corresponding IS against the concentration of each compound. The data from the standard curve were analyzed using regression analysis to obtain the slope, the intercept, and the correlation coefficient (r^2). The acceptance criteria of the recalculated measured values of each calibration point were ≤ 15%.

The within-run precision was assessed by five replicate analyses of low, medium, and high isavuconazole concentrations of a serumpool on one day. The between-run precision was determined by replicate analyses of low, medium, and high isavuconazole concentrations of the same serumpool on 5 consecutive days.[11] The precision goal for each concentration was not to exceed 15% of the coefficient of variation (CV).[12]

The recovery of isavuconazole was determined three times at three different concentrations (0.5, 2.5, and 10 mg/L) by calculating the percentage difference between the area of analytes of authentic pure substance added to a patient pool before and after precipitation.

The LOD was defined as the lowest concentration, which produced a signal at least three times higher than the average background noise. The LOQ was defined as the lowest concentration possible to quantify imprecision of < 10%.[13,14]

This study was performed in accordance with the latest version of the declaration of Helsinki (Fortaleza 2013) and was approved by the Ethical Committee of the Medical University of Graz (Graz, Austria).

Preanalytical Analyses

Since specimens are usually handled and transported to the laboratory at RT, we performed isavuconazole stability measurements at constant 25°C RT in our laboratory. We stored a patient pool (n = 10) at RT with daylight exposure. For 3 days, one portion of the pool was frozen at -80°C on each day until batch analyses of all frozen samples were performed immediately after this period.

To detect if separator gel absorbs isavuconazole, we incubated the triplicate heparinized patient samples spiked with different concentrations of isavuconazole (1.7, 4.6, and 12.8 mg/L) for 18 hours in serum tubes with separator gel at RT and compared the measured concentrations after the incubation with the initial measurements before incubation.
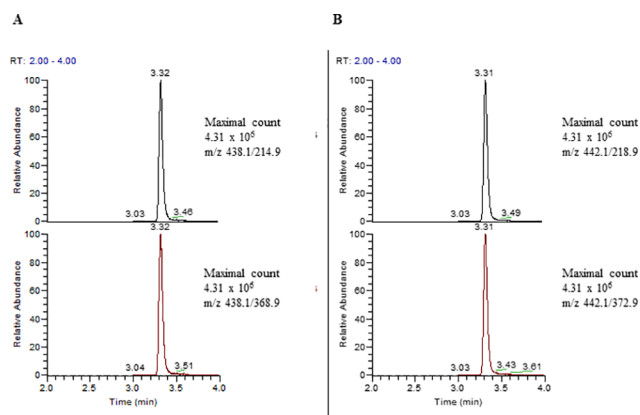
Fig. 1 Representative chromatogram of a patient with 3.63 mg/L isavuconazole. Every peak has over 20 measuring points. Peaks are shown without smoothing. (**A**) Isavuconazole: m/z 438.1→214.9 as the quantifier and 438.1→368.9 as a second qualifier; (**B**) Internal standard isavuconazole d4: m/z 442.1→218.9 as the quantifier and 442.1→372.9 as a second qualifier.
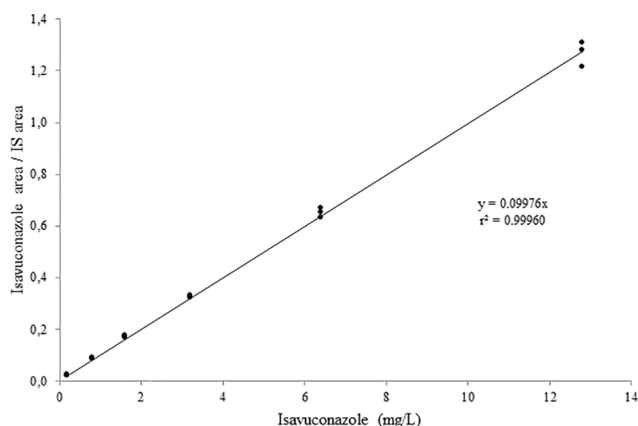

Fig. 2 Calibration curve of isavuconazole: $y = 0.0997x$, $r^2 = 0.999$. IS, internal standard.

Statistical Analysis

The paired t-test was calculated to assess the differences of mean isavuconazole concentrations at different serum levels (1.7, 4.6, and 12.8 mg/L) before and after incubation in serum tubes with separator gel. A p-value < 0.05 was considered statistically significant. The analyses were performed using SPSS 25.0 statistical software (SPSS Inc., Chicago, Illinois, United States).

Results

Analytical Performance

The chromatographic separation of isavuconazole with the LC-MS/MS method in human serum is illustrated in ►**Figs. 1A** and **B**. The calibration curve was linear over the range from 0.2 to 12.8 mg/L (►**Fig. 2**). The evaluation results of the six-point calibration curve measurements are shown in ►**Table 1**. The coefficient of determination (r^2) always exceeded 0.999.

The characteristics of the analytical method are presented in ►**Table 2**. The within-run CVs varied between 1.4 and

2.9% and the between-run CVs ranged between 1.5 and 3.0%. The recovery result was highly satisfactory. The LOQ and LOD were below the lowest measured values. On the LOQ (0.1 µg/mL) the CV was < 10%.

Preanalytical Measurements

As shown in ►**Fig. 3**, isavuconazole serum samples were stable for at least 3 days at RT. The mean (± standard deviation) serum isavuconazole concentration of all measured time points ($n = 4$) was 3.63 (± 0.13), respectively.

Isavuconazole concentrations were determined at different serum levels before and after incubation (18 hours) in serum tubes with separator gel at RT. The measured mean concentrations (± standard deviation) at the low (1.7 mg/L), medium (4.6 mg/L), and high (12.8 mg/L) serum level were significantly lower after incubation (1.60 ± 0.03, 4.23 ± 0.10, and 11.12 ± 0.29 mg/L) compared with measurements before (1.75 ± 0.01, 4.62 ± 0.02, 12.58 ± 0.28 mg/L) (p-values: 0.001, 0.003, and 0.003) incubation in blood collection tubes with separator gel, respectively.

Discussion

Herein, we described the development and evaluation of a dual-column LC-MS/MS method for the measurement of the new triazole isavuconazole in human serum. The method was linear within the range of 0.2 to 12.8 mg/L, which comprises the clinically expected isavuconazole through blood levels (median: 2.93 mg/L; range: 0.81–9.95 mg/L).[15,16] We prepared the calibration curve with six different isavuconazole concentrations in triplicate and fulfilled the recommended criteria, which propose the determination of five to seven calibrators at least tested in duplicate at each level.[10,17] The within- and between-run imprecisions varied between 1.4 and 3.0%. These results are in line with the LC-MS/MS acceptance criteria of ≤15% in the guidelines of biological method validation of the European Medicines Agency and the FDA.[12,17]

Here, we used the LC-MS/MS method for isavuconazole quantification. This technique was shown to be more capable of determining mixtures of drugs compared with the
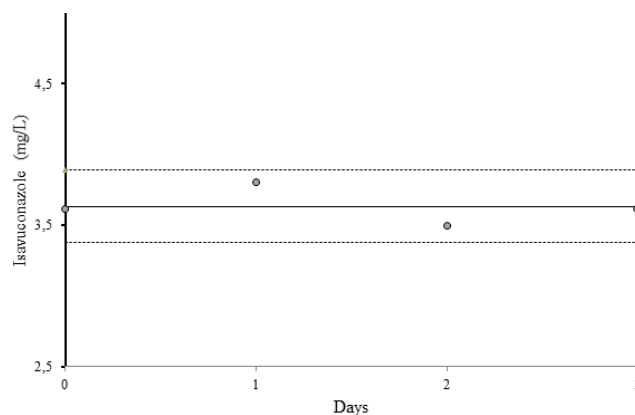

Fig. 3 Isavuconazole stability measurements of a patient pool (n = 10) at room temperature for 3 days. The solid line shows the mean isavuconazole concentration of all measurements (3.63 ± 0.13 mg/L), the dotted lines represent the twofold standard deviation (± 2 SD).

Table 1 Calibration curve for isavuconazole in triplicate

Isavuconazole (mg/L)	Mean	SD	CV (%)	MaxD (%)
Calibrator 1 (0.2)	0.21	0.002	1.00	8.0
Calibrator 2 (0.8)	0.85	0.020	2.29	9.3
Calibrator 3 (1.6)	1.69	0.045	2.64	8.4
Calibrator 4 (3.2)	3.21	0.027	0.84	1.2
Calibrator 5 (6.4)	6.42	0.192	3.0	−2.9
Calibrator 6 (12.8)	12.51	0.463	3.7	−6.3

Abbreviations: CV, coefficient of variation; MaxD, maximum deviation of the recalculated single values of the determination in triplicate; SD, standard deviation.

Table 2 Characteristics of the isavuconazole LC-MS/MS method

	Isavuconazole		
Working range (mg/L)	0.2–12.8		
Calibration curve			
Slope	0.0997		
Intercept	0		
Correlation r^2	0.999		
Within-run precision ($n = 5$)	Low	Medium	High
Mean (mg/L)	2.85	4.78	6.62
SD (mg/L)	0.09	0.07	0.12
CV (%)	2.96	1.4	1.78
Between-run precision ($n = 5$)	Low	Medium	High
Mean (mg/L)	2.89	4.79	6.59
SD (mg/L)	0.09	0.09	0.1
CV (%)	3.01	1.81	1.53
Recovery (range, %)	93.9–102.7		
Limit of quantification			
LOQ (mg/L)	0.1		
SD (mg/L)	0.006		
CV (%)	5.7		
Limit of detection			
LOD (mg/L threefold SD of the baseline noise)	0.04		
Stability measurements			
Stability at RT	3 d		

Abbreviations: CV, coefficient of variation; LOQ, limit of quantification; LOD, limit of detection; RT, room temperature; SD, standard deviation.

HPLC.[18] The HPLC technique is more time-consuming, has a lower throughput, and different substances may interfere with the analyte determination. Since quantification of isavuconazole serum concentrations is quite often recommended for critically ill patients with multidrug therapy, the LC-MS/MS is considered the method of choice in this patient setting.[19]

The selection of adequate LC columns is required for the development of an optimal quantitative LC-MS/MS method.[20] In the present study, we implemented a dual-column LC-MS/MS. The serial combination of two different columns is known to be more effective in view of

separating drugs and metabolites in complex matrices compared with single column methods.[21-23]

To obtain valid results for TDM of isavuconazole, preanalytical influencing factors such as blood collection tubes, handling procedures, and storage conditions must be considered. Here, we performed stability measurements at RT, which is known the worst encumbering condition for specimens of blood. At ambient RT, which was constant at 25°C and monitored by continuous record of air conditioner in our laboratory, isavuconazole was observed to be highly stable for 3 days. In comparison, a recently published study reported good results of stability experiments at RT, which were performed for up to 6 hours only.[24] The same authors

observed isavuconazole measurements to be stable at –20°C and –80°C for at least 5 weeks.[24] Another study found unextracted isavuconazole to be stable for 15 days at RT and 4°C.[25]

In this work, serum isavuconazole concentrations were significantly lower after incubation in blood collection tubes with separator gel at RT. The absorption of therapeutic drugs by barrier gel has been known as a potential reason of clinically important error in drug measurements for a long time.[26-29] Nevertheless, in the current literature, we could not find studies investigating separator gel effects on serum isavuconazole concentrations. A recently published study, which evaluated the impact of serum separator blood collection tubes on the stability of a panel of 167 drugs, demonstrated, that tubes without separator gel cause less drug interferences.[30] Therefore, these tubes are recommended for the quantification of drugs.[30]

The major limitation of this study is that individual serum concentrations of isavuconazole were not assessed in a patient setting.

Conclusion

The evaluated dual-column isavuconazole LC-MS/MS method described here shows a broad analytical range and meets the imprecision acceptance criteria of ≤ 15%. These data are indicative for a precise and reliable diagnostic tool for the TDM of isavuconazole in daily clinical routine. Isavuconazole serum samples should be collected in tubes without separator gel and are stable at RT for at least 3 days.

Compliance with Ethical Standards
This study was performed in accordance with the latest version of the declaration of Helsinki (Fortaleza 2013) and was approved by the Ethical Committee of the Medical University of Graz (Graz, Austria).

Authors' Contribution
D.E., S.Z., M.H., R.K., and A.M. designed the study. D.E. and A.M. collected, analyzed, and interpreted the data. D.E. wrote the first draft of the manuscript and A.M. supervised this study project. All listed authors revised the manuscript critically and approved the last version of this manuscript.

Conflicts of Interest
The authors declare that they have no conflicts of interest.

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