A sensitive and validated HPLC-UV method for the quantitative determination of the new antifungal drug isavuconazole in human plasma

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Abstract

Isavuconazole is a broad-spectrum triazole antifungal drug recently approved for the therapy of both invasive aspergillosis and mucormycosis. To support a widespread therapeutic drug monitoring (TDM) of isavuconazole, a simple, sensitive, and precise high-performance liquid chromatography (HPLC) method with UV detection was developed and fully validated for the quantification of this drug in human plasma. The method involved a combined protein precipitation–solid-phase extraction and a chromatographic separation on a Waters XTerra RP18 (150 mm × 4.6 mm, 3.5 μm) column using an isocratic mobile phase of ammonium acetate buffer (pH 8.0, 10 mM) and acetonitrile (45:55, v/v). The UV detection was performed at 285 nm. This method was linear (correlation coefficients ≥ 0.998), specific (no interference with plasma components or various potentially co-administrated drugs), sensitive (lower limit of quantification of 0.025 μg/mL), reproducible (coefficients of variation were ≤ 7.9%), and accurate (deviations ranged from -5.0% to 8.0%) over the range of 0.025–10 μg/mL. The method fulfilled all the FDA guidelines validation criteria and performed well in an international proficiency testing program. The assay was also successfully applied to a routine TDM of patients and to drug stability investigations under various conditions.

1. INTRODUCTION

Isavuconazole (ISC, formerly termed BAL4815) is a new triazole antifungal drug recently licensed (in 2015) for the treatment of invasive aspergillosis and mucormycosis (Astellas Pharma US, 2015). ISC acts on a wide range of clinically relevant yeasts, molds, and dimorphic fungi by blocking the synthesis of ergosterol and leading to the production of aberrant and potentially toxic sterols on the fungal cell membrane (Guinea et al., 2008; Miceli and Kauffman, 2015).

ISC is administered as a prodrug (isavuconazonium sulphate, also known as BAL8557) available in an intravenous formulation and also as oral capsules, since its has high oral bioavailability (nearly 100%). The water-soluble prodrug is rapidly hydrolyzed by plasma esterases and almost completely converted to ISC (~98%) (Miceli and Kauffman, 2015; Schmitt-Hoffmann et al., 2006a; Schmitt-Hoffmann et al., 2006b). The recommended dosing regimen of ISC in both oral and IV formulations is 200 mg every 8 hours for 2 days as a loading dose followed by a maintenance dose of 200 mg once daily (Astellas Pharma US, 2015).

Although ISC has a better tolerability and fewer CYP-mediated drug interactions than other triazole drugs, therapeutic drug monitoring (TDM) of ISC is required, especially in certain clinical cases, to optimize clinical outcome in terms of improving efficacy and reducing drug-related adverse effects (Stott and Hope, 2017; Bellmann and Smuszkiewicz, 2017). As highlighted at the 6th European Conference on Infections in Leukaemia (ECIL), TDM of ISC is indicated in unresponsive infections, in the treatment of pathogens with reduced susceptibility, or in the case of drug-drug interactions (Lewis et al., 2015). In fact, since ISC is a moderate CYP3A4 inhibitor, some drug-drug interactions were already observed (Bellmann and Smuszkiewicz, 2017). For example, an increase of ISC levels has to be considered under treatment with lopinavir and ritonavir, while concomitant therapy with rifampin, carbamazepine, barbiturates or St John's wort is contra-indicated because of resulting sub-therapeutic ISC levels (Bellmann and Smuszkiewicz, 2017). TDM of ISC is also recommended in subjects with severe hepatic impairment, as little is

known about the drug pharmacokinetics properties in this group of patients (European Medicines Agency, 2015; Desai et al., 2016).

Some analytical methods have been reported so far for the determination of ISC in human plasma and most of them employed mass spectrometry or fluorescence detection (Schmitt-Hoffmann et al., 2006a; Schmitt-Hoffmann et al., 2006b; Farowski et al., 2010; Toussaint et al., 2017; Jørgensen et al., 2017; Müller et al., 2017; Fatiguso et al., 2017; McShane and Wang 2017). Alternatively, a method based on ultra-performance liquid chromatography (UPLC) coupled with UV detection has also been developed (Verweij-van Wissen et al., 2012). However, this method requires specialized equipment that is not available in all clinical laboratories. To promote widespread TDM of ISC, alternative methods which employ standard equipment and are simpler and easier to set up, would be desirable. To the best of our knowledge, no validated high-performance liquid chromatography (HPLC)-UV method has been yet developed to measure ISC concentrations in human plasma.

Thus, the aim of this work was to develop a widely applicable, very simple and still robust HPLC-UV method, eligible for a clinical routine use. This paper describes the development and validation of the first HPLC-UV method for the quantification of ISC in human plasma, which employs a rapid precipitation of plasma proteins with acetonitrile followed by a solid-phase extraction (SPE) procedure and an isocratic elution. This assay is highly precise, accurate, selective, sensitive and suitable for a routine TDM of ISC in a clinical laboratory with standard equipment. Moreover, this study reports the stability of ISC under several common conditions which can occur during the sample handling and analysis in the whole TDM process, including thermal inactivation of pathogenic microorganisms and exposure to ambient light.

2. EXPERIMENTAL

2.1 Chemicals and reagents

ISC powder was kindly provided by Basilea Pharmaceutica Ltd. (Basel, Switzerland) and its chemical purity was above 99%. Acetonitrile (HPLC Gradient Grade, LiChrosolv) was obtained from Merck (Darmstadt, Germany), methanol for chromatography was from Carlo Erba (Milan, Italy). All other chemicals were of analytical or HPLC grade and purchased from J. T. Baker (Deventer, The Netherlands). Ultrapure water used in all experiments was supplied by a Milli-Q water purification system (Millipore, Bedford, MA, USA). SPE cartridges (Oasis HLB 1cc) were obtained from Waters (Milford, MA, USA). Blank human plasma used for the preparation of quality controls (QCs) and calibrators was obtained from Seracare Life Sciences (Basematrix 53, Defibrinated human plasma, Milford, MA).

2.2 Equipment and chromatographic conditions

The HPLC system consisted of an Alliance 2695 Separation Module and a Photodiode Array Detector (PDA, 2996) connected to the Empower data acquisition software (version 2.0) (Waters, Milford, MA, USA). Chromatographic separation was carried out on an XTerra RP18 (150 mm × 4.6 mm, particle size 3.5 μm; Waters) analytical reversed-phase column equipped with an XTerra RP18 (20 mm × 3.9 mm, particle size 5 μm; Waters) guard column, both thermostated at 40°C. Samples were separated using an isocratic mobile phase consisting of acetonitrile - ammonium acetate buffer (pH 8.0, 10 mM) (55:45, v/v), previously filtered through a 0.22 μm membrane filter (Millipore, Bedford, MA, USA). The flow rate was 1.0 mL/min and the total assay run time was 12 min. UV detection was set at 285 nm. An Extraction Manifold (Waters) liquid handling system was used to perform the sample preparation. A DRI-BLOCK DB-3 evaporator (Techne, Stone, United Kingdom) was used for drying eluates from SPE.

2.3 Stock solutions, working solutions, calibration standards and quality control (QC) samples preparation

Stock solutions of ISC, at the concentration of 1 mg/mL, were prepared in methanol. Working solutions, at the concentration of 500, 200, 100, 50, 20, 10, 5, and 2.5 µg/mL, were then obtained through scalar dilutions from the ISC stock solution in methanol. Both the stock and working solutions were stored at –20°C. Plasma calibration standards at 10, 5, 2, 1, 0.5, 0.2, 0.1, 0.05, and 0.025 µg/mL were freshly prepared by 1:100 dilution of the respective stock or working solution in human plasma and analyzed on the same day. Of note, the total added volume of organic solvent spiked in all samples corresponded to 1% of the biological sample, a value below the threshold level of 2% recommended for non-biological matrix by the FDA guidelines (US Food and Drug Administration, 2013). Quality control (QC) samples at low (0.1 µg/mL), medium (1 µg/mL), high (7.5 µg/mL) concentration levels as well as the lower and the upper limit of quantification (LLOQ = 0.025 µg/mL and ULOQ = 10 µg/mL, respectively) were prepared by diluting the working solutions in plasma. The QC samples were prepared in batches at the same occasion, stored at –20°C, and then thawed and thermized at 60°C for 60 min on the day of analysis.

2.4 Sample preparation

Blood samples (5 mL) were withdrawn from patients and collected in tubes with K_3EDTA as an anticoagulant. Plasma samples were obtained after centrifugation of blood samples at 1,600 x g for 10 min at 4°C (Sigma Centrifuge, Model 2K15) and then stored at –80°C until analysis. On the day of analysis, plasma samples were thawed and heated at 60°C for 60 min. Three hundred and fifty µL of heat-inactivated plasma samples were deproteinized by addition of 350 µL of acetonitrile, vortexed thoroughly, and then centrifuged at 15,000 x g for 5 min at room temperature (RT). An aliquot of 500 µL of supernatants was transferred to clean tubes and diluted 1:1 with 500 µL of Milli-Q water. Samples clean-up was then achieved using Oasis HLB 1cc cartridges (Waters) following the SPE procedure suggested by the manufacturer's instructions, with minor modifications. Briefly, the sample mixtures were loaded into Oasis HLB 1cc cartridges, already

activated and equilibrated with 1 mL of methanol and 1 mL of water, respectively. After a

washing step with 1 mL of 5% methanol diluted in water (v/v), the analyte was eluted from the

cartridges with 0.5 mL of 100% methanol, collected and finally evaporated to dryness under a N_2

stream at 40°C. The dried residues were then reconstituted with 62.5 µL of mobile phase and

centrifuged at 15,000 x g for 5 min at RT. The supernatants were transferred to HPLC vials and

kept at 4°C in the autosampler until analysis. The injection volume for each sample was 20 µL.

2.5 Method validation

This method was validated to meet the acceptance criteria of the FDA guidelines for bioanalytical

method validation (US Food and Drug Administration, 2013). Assay validation was performed to

assess the recovery, limit of detection (LOD) and LLOQ, selectivity, specificity, linearity,

accuracy, and precision of the method.

2.5.1 Extraction recovery

The mean extraction recovery of ISC from human plasma was determined by analyzing four

replicates at the LLOQ, low-, medium-, high-QC, and ULOQ level. The ISC peak area response

of extracted QC samples was compared to that of the equivalent concentration level of the analyte

directly spiked into the mobile phase and not subjected to extraction, which represents 100%

recovery. The extraction recovery of ISC from plasma was expressed as a percentage of the

response of ISC diluted in mobile phase. Since the extraction procedure resulted in an analyte

concentration of 4 times (250-µL plasma samples are extracted, evaporated to dryness and then

reconstituted with 62.5 µL of mobile phase), a concentration factor of 4 had to be applied to the

ratio of the peak area response, as described previously (Loregian et al., 2006).

2.5.2 Specificity and selectivity

Ten different batches of human plasma were evaluated as blank and spiked with ISC at LLOQ

level to check for possible interferences from endogenous plasma components. Chromatograms of

blank samples were overlapped with those of LLOQ QC samples to verify the lack of analytic

interference at the retention time of ISC. Potential interferences due to concomitantly

administrated drugs were also investigated by analyzing both samples from several patients and

chromatograms obtained by injecting the drugs listed in Table 1 and diluted in the mobile phase (at

10 µg/mL).

2.5.3 Precision and accuracy

The ISC concentration was back-calculated from all calibrator points of the calibration curves and

accuracy and precision of these measurements were determined. Five replicates of each QC

sample spiked with five different concentrations of ISC, corresponding to the LLOQ, low-,

medium-, high-QC, and ULOQ were also analyzed to determine intra-day precision and accuracy.

Inter-day values were obtained by duplicate analyses of the QC samples repeated on 5 different

days. Precision was calculated as the percentage of the coefficient of variation (CV) of the

measured values, whereas the accuracy represents the percentage of deviation between nominal

and measured concentration (% bias). The acceptance criteria for all precision and accuracy

parameters were deviations within 15% at each QC level, except for the LLOQ, where could not

exceed by more than 20%, as requested by FDA guidelines (US Food and Drug Administration,

2013).

2.6. Effect of dilution and carry-over effect

The effect of dilution of plasma samples was validated with a ULOQ diluted to half, and analyzed

five times. Carry-over effect was tested by injecting three blank plasmas after the ULOQ:

percentage of disappearance of analyte was evaluated.

2.7. Stability evaluation

ISC stability in human plasma was assessed by analyzing six replicates of QC samples at the low-, medium-, high-QC concentrations. Three series were immediately analyzed, whereas the three other series were subjected to different conditions of storage/treatment that clinical samples can experience during TDM analysis. The ISC peak area of both samples groups was then compared. The spiked plasma samples were exposed to the following different conditions: (a) storage at –80°C for 5 months; (b) storage at RT for 48 h in the dark; (c) storage at RT for 48 h under ambient light (d) after three freeze-thaw cycles; and (e) after the thermization process (60°C for 60 min). Additionally, post-preparative sample stability of dried extracts (i.e., after SPE) at –20°C for 168 h and of extracts reconstituted in the mobile phase and incubated in the autosampler at 4°C for 96 h were analyzed. The stability of both stock and working ISC solutions in methanol stored at −20°C was also investigated.

2.8. Statistics

Statistical analysis was performed by the GraphPad Prism 6 (GraphPad Software Inc.) and Microsoft Excel. In stability studies, comparisons of assay values were made by one-way analysis of variance (ANOVA).

3. RESULTS

3.1 Sample preparation

The purpose of sample extraction optimization was mainly to achieve high extraction recoveries and minimize interferences in order to improve the method sensitivity and the reliability of HPLC–UV analysis. Since ISC is highly bound (>99%) to human plasma proteins (Astellas Pharma US, 2015), we first assessed protein precipitation methods using two different precipitating agents that are most commonly used (methanol and acetonitrile) at different dilution factors. Protein plasma

precipitation with acetonitrile, when added at a ratio of 1:1, gave the best results (data not shown), achieving a high recovery of ISC (around 100%), but an unsatisfactory clean-up efficiency. Other approaches were then tested such as liquid–liquid extraction using different solvents (i.e., chloroform, ethylacetate, ether, dichloromethane, and dichloroethane) and SPE using various cartridges (e.g., Oasis HLB, Oasis WAX, and Oasis MAX cartridges from Waters; Bond-Elut phenyl boronic acid and Bond-Elut C18 cartridges from Varian). However, all these extraction approaches resulted in low clean-up efficiency and/or low recovery (data not shown). In particular, a suboptimal recovery was in general observed in SPE, probably because the high extent of drug-plasma proteins binding may have negatively affected the extraction recovery. Therefore, we attempted the combination of the deproteinization using acetonitrile with a standard SPE protocol employing Waters Oasis HLB 1cc cartridges, as this strategy was previously found by our group to succeed in improving the extraction recovery of other drugs (e.g. simeprevir and daclatasvir) extensively bound to plasma proteins (Nannetti et al., 2016; Nannetti et al., 2017). SPE was carried out following the protocol recommended by the producer, but the elution step was modified by decreasing the volume of the eluting solution (0.5 mL of methanol instead of 1 mL), as this allowed shortening the step of SPE eluates drying, while maintaining a high drug recovery. This combined protein precipitation-SPE procedure allowed to achieve a high and constant ISC recovery from human plasma at different drug levels (see Section 3.3.2), as well as to concentrate the samples in order to reach a high sensitivity, without having interferences at the retention time of the analyte (see Section 3.3.3).

3.2 Optimization of chromatographic conditions

The chromatographic conditions were optimized by testing different compositions of mobile phases and various chromatographic columns, including a Waters Atlantis dC18 (150 mm × 3.9 mm,5 μm) column, a Symmetry C18 (75 mm × 4.6 mm, 3.5 μm) column, and an XTerra RP18 (150 mm × 4.6 mm, 3.5 μm) column. The best chromatographic separation was achieved using an

isocratic mixture of ammonium acetate buffer (pH 8.0, 10 mM) and acetonitrile (45:55, v/v) at a constant flow rate of 1.0 ml/min on an XTerra RP18 (150 mm × 4.6 mm, 3.5 μm; Waters) column. These conditions allowed the elution of ISC at a retention time of 8.7 min with a good resolution from plasma endogenous interferences, as shown in Figure 1. The run time was 12 min, a time sufficient to remove all plasma components from the column. Since adsorption of some azoles (e.g., posaconazole and itraconazole) to glassware was previously observed by us and others (Verweij-van Wissen et al., 2012 and unpublished data), the possible adsorption of ISC to glassware was investigated by using Waters silanized glass vials along with untreated glass vials for the reconstituted samples in the autosampler. However, no difference was observed in ISC levels when using either type of vial (data not shown). The UV detection was set at 285 nm, wherein ISC has the maximal absorbance (Figure 2) and the highest signal-to-noise ratio was recorded in chromatograms. The detection at such wavelength allowed reaching a high sensitivity (see Section 3.3.1) resulting in a LLOQ two-fold lower than that reported by a UPLC-UV method that performed ISC detection at 260 nm (Verweij-van Wissen et al., 2012), wherein ISC absorbance is approximately half of that at 285 nm (Figure 2). Figure 1 reports typical chromatograms obtained from (A) blank (drug-free) plasma; (B) blank plasma spiked with ISC at LLOQ (0.025 μg/ml), and (C) blank plasma spiked with ISC at ULOQ (10 μg/ml).

3.3 Method validation

The proposed analytical method was validated according to the FDA guidelines in terms of linearity, sensitivity, recovery, selectivity, specificity, precision, and accuracy (US Food and Drug Administration, 2013).

3.3.1. Linearity and sensitivity

A nine-point calibration standard curve of ISC in plasma, ranging from 0.025 to 10 μg/mL, was prepared in triplicate in five independent runs. This calibration curve covers the whole range of

ISC concentrations that have been so far observed in human plasma from clinical patients (Maertens et al., 2016). The method was linear over the tested concentration range with correlation coefficients (r^2) greater than 0.998 for each calibration curve. The LOD, defined as the drug concentration that produces a signal-to-noise ratio (S/N) of 3, was 0.012 μg/mL. The LLOQ was found to be 0.025 μg/mL, with an S/N of 6.3. A representative chromatogram of this calibration point is shown in Figure 1B. The lowest C_{trough} values measured in patients at steady-state (Maertens et al., 2016) are well within the concentration range of the calibration curve and considerably higher than the LLOQ. These data confirmed the suitability of this method for routine TDM analysis of ISC in the clinical setting, suggesting the assay potential for dosing ISC even in patients with reduced compliance and in the case of low ISC concentrations due to drug-drug interactions.

3.3.2. Extraction recovery

The absolute recovery of ISC from human plasma ranged from 93.1% to 97.5% in the tested concentration range (0.025-10 μg/mL), with CV values varying from 2.4% to 4.9%. Thus, differently from a previous extraction method for ISC from human plasma that reported a trend toward lower recovery at lower ISC concentrations (Jørgensen et al., 2017), our extraction procedure provides a constant recovery across the whole concentration range, also when using different lots of SPE cartridges. Taken together, these data demonstrate that the sample preparation step is an efficient procedure, which concurs to the reliability and precision of this method.

3.3.3. Selectivity and specificity

Analyses of ten different batches of commercial control human plasma were carried out to assess the selectivity and specificity of the method. A representative chromatogram of drug-free plasma,

reported in Figure 1A, shows that no interfering peaks from endogenous plasma substances are detected within the retention time window of ISC (see also Figure S1 of Supporting Information).

Method specificity was also evaluated by checking possible interferences at the analyte retention time after direct injection of 10 µg/mL of several other drugs (listed in Table 1 along with their retention time) diluted in the HPLC mobile phase. None of the tested drugs interfered with ISC detection. In addition, no analytical interference was observed during routine TDM analysis of clinical plasma samples from patients co-treated with ISC and other drugs. The lack of analytic interference was also confirmed by the use of the peak purity checking system and the library matching of the Empower software.

3.3.4. Precision and accuracy

Method precision (expressed as % CV) and accuracy (expressed as % bias) were evaluated in plasmas spiked with ISC at 5 concentrations (see also Section 2.5.3) as well as from the back-calculated concentrations of ISC calibration standards; the results are reported in Table 2 and Table S1 of Supporting Information, respectively. The intra-day and inter-day CVs were always equal or lower than 6.3% and 7.9%, respectively, in the tested concentration range. The intra-day and inter-day deviations (% bias) of the means of the measured concentrations from their nominal concentrations ranged from -5.0% to 3.8% and from – 4.0% to 8.0%, respectively. Overall, all accuracy and precision parameters were well within the acceptance criteria (<15%) set by FDA guidelines for method validation (US Food and Drug Administration, 2013), thus demonstrating that this method is highly reliable and reproducible for the determination of ISC levels in human plasma samples.

3.4. Effect of dilution and carry-over effect

Quantification of half diluted ULOQ gave reliable results: 4.2% for bias and 4.9% for CV. No carry-over effect was detected, since after injection of the ULOQ no ISC was quantifiable in three following plasma blanks.

3.5 Participation in an external quality control (proficiency testing) program

To further assess the precision and reliability of the method, we participated in an international proficiency testing program ("round robin test"). Four single-blind QC samples of plasma containing ISC at unknown concentrations were prepared and distributed by Basilea Pharmaceutica Ltd. (Basel, Switzerland) to thirty analytical laboratories across Europe for an external quality assessment. For each QC, the percentage of deviation (% bias) between the measured ISC level and the nominal concentration was calculated by Basilea Pharmaceutica. Feedback of the results was then given to the participants to enable them to improve their method when necessary. The accuracy of our method ranged from 1.3% to 4.2% for all QC samples, thus well within the 15% of deviation requested by the FDA guidelines.

3.6 Stability data

Stock and working solutions of ISC dissolved in methanol were proved to be stable at -20°C for at least 6 months (reduction of ISC peak areas below than 15%), in accordance with data already published (Verweij-van Wissen et al., 2012; Fatiguso et al., 2017). The stability of ISC was also investigated in plasma samples spiked with the drug at three different concentration levels and subjected to different storage and treatment conditions which clinical samples can commonly experience during the whole TDM process. The ISC peak areas measured in treated QC samples were compared with those of immediately analyzed QCs at the respective concentration. Each QC sample of both groups was assayed in triplicate. ISC was considered stable when the deviation of ISC peak areas between these two groups was within 15%, as indicated in the FDA guidelines for bioanalytical methods validation (US Food and Drug Administration, 2013). As summarized in

Table 3, ISC was found to be stable in human plasma under all tested stability conditions (one-way analysis of variance, not significant). Specifically, stability was confirmed for ISC in plasma samples stored at RT for 48 h and after three freeze-thaw cycles, as reported in previous studies (Farowski et al., 2010; Toussaint et al., 2017; Jørgensen et al., 2017; Müller et al., 2017; Fatiguso et al., 2017; Verweij-van Wissen et al., 2012). Stability of the analyte was also found in plasma stored at –80°C for 5 months, and when heated at 60°C for 60 min (Table 3), a safety procedure which is recommended to inactivate pathogenic microorganisms, including *Aspergillus* spp. fungi, in biological samples (US Environmental Protection Agency, 1979) and which we usually apply to all plasma samples received by our TDM unit (Loregian et al., 2006: Loregian et al., 2007). Since we recently experienced the photochemical degradation of some drugs in plasma under daylight condition (Nannetti et al., 2017) and to the best of our knowledge, no detailed investigation on ISC photo-stability in human plasma under common light conditions that clinical samples can experience has been published, we also investigated the stability of ISC in plasma samples stored at RT for 48 h following exposure to ambient light (bench top). No significant difference in ISC levels between plasma samples stored at RT for 48 h either in the dark or under ambient light was observed (Table 3). Finally, the post-preparative stability of ISC both in dried and in reconstituted extracts was evaluated. Table 3 shows that ISC was stable in dried and reconstituted extracts at -20°C for at least 1 week and in autosampler at 4°C for 96 h, respectively.

3.7 Analysis of patients samples

The applicability of the method in the clinical setting was evaluated by analyzing plasma samples obtained from 10 patients treated with ISC. The measured ISC plasma level ranged from 1.33 µg/mL to 4.22 µg/mL (median of 2.38 µg/mL), which were within the range reported in the literature (Maertens et al., 2016). Figure 3 shows two representative chromatograms of plasma samples of patients at steady-state receiving the recommended maintenance oral dose of 200 mg daily of ISC. 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 ISC in our diagnostic Unit.

4. DISCUSSION AND CONCLUSIONS

The present paper describes the development and validation of a simple, accurate, selective, and sensitive HPLC assay with UV detection for the quantification of ISC in human plasma. Waiting to define the precise role of TDM for ISC, the determination of drug exposure is already required in selected clinical cases to adjust the drug dosage, in order to guarantee the effectiveness of therapy and to minimize the toxicity (Stott and Hope, 2017). Previously published methods for the determination of ISC levels in plasma involved the use of UPLC, LC-MS/MS, or fluorescence detectors, which are not always available in analytical laboratories (Schmitt-Hoffmann et al., 2006 a; Schmitt-Hoffmann et al., 2006 b; Farowski et al., 2010; Verweij-van Wissen et al., 2012; Toussaint et al., 2017; Müller et al., 2017; Fatiguso et al., 2017; McShane and Wang 2017). To the best of our knowledge, this is the first HPLC method with UV detection developed for the determination of ISC in patients plasma, which uses commonly available equipment and still offers a sensitivity two or eight times higher (0.025 μg/mL versus 0.05 μg/mL and 0.2 μg/mL, respectively) than that of two previously published chromatographic methods not coupled with mass spectrometry detection (Verweij-van Wissen et al., 2012; Jørgensen et al., 2017). The sensitivity of this method is also comparable or even higher (0.025 μg/mL versus 0.031 μg/mL - 0.2 μg/mL) than that of some LC-MS/MS methods developed for TDM of ISC (e.g., Toussaint et al., 2017; Fatiguso et al., 2017; McShane and Wang 2017). This method also exhibits great robustness and reproducibility, having intra-day and inter-day bias and CV values comparable to or better than those of other published methods (e.g., Toussaint et al., 2017; Fatiguso et al., 2017). In addition, another major advantage of this assay is that it employs an isocratic elution, since gradient elution requires control by a gradient HPLC pump system, re-equilibration time, perfect solvent mixing, etc. Although this can imply a longer sample run than gradient-based methods, the run time of this assay is similar (12 minutes versus 6 - 13 minutes) to that reported from others

(e.g., Farowski et al., 2010; Verweij-van Wissen et al., 2012, Jørgensen et al., 2017; Fatiguso et al., 2017). Nevertheless, the introduction of a gradient with an increasing acetonitrile concentration could speed up the ISC elution and reduce the run time. Furthermore, taking into account the high precision and reproducibility of this assay along with the constant recovery of the extraction procedure, an internal standard was not required and hence not used in order to further simplify the method. This method was fully validated according to the FDA recommendations and exhibited very good performance in an international quality assessment program involving thirty analytical laboratories across Europe.

Stability studies of ISC under different common conditions to which the drug can be subjected during the TDM process showed that ISC concentration remains stable both in plasma and in processed samples under all tested conditions, including in plasma samples exposed to ambient light, a condition in which, to our knowledge, stability studies have not been previously published. In addition, for the first time we investigated the stability of ISC to a thermal microbial inactivation at 60°C for 60 min, a safety procedure which is recommended to inactivate pathogenic microorganisms in biological samples. Indeed, Fatiguso *et al*. recently reported the investigation of ISC stability at 58°C for 35 min, which corresponds to inactivation conditions for HIV samples (Fatiguso et al., 2017); however, more aggressive procedures have been recommended to inactivate pathogenic fungi, including *Aspergillus* spp. (US Environmental Protection Agency, 1979). Thus, the results of our studies of drug stability upon thermal inactivation could be most useful for properly handling ISC-containing plasma samples during routine clinical analyses of infected plasma samples from patients with invasive aspergillosis, wherein the fungus is present in the blood.

Finally, the clinical applicability of the method and the appropriateness of the validated concentration range have been demonstrated in the analysis of plasma samples of patients receiving ISC.

Overall, our method provides a simple, sensitive, precise, and reproducible assay for routine therapeutic drug monitoring of ISC and can be most useful in conventional hospital laboratories with standard equipment, as well as suitable for pharmacokinetic investigations.

CONFLICT OF INTEREST

The authors disclose no conflicts.

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FIGURE 1 Typical chromatograms of (A) blank plasma sample, (B) plasma sample spiked with

ISC at 0.025 μg/mL (LLOQ), and (C) plasma sample spiked with ISC at 10 μg/mL (ULOQ).
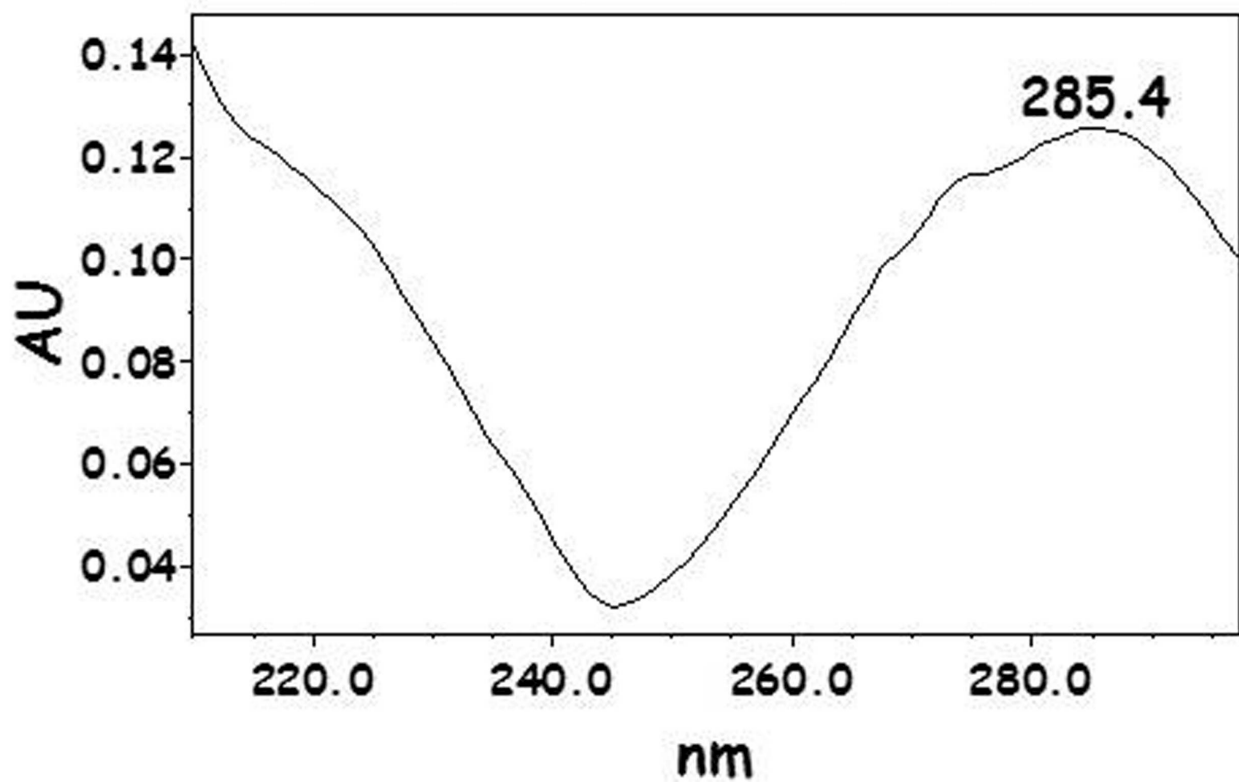
LLOQ exhibited an S/N of 6.3.

Figure 1
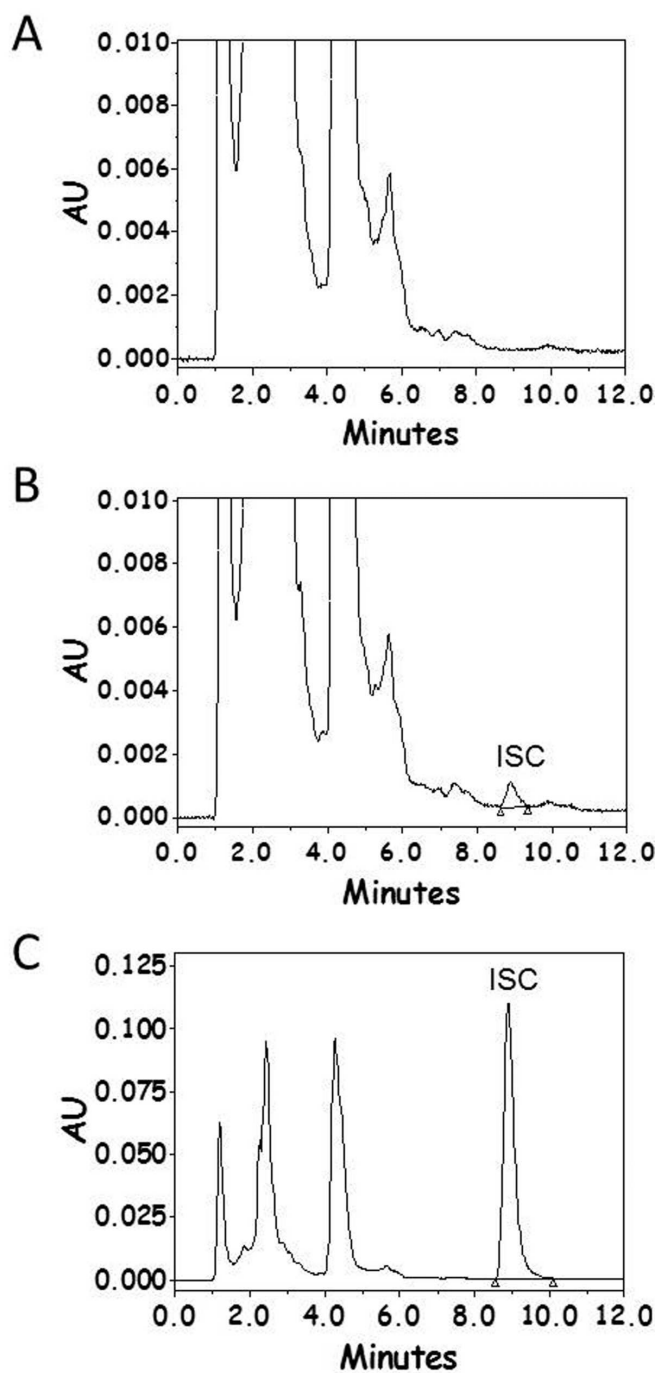
FIGURE 2 The UV spectrum of ISC.

Figure 2

FIGURE 3 Chromatograms of plasma samples from patients treated with ISC in combination with (A) omeprazole, bisoprolol, lorazepam, cyclosporine A, prednisone, trimethoprim, and sulfamethoxazol; or (B) acyclovir, omeprazole, and prednisone. The measured concentration of ISC was 1.81 µg/mL in sample (A) and 2.51 µg/mL in sample (B).

Figure 3
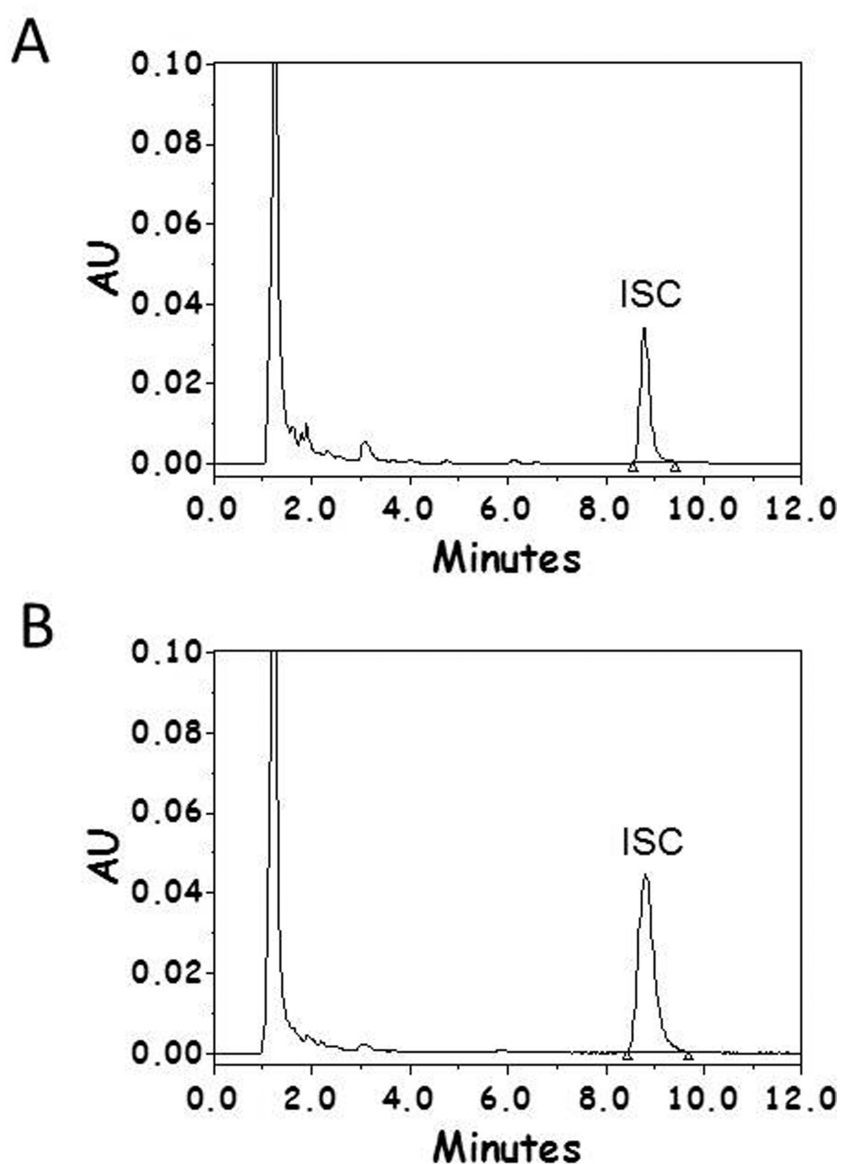
Supplementary Figure S1

Overlapped chromatograms of LLOQ (black line) and blank plasma (red line).
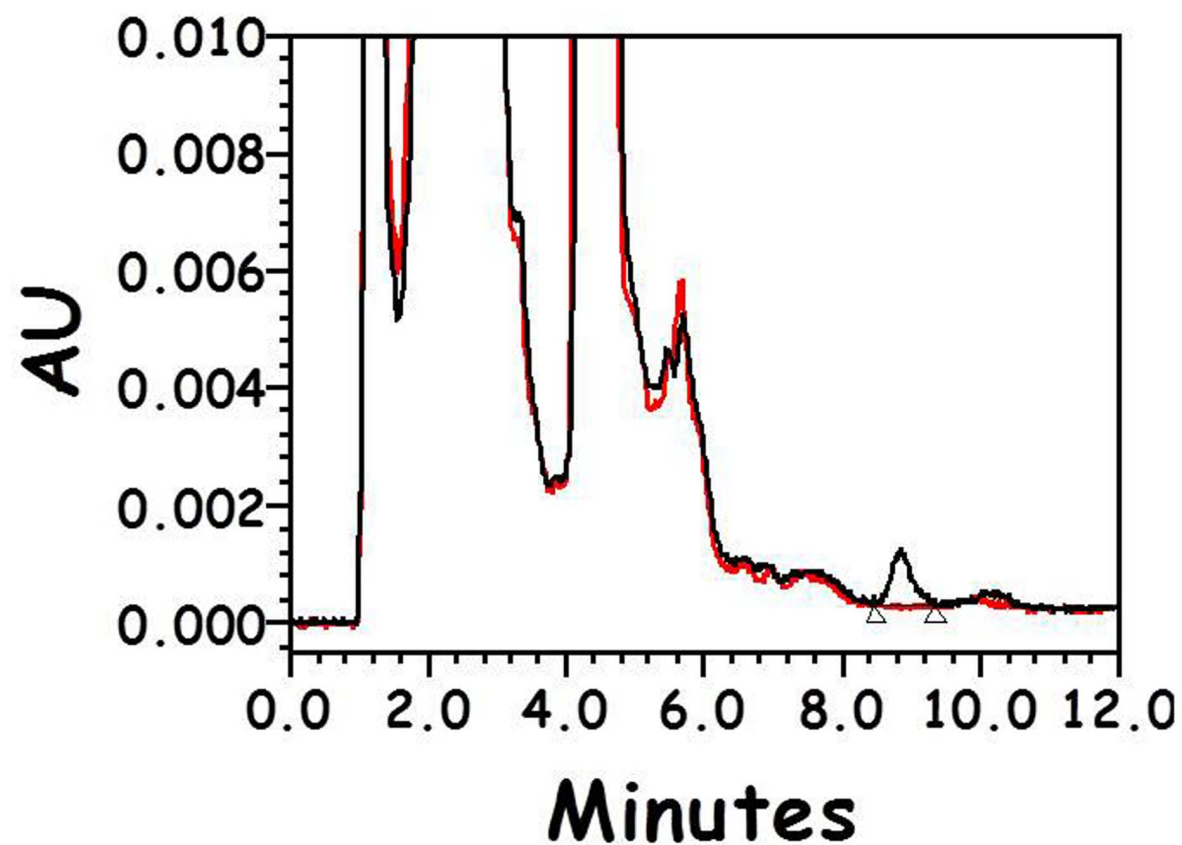
TABLE 1

List of potentially co-administrated drugs tested for specificity with their relative retention time.

Drug name	RT (min)	Drug name	RT (min)	Drug name	RT (min)
abacavir	1.9	ganciclovir	1.6	simeprevir	N.D.
acyclovir	1.7	gentamicin	2.9	sofosbuvir	2.6
ampicillin	1.3.	ibuprofen	N.D.	sofosbuvir metabolite GS-331007	1.6
amprenavir	4.3	imipenem	1.4	Stavudine	N.D.
atazanavir	6.9	indinavir	N.D.	streptomycin	N.D.
bisoprolol	N.D.	kanamycin	N.D.	sulfamethoxazole	N.D.
carbenicillin	1.3	lamivudine	1.6	teicoplanin	1.6
chloramphenicol	2.4	linezolid	2.1	tenofovir	N.D.
cyclosporine A	N.D.	lopinavir	4.0	tetracycline	1.8
daclatasvir	3.6	lorazepam	N.D.	tigecycline	2.4
daptomycin	1.4	paracetamol	N.D.	trimethoprim	N.D.
didanosine	1.6	posaconazole	7.1	vancomycin	N.D.
efavirenz	11.3	prednisone	N.D.	voriconazole	3.3
erythromycin	N.D.	ribavirin	N.D.	Zalcitabine	1.6
fluconazole	N.D.	rifampicin	2.4	Zidovudine	1.7
foscarnet	N.D.	ritonavir	N.D.		

N.D. = Not detected

TABLE 2

Intra-day and inter-day accuracy and precision results in QC plasma samples.

Nominal concentration (µg/mL)	Intra-day (n = 5)			Inter-day (n = 5)		
	Mean measured concentration (µg/mL)	Accuracy[a] (% bias)	Precision (% CV)	Mean measured concentration (µg/mL)	Accuracy[a] (% bias)	Precision (% CV)
0.025 (LLOQ)	0.024	-4.0	6.3	0.027	8.0	7.9
0.1 (low QC)	0.095	-5.0	5.2	0.096	-4.0	5.8
1.0 (medium QC)	0.994	-0.6	1.9	1.063	6.3	5.0
7.5 (high QC)	7.785	3.8	3.8	7.696	2.6	4.1
10.0 (ULOQ)	10.147	1.5	4.8	10.394	3.9	5.1

[a]Accuracy = [(measured concentration – nominal concentration)/nominal concentration] x 100

TABLE 3

ISC stability in plasma samples and in dried or reconstituted extracts under different treatment/storage conditions.

	Nominal concentration (µg/mL)		
	0.1	**1.0**	**7.5**
(A) In plasma incubated at 60°C for 60 min			
Deviation (%)	3.9	2.8	-3.5
CV (%)	5.7	3.3	3.0
(B) In plasma stored at room temperature for 48 h in the dark			
Deviation (%)	-4.3	-3.3	-1.8
CV (%)	5.0	2.1	3.0
(C) In plasma stored at room temperature for 48 h exposed to daylight			
Deviation (%)	-10.2	-8.8	-5.8
CV (%)	6.9	6.3	5.5
(D) In plasma stored at – 80°C for five months			
Deviation (%)	3.7	-5.4	-3.5
CV (%)	4.2	2.8	4.4
(E) In plasma after three freeze/thaw cycles			
Deviation (%)	5.9	6.5	-0.7
CV (%)	5.5	4.8	2.0
(F) In reconstituted extracts stored at 4°C for 96 h			
Deviation (%)	5.3	-1.6	4.0
CV (%)	5.7	2.5	5.3
(G) In dried extracts stored at – 20°C for 1 week			
Deviation (%)	-2.9	-2.4	-0.6
CV (%)	1.8	2.9	2.8

Supplementary Table S1

Accuracy and precision results of back-calculated calibration standards

Nominal concentration (μg/mL)	Mean measured concentration (μg/mL)	Accuracy[a] (% bias)	Precision (% CV)
0.025	0.026	4.0	7.6
0.05	0.047	-6.0	5.7
0.1	0.105	5.0	5.8
0.2	0.207	3.5	3.9
0.5	0.484	-3.2	2.1
1.0	0.973	-2.7	3.2
2.0	2.058	2.9	2.5
5.0	5.071	1.4	2.2
10.0	10.138	1.4	4.0

[a]Accuracy = [(measured concentration – nominal concentration)/nominal concentration] x 100