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ORIGINAL

High Performance Liquid Chromatography–Tandem Mass Spectrometry Method for Simultaneous Quantification of Caspofungin, Anidulafungin and Micafungin in Human Plasma for Feasible Applications in Pediatric Haematology/Oncology

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Abstract A liquid chromatography–tandem mass spectrometry method was validated for the simultaneous quantification of anidulafungin (ANF), caspofungin (CSF) and micafungin (MCF) in human plasma. The triple quadrupole mass spectrometer was equipped with a heated-electrospray ionization probe and a Zorbax SB-C18 column. The calibration curves were generated using a weighted ($1/x$) linear regression for caspofungin, a weighted ($1/x$) quadratic function for micafungin and a log–log transformed linear regression for anidulafungin. The coefficients of variation of the normalized matrix factor were 11.2, 6.1 and 11.6% at low quality control level (QcL) and 11.5, 12.7 and 11.3% at high quality control level (QcH), for ANF, CSF and MCF, respectively, confirming the absence of matrix effects. The resulting method is sensitive (LLOQ 0.2 μg mL^{-1} for the echinocandins; LOD 0.05 μg mL^{-1} for MCF and 0.01 μg mL^{-1} for CSF and ANF), precise (within- and between-run from 2.9 to 15.0% and from 6.3 to 12.8%, respectively) and accurate (within- and between-run from 93.2 to 104% and from 95.9 to 109%, respectively) covering clinical concentration ranges acceptable for pediatric patients (0.2–10 μg mL^{-1}). This analytical method could represent an efficient tool for monitoring the real-time efficacy and the safety of different recommended antifungal regimes in the pediatric population.

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Introduction

Caspofungin acetate (Cancidas; Merck) was the first echinocandin product approved in the United States by Food and Drug Administration (2001) and licensed for the treatment of patients with invasive candidiasis, aspergillosis refractory to amphotericin B and empiric treatment of febrile neutropenia in immunocompromised patients as it has shown efficacy in the treatment of a variety of fungal infections [1]. Micafungin (2005) is, at present, the only echinocandin approved as prophylaxis for Candida infections in hematopoietic stem cell transplant (HSCT) patients and in children with prolonged neutropenia, even in neonates. There are also several lines of evidence to support anidulafungin's role (2006) as the primary therapy for the treatment of invasive candidiasis in non-neutropenic adult patients, and as an alternative therapy to fluconazole in patients with esophageal candidiasis with azole intolerance or triazole-resistant *Candida* [2, 3].

Due to their large molecular weight, none of these antifungal agents is significantly absorbed when administered orally, therefore, they are available for intravenous administration only; they are highly bound to plasma proteins and achieve negligible concentrations in cerebrospinal fluid, intravitreal fluid and urine [4].

Their mechanism of action is unique among antifungal therapies, since they inhibit the synthesis of (1,3) β-D-glucan, an essential component of the fungal cell wall, by acting as noncompetitive inhibitors of glucan synthase. Fungal cells that lack sufficient glucan are osmotically fragile and easily lysed [5, 6].

While their antifungal activity overlaps, there are substantial differences in the pharmacokinetics (PK) of the three drugs. Inadequate initial antifungal dosing contributes not only to suboptimal outcomes, but also to the emergence of resistance [7], whereas the effects of incorrect dosing might impair their efficacy and safety [8]. Several studies, also conducted on ICU hospitalized patients [7, 8], demonstrate that effectiveness of the antifungal therapy depends on the large intra- and inter-individual variability of the echinocandins pharmacokinetics. Numerous factors affect PK parameters: age (pediatrics vs adults), chemotherapy, HSCT, body composition (weight and protein/albumin content), drug–drug interactions, hepatic and renal impairment, and higher risk of infections due to indwelling catheters. All these aspects have to be considered for a therapy tailored to the individual needs. Even though specific reference ranges have not yet been clearly identified as a suitable guide for therapeutic echinocandin monitoring, there is increasing clinical evidence regarding the need to detect their plasma concentrations in specific situations [9], when altered pharmacokinetics may be expected and when dosing guidelines are conflicting [10], especially in severely ill patients or in cases when standard dosing regimens are ineffective.

Based on these considerations, we aimed to develop a simple, sensitive, rapid and robust HPLC–MS/MS method for the simultaneous analysis of caspofungin, anidulafungin and micafungin in human plasma, in a single analytical run, for analyses performed within 72 h from blood sample collection.

Materials and Methods

Chemicals and Reagents

Anidulafungin (A673000; Lot: 23-GHZ-133-2) and micafungin sodium (M342350; Lot:3-EOD-66-1) were purchased from Toronto Research Chemicals (TRC, ON, Canada), and caspofungin diacetate (SML0425-25MG) was purchased from Sigma Aldrich (Sigma-Aldrich S.r.l., Milan, Italy). Ultrapure water was obtained from a Direct-Q apparatus (Millipore Corp., Burlington, MA, USA). Formic Acid (33015-1L), acetonitrile (34851-2.5L), methanol (34860-2.5L-R) and 2-propanol (33539-L-R) were purchased from Sigma Aldrich. All chemicals were analytical grade.

Blank plasma used for the preparation of calibrators and control samples was purchased from SeraCare (Milford, MA, USA) (HB-100; Lot: 10112103). All plasma samples (calibrators, quality controls as well as patients' blood samples) contained sodium heparin as anticoagulant. Patient samples were centrifuged within 1 h from their collection at 3200*g*, for 10 min at +4 °C (Labofuge 400R, HERAEUS—Germany). Plasma was separated and transferred to 2.0 mL cryovials before being stored at −80 °C and analyzed within 72 h.

Samples

Three different stock solutions of anidulafungin (ANF: 0.5 mg mL^{-1}, in methanol), micafungin (MCF: 1 mg mL^{-1} in water) and caspofungin (CSF: 1 mg mL^{-1} in water) were prepared by carefully weighing the corresponding standard powders. The standard working solution "A" was obtained adding 2, 1 and 1 mL of the above respective stock solutions, to 6 mL of methanol (final volume—Vf 10 mL) to obtain a final concentration of 100 µg mL^{-1}. The other working standard solutions were obtained by serial dilution, with methanol, from "A" (100 µg mL^{-1}) to "F" (2 µg mL^{-1}). Calibrators were prepared by adding 10 µL of each working solution to six different plasma drug-free samples (90 µL). Quality controls (QCs) were similarly prepared, starting from another "A_{QC}" working solution to obtain final concentrations of 80, 40 and 4 µg mL^{-1} for QcH, QcM and QcL, respectively. The internal standard working solution (reserpine: 50 ng mL^{-1}) was obtained by adding 25 µL of 1 mg mL^{-1} stock solution in CH_3OH to 50 mL of CH_3CN: the resulting concentrated solution was then tenfold diluted to a final concentration of 50 ng mL^{-1}, with CH_3CN containing 0.05% HCOOH. All stock solutions were stored at −20 °C.

Calibration Standards and Quality Controls (QCs) Preparation

In 1.5-mL Eppendorf tubes, plasma samples, calibrators and controls (100 µL) were spiked with 300 µL of $(CH_3CN)_{0.5\%\ HCOOH}$ containing IS (reserpine: 50 ng mL^{-1}) and 50 µL of ethanol [11], to promote protein precipitation. The mixtures were vortex-mixed (15 s) and centrifuged for 10 min at 10,400*g*, at room temperature. The clear supernatant was then directly injected into the HPLC–MS/MS system (15 µL).

Each analytical run consisted of a blank sample (θ: blank plasma sample without analyte and without IS), a zero sample (θ_{IS} blank plasma sample with IS), a standard sample (θ_{WS}: blank plasma sample with one of the working solutions containing each echinocandin), calibration standards (at six concentration levels), and three levels of Qc samples (low, medium and high). The back calculated concentrations of the calibration standards had to be within ±15% of the nominal values, except for the LLOQ, for which had to be within ±20%. At least 75% of the calibration standards, with a minimum of six levels, had to fulfil this criteria. The accuracy values of the Qc samples had to be within ±15%

of the nominal values. At least 67% of the Qc samples and at least 50% at each concentration level, had to comply with this criterion. In case these criteria were not fulfilled, the analytical run was rejected, and the study samples re-extracted and analysed [12].

Liquid Chromatography and Mass Spectrometry

Separation of the three echinocandins was performed using a Thermo Scientific Accela system, a quaternary pump coupled to an autosampler, and an Agilent Zorbax SB-C18 (4.6 × 75 mm; 3.5 µm) column heated and maintained at 45 °C. Elution was carried out in gradient mode, at a flow rate of 0.8 mL min^{-1} with acidified water (0.1% HCOOH) (mobile phase A) and CH_3CN (mobile phase B). Fresh solvents were prepared daily for each analytical series: the injection volume was 15 µL.

A TSQ Quantum Access triple quadrupole mass spectrometer (Thermo Scientific) was employed with a heated-electrospray ionization probe (HESI), operating at 300 °C. The source parameters included a spray voltage of 3.5 kV, vaporizing temperature of 300 °C, sheath gas pressure of 60 (arbitrary units: au), auxiliary gas pressure 20 (arbitrary units: au) and capillary temperature of 350 °C. Multiple reaction monitoring (MRM) mode was utilized for all of the echinocandins and for the internal standard (IS): collision energy was optimized individually for each of them. The optimal instrument parameters and MS/MS transitions were determined by direct infusion at a flow rate of 15 µL min^{-1} for each compound solution, separately, into the MS/MS detector, at a concentration of 1 µg mL^{-1} in the mobile phases A:B = 50:50 (v/v). An example of a typical chromatogram, indicating also the selected *m/z* transitions and the collision energy (CE) for each analyte and IS, is reported in Fig. 1.

LC–MS/MS Assay Validation

All of the main bioanalytical method characteristics, essential to ensure the acceptability of the performance and the reliability of analytical results (i.e. selectivity, carryover, lower limit of quantification LLOQ, limit of detection LOD, calibration range, accuracy, precision, dilution integrity, stability and matrix effects) were analyzed for each echinocandin over more than 5 days, in accordance with the European Medicines Agency (EMA) "Guidelines on bioanalytical method validation" [12].

Standard calibration curves were prepared using six plasma calibrator points (A: 10 µg mL^{-1}; B: 5 µg mL^{-1}; C: 2.5 µg mL^{-1}; D: 1.0 µg mL^{-1}; E: 0.5 µg mL^{-1}

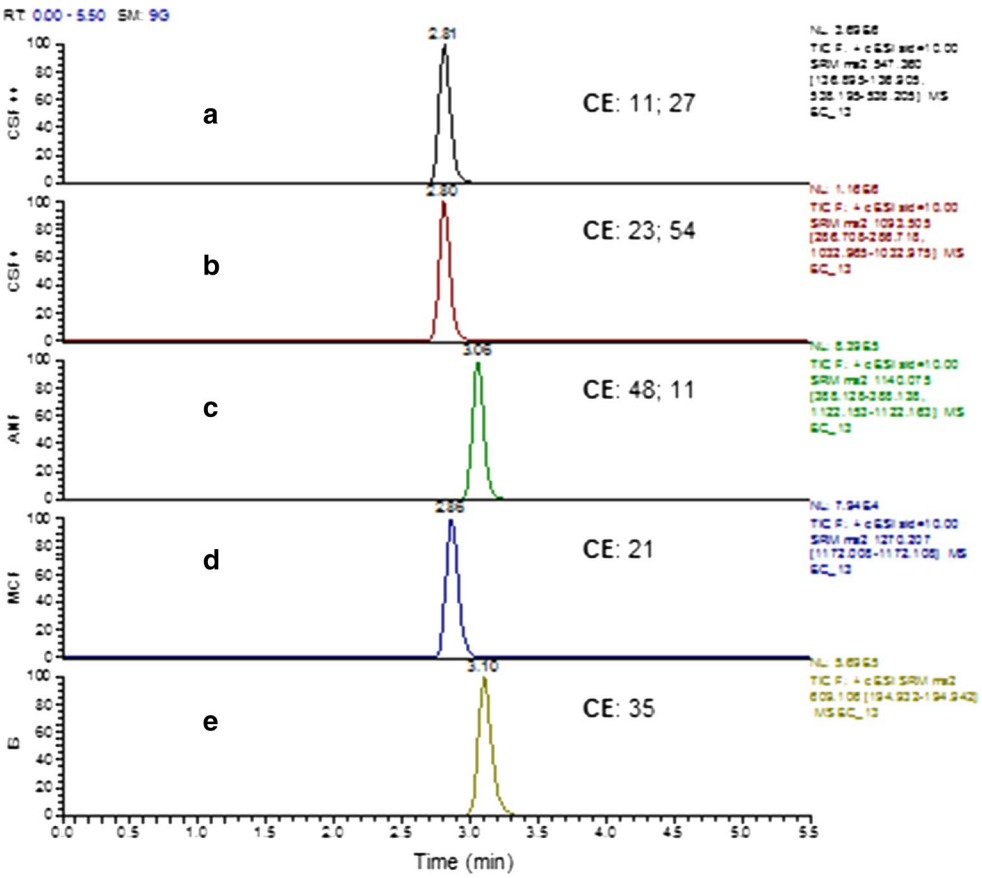

Fig. 1 A representative chromatogram of the three echinocandins (**a** CSF^{++}, *m/z* 547.36 → 538.20, 136.90; **b** CSF^{+}, *m/z* 1093.50 → 1032.97, 286.71; **c** ANF^{+}, *m/z* 1140.07 → 388.13,1122.16; **d** MCF^{+}, *m/z* 1270.21 → 1172.06; **e** IS; *m/z* → 609.11 → 194.94). Collision energies (CE: eV) are reported on the *right side* of the picture, respectively. Quantitative analysis were performed using only the singly charged protonated molecular ions

and F: 0.2 μg mL^{-1}) and three quality controls (QcH: 8.0 μg mL^{-1}; QcM: 4.0 μg mL^{-1}; QcL: 0.4 μg mL^{-1}). The LOD was the lowest concentration of the analytes detectable in a sample—by visual evaluation and with a signal to noise ratio (S/N) around 3—but not necessarily quantifiable as an exact value: it was evaluated as 0.05 μg mL^{-1} for micafungin and 0.01 μg mL^{-1} for caspofungin and anidulafungin.

LLOQ was the lowest concentration of each analyte in a sample, which could be reliably quantified, with acceptable accuracy (within 20% of the nominal value) and imprecision (a coefficient of variance better than or equal to 20%). It was chosen as the lowest calibration standard (F: 0.2 μg mL^{-1}) for all of the antifungal agents.

After defining all of the chromatographic parameters and mass spectrometer settings, selectivity (specificity) was evaluated by separately analyzing the mobile phases (A and B), the precipitant solution containing IS and six different drug-free extracted plasma lots (provided by healthy volunteers from the Immunohaematology and Transfusion Medicine Service of our Institute), to verify the absence of analytes and internal standard interfering peaks at the expected retention times.

The absence of carry-over was also assessed by injecting CH_3CN (0.1% HCOOH) after the calibration standards at the upper limit of quantification (ULOQ: 10 μg mL^{-1}), for all analytes.

Accuracy was assessed on samples spiked with known amounts of the three analytes at the quality control (Qcs and LLOQ) concentration levels. The Qc samples were spiked independently from the calibration standards, using separately prepared stock solutions and were analyzed against the corresponding calibration curves. The concentrations obtained were compared with the nominal values and accuracy was reported as the percent of the nominal value. Precision was demonstrated for LLOQ, in Low, Medium and High Qc samples, within a single run and between different runs, using the same runs and data as previously reported for accuracy.

To assess the stability of micafungin, anidulafungin and caspofungin in plasma, quality control samples were prepared and measured after 72 h at −20 °C and after three freeze–thaw cycles (−20 °C—room temperature): in details, Qc samples were stored and frozen in the freezer at −20 °C and allowed to thaw at room temperature for approximately 3 h. After complete thawing, were refrozen again, applying the same conditions. At each cycle, samples were frozen for at least 12 h, before they had been thawed again. Stability tests in whole blood samples were also performed in specimens collected and stored at +4 °C for 24 h. Processed sample stability was also evaluated after resting 24 h in the autosampler, at 10 °C. Stabilities were tested by comparing the quantitative results with the nominal concentrations (plasma) or with previous results: the mean concentration at each level had to be within ±15% of the expected result.

Matrix effects were first examined qualitatively: three specimens of extracted blank matrix were injected into the HPLC system by the autosampler, while three subsequent solutions (5 μg mL^{-1}) of caspofungin (5 μg $mL^{-1)}$, anidulafungin (5 μg mL^{-1}) and micafungin (10 μg mL^{-1}) were separately and continuously infused post-column, and mixed with the column effluent through a tee, before entering the electrospray interface (HESI). This type of infusion allows to raise the background level so that the matrix suppressions are showed as negative peaks.

The matrix effect was quantified using at least six different lots of different drug-free plasma matrix samples from individual donors: a pooled matrix was not used. The matrix factor (MF) was calculated for all three echinocandins and IS, in each matrix lot, by calculating the ratio of the peak area in the presence of matrix (measured by analyzing blank matrix spiked with the working solutions), to the peak area in absence of matrix (water instead of plasma). The IS normalized MF was also calculated by dividing the MF of the analyte by the MF of the IS [12, 13]. The matrix effect was investigated at Low and at High concentration levels (QcL, QcH) in six normal plasma samples and in two specific matrices: hemolyzed and lipemic plasma samples. The overall CV calculated for the concentration should not be greater than 15% [12]. Quality controls for the matrix effect investigation were prepared as previously reported.

Data Analysis

Xcalibur 2.07 and LCquan 2.5.6 software from Thermo Scientific (S. Jose, CA) were utilized for the LC–MS/MS system control, data acquisition and data analysis. Calibration curves were generated using a weighted ($1/x$) linear regression curve for Caspofungin, a weighted ($1/x$) quadratic function for Micafungin and a log–log transformed linear regression for Anidulafungin [14]. Analytes peaks were identified with a combination of retention times and the specific MRM transitions: the corresponding amounts were quantitated by normalizing the peak area to the internal standard and concentrations were calculated from the respective calibration curves.

Results

Chromatography

Using high performance liquid chromatography mass spectrometry, all three of the echinocandins in human plasma

were simultaneously separated and detected in a 6-min run. A representative chromatogram of spiked plasma is shown in Fig. 1. The three compounds have different parent mass-to-charge ratios and unique MRM transitions, so they are spectrally distinguishable.

Selectivity and Specificity

The selectivity of this method was evaluated by analyzing six different lots of drug-free extracted plasma (blank plasma) and lipemic and hemolyzed plasma samples from eight individual donors (pooled matrix was not used). Absence of interfering components at the expected retention times was also verified for the mobile phases (A, B) and the precipitant solution containing the IS: all responses were always less than 20% of the LLOQ for the analytes, and 5% for the internal standard.

Matrix Effects

Matrix effects were first examined qualitatively by simultaneous post-column infusions of antifungals into the MS/MS detector during the chromatographic analysis of blank plasma extracts. As illustrated in Fig. 2, during the chromatography of blank matrices, no significant drifts or shifts of the selected transition signals were apparent at the retention times for the three echinocandins.

For caspofungin, anidulafungin, micafungin and IS, the matrix factor (MF) was also evaluated in six lots of different drug-free plasma matrices, haemolysed and hyperlipidaemic plasma samples, by calculating the ratio of the peak area in the presence of matrix (blank matrix spiked with the analytes), to the peak area in absence of matrix (pure solutions of the analytes). The IS normalized MF was also calculated by dividing the MF of the analytes by the MF

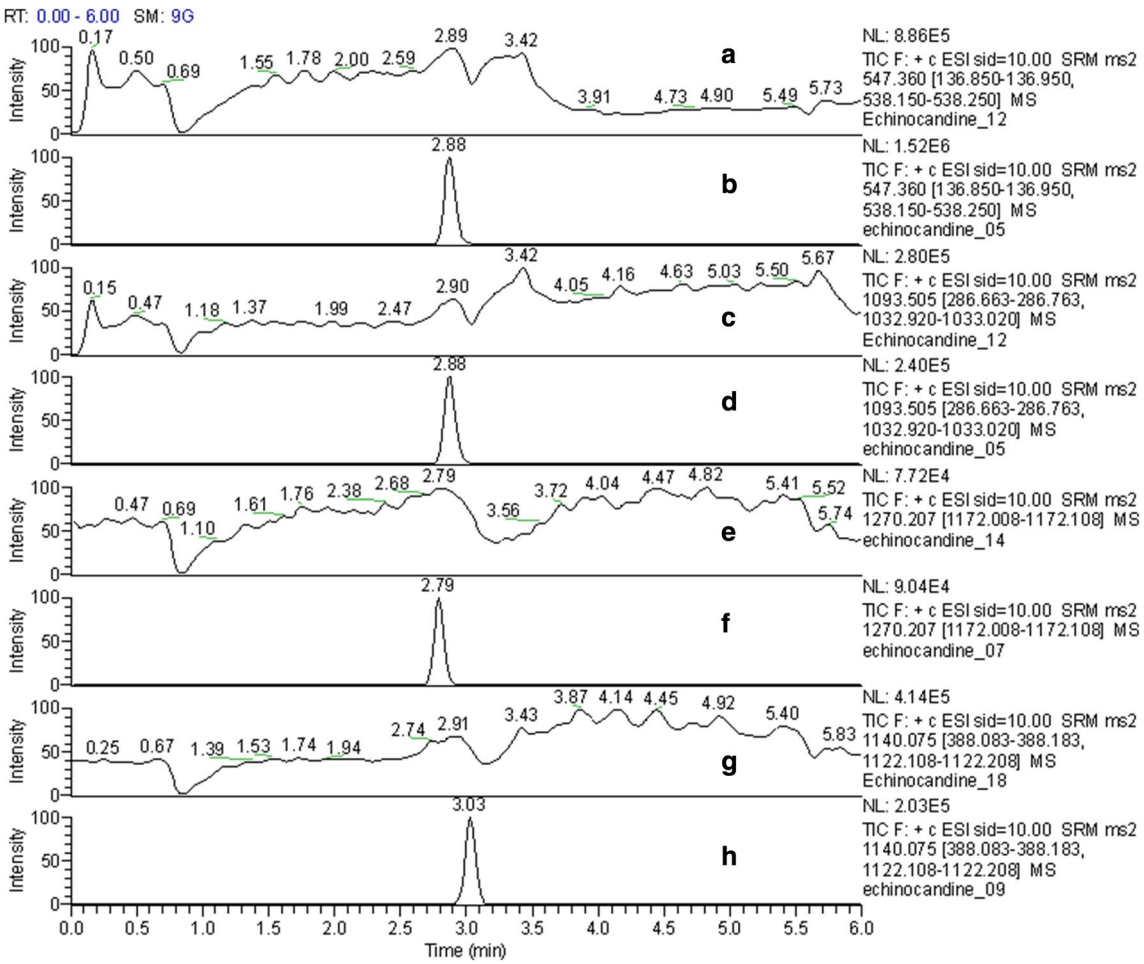

Fig. 2 Post-column infusion of antifungals into the MS/MS detector during the chromatographic analysis of blank plasma extracts (**a**, **c**, **e**, **g**) and typical expected peaks (**b** CSF; **d** CSF; **f** MCF; **h** ANF)

of IS. The resulting coefficients of variation (CV%) were 11.2, 6.1 and 11.6% at the low quality control level (QcL) and 11.5, 12.7 and 11.3% at the high quality control level (QcH), for anidulafungin, caspofungin and micafungin, respectively. Acceptance criteria were met for all of the echinocandins [12].

The extraction yields were calculated for all the three echinocandins as percentage ratio of the peak area in blank matrix, to the peak area in pure solutions: they were 102.7 and 99.4% for ANF, 98.8 and 102.1% for CSF, 103.6 and 96.9% for MCF, at QcL and QcH levels, respectively.

Calibration Curves

Six-point standard calibration curves were calculated and fitted by weighted $1/x$ linear regression for caspofungin, by quadratic function for micafungin and by a log–log transformed linear regression for anidulafungin [14] of the peak area ratio for each antifungal drug to IS, versus the nominal concentration of the respective compounds in each standard sample. The calibration concentration ranges (0.2–10 μg mL^{-1}) were selected to cover the antifungal concentrations expected for pediatric patients, according to pharmacokinetic studies and the spectrum of the MICs for each antifungal drug, for the most frequent fungal pathogens [15–17]. Each curve exhibited consistent linearity and reproducibility in the specific concentration range: regression coefficients (r) were higher than 0.99 for all the analytes (Table 1).

Table 1 Calibration curves parameters

				Mean	σ
MCF					
$y = a + bx + cx^2$					
a	0.000249	−0.000385	0.000319	0.00006	0.00032
b	0.02361	0.02185	0.02015	0.0219	0.00141
c	−0.000621	−0.000661	0.000447	−0.0003	0.00051
r^2	0.995	0.999	0.999	0.998	0.0020
CSF					
$y = a + bx$					
a	−0.01149	−0.005206	0.000246	−0.0055	0.00480
b	0.3207	0.2974	0.2509	0.2897	0.02902
r^2	0.999	0.997	0.996	0.997	0.0010
ANF					
$y = a + b\log x + c\log x^2$					
a	−0.53654	−0.50357	−0.52347	−0.5212	0.01356
b	0.90403	0.89634	0.8951	0.8985	0.00395
c	−0.12428	−0.18888	−0.16239	−0.1585	0.02651
r^2	0.996	0.995	0.998	0.996	0.0013

Dilution Integrity

The dilution integrity experiment was performed for caspofungin and micafungin and anidulafungin, to determine the accurate quantification within the calibration range, when echinocandin concentrations were greater than the ULOQ (upper limit of quantitation: 10 μg mL^{-1}), and then properly diluted with the same matrix.

Five samples containing both caspofungin, micafungin and anidulafungin at a concentration of 40 μg mL^{-1} were prepared and diluted (1:10) to 4 μg mL^{-1} with drug-free plasma: acceptable accuracy was expected to be within ±15% of the nominal concentration and imprecision (coefficient of variance) equal to or better than 15%. Results are reported in Table 2.

Dilution integrity was confirmed for CSF, MCF and ANF.

Carry-Over

The absence of carry-over was assessed by injecting CH_3CN after the calibration standards at the upper limit of quantification (10 μg mL^{-1}) for all of the echinocandins. Residual traces were below 20% of the LLOQ signal.

Accuracy and Precision

Within-run accuracy was determined by analyzing for each echinocandin, in a single run, five specimens at four different concentrations (LLOQ, QcL, QcM and QcH), whereas the between-run accuracy was evaluated from nine runs on two different days. The mean calculated concentrations were within ±15% (85–115%) of the nominal values for the Qc samples, and within ±20% (80–120%) of the nominal value for the LLOQ. Results are summarized in Table 3.

Precision was expressed as the coefficient of variation (CV%: SD/average × 100): the within-run and the between-run precision were less than 15% for the QC samples and less than 20% for the LLOQ. Results are reported in Table 4.

Stock Solutions Stability

The stability of ANF, MCF and CSF stock solutions (0.5 and 1 mg mL^{-1}) were tested by comparing the analytical response of the stored solutions (2 weeks at −20 °C) to that of freshly prepared reference solutions. For all the three antifungal drugs, the acceptable differences between the responses of fresh and stored solutions did not exceed ±15% (−13.8; −9.7; 11.3%, respectively). Working solutions were prepared fresh daily.

Table 2 Dilution Integrity

Nominal concentration	Micafungin (μg mL^{-1})	Caspofungin (μg mL^{-1})	Anidulafungin (μg mL^{-1})
(4 μg mL^{-1})	4.805	3.898	3.496
	4.548	3.913	3.510
	4.189	3.473	3.199
	4.999	3.582	3.689
	4.245	3.265	4.147
Mean	4.557	3.626	3.608
SD	0.35	0.279	0.349
CV%	7.68	7.7	9.7
Accuracy	113.9	90.7	90.2

Table 3 Within-run ($n = 5$) and between-run ($n = 9$) accuracy

Compound	Accuracy							
	Within-run				Between-run			
	LLOQ	QcL	QcM	QcH	LLOQ	QcL	QcM	QcH
Caspofungin	100.8	104.00	99.60	104.4	109.0	107.0	100.7	106.4
Anidulafungin	101.0	101.6	100.4	106.2	103.7	105.6	104.0	104.3
Micafungin	98.6	101.1	93.2	98.1	106.0	102.6	95.9	96.2

LLOQ: 0.2 μg mL^{-1}; QcL: 0.4 μg mL^{-1}; QcM: 4 μg mL^{-1}; QcH: 8 μg mL^{-1}

Table 4 Within-run ($n = 5$) and between-run ($n = 9$) precision

Compound	Precision							
	Within-run				Between-run			
	LLOQ	QcL	QcM	QcH	LLOQ	QcL	QcM	QcH
Caspofungin	11.8	4.7	7.3	2.9	12.8	6.5	7.0	7.0
Anidulafungin	11.2	7.9	5.4	4.4	8.9	8.5	6.3	7.0
Micafungin	15.0	4.9	5.4	4.1	10.3	10.9	8.6	9.7

LLOQ: 0.2 μg mL^{-1}; QcL: 0.4 μg mL^{-1}; QcM: 4 μg mL^{-1}; QcH: 8 μg mL^{-1}

Samples Stability

Echinocandin concentrations (at QcH and QcL levels), in frozen plasma samples (−20 °C), were stable for at least 72 h and minimal variations (within 15%) were detected.

For the freeze–thaw stability test, 5 QcL and 5 QcH plasma samples were stored at −20 °C, and after three cycles of freeze–thaw at room temperature, their concentrations were compared with the nominal values. Negligible changes were observed; all concentrations remained within 85–115% of the expected results.

Antifungal stability in blood after 24 h of storage in the refrigerator at +4 °C was not confirmed: concentrations changed more than ±15% in most reanalyzed samples.

The absolute concentrations of micafungin and anidulafungin also changed significantly (more than ±15%) when reanalyzed in processed samples, stored for 24 h in the refrigerated autosampler of the HPLC-system (10 °C): treated specimens could not be reanalyzed on the next day. Finally, blood samples should be centrifuged quickly after collection and plasma immediately frozen. The frozen plasma sample should be shipped to the laboratory on dry ice and kept at −20 °C until analysis within the next 72 h.

Clinical Application

According to the ESCMID 2012 [18] guidelines for the management of Candida infections, echinocandins (CSF and MCF) are approved for the treatment of invasive candidiasis (IC) in neonates, infants and children; MCF is also indicated for IC prophylaxis in neutropenic children with acute myeloid leukemia and recurrent

leukemia (grade A-II) and from the preparatory regimen until engraftment in those receiving an allo-HSCT. The Fourth European Conference on Infections in Leukaemia (ECIL-4) [19] guidelines for antifungal treatment in children with leukemia or post-HSCT also report that micafungin may be considered for patients undergoing allogenic HSCT as primary prophylaxis during the neutropenic phase and until immune recovery (grade CI), or in the post-transplant period in case of GVHD requiring immunosuppressive treatment. To date, ANF has not been approved for pediatric patients but the manufacturer's pediatric development program is at an advanced stage [20].

Figures 3 and 4 report two clinical examples of CSF and MCF chromatograms obtained in plasma from leftover pediatric blood samples taken for routine screening purposes, previously made anonymous and irreversibly unlinked from the source.

Discussion

To the best of our knowledge this is the first report on a simple, sensitive, rapid and robust HPLC–MS/MS method for the simultaneous analysis of caspofungin, anidulafungin and micafungin in human plasma in a single analytical run. Recently, different liquid chromatography tandem mass-spectrometry methods have been proposed, but they have some limitations.

Farowski [9] proposed a time-consuming sample preparation method for the simultaneous quantification of echinocandins and azoles in which the results showed low signal intensities, particularly for MCF and asymmetric chromatographic peaks. Martens-Lobenhoffer [21] quantified micafungin and anidulafungin only and could not achieve a stable and precise MS quantification for MCF. Decosterd et al. [10] and van Wanrooy [22] did not quantify MCF; moreover, voriconazole-d3 was used

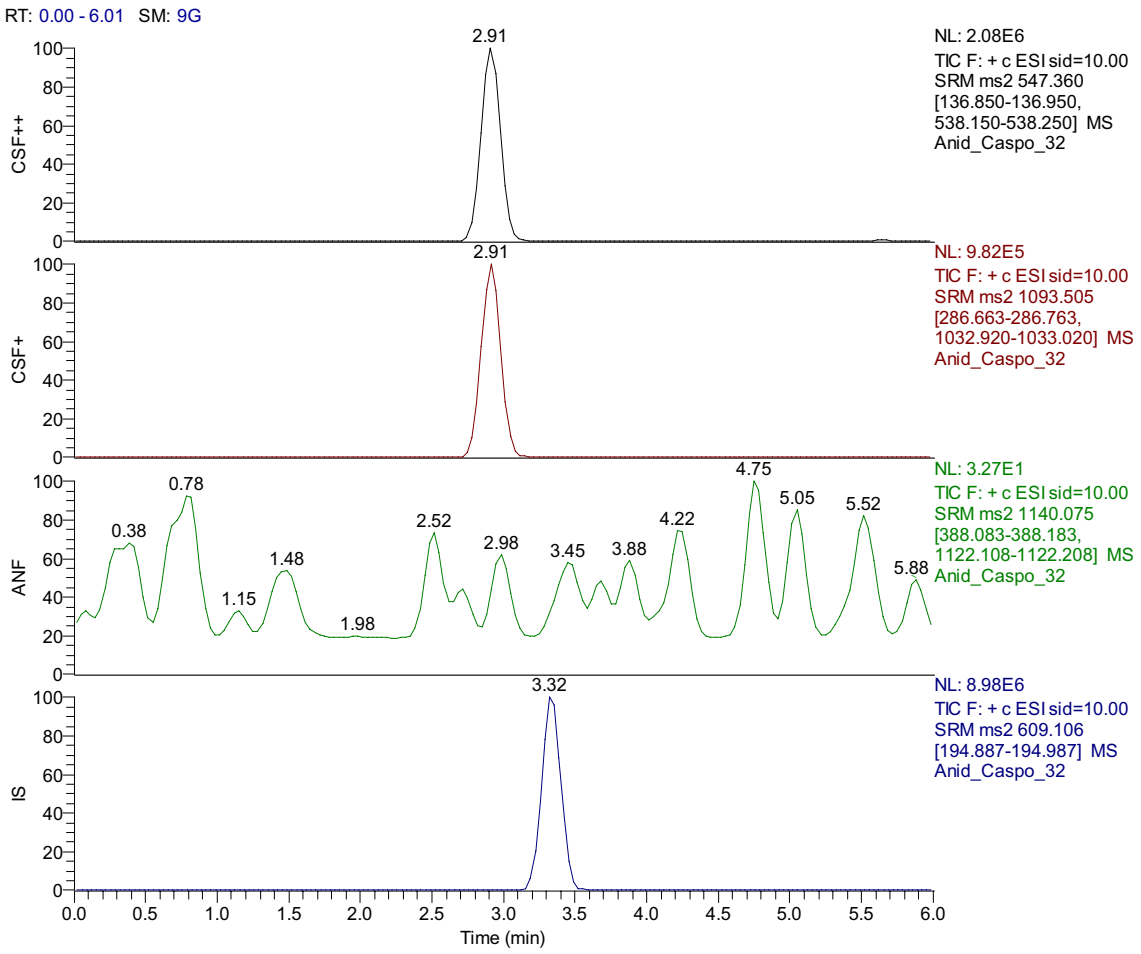

Fig. 3 A representative chromatogram of CSF plasma concentration (13.7 μg mL^{-1}) from leftover blood sample taken for routine screening purposes, made anonymous and irreversibly unlinked from the source

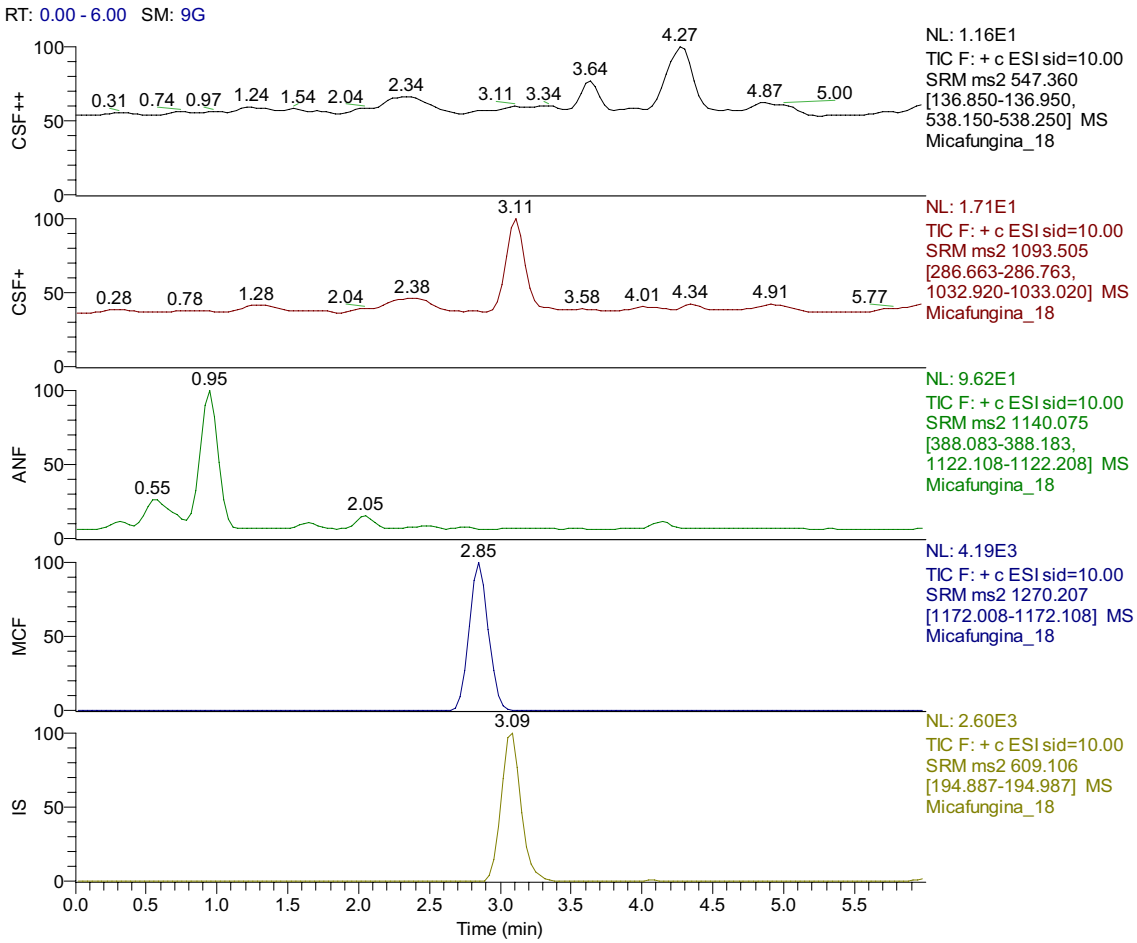

Fig. 4 A representative chromatogram of MCF plasma concentration (9.82 μg mL^{-1}) from leftover blood sample taken for routine screening purposes, made anonymous and irreversibly unlinked from the source

to quantify both caspofungin and anidulafungin [10], while, Nakagawa [11], Cangemi [23] and Uranishi [24] focused on MCF detection only.

Our method was validated according to the European Medicines Agency's Guidelines [12]. However, due to the limited available volume of samples and the instability of echinocandins, as already reported for anidulafungin [25], incurred sample re-analysis was not performed. Therefore, we propose this analytical procedure for TDM or daily supportive analysis.

Conclusions

This method is specific, accurate, precise and reproducible without the need for diluting steps, the use of on-line solid-phase extraction or expensive ultra-performance liquid chromatography. Since deuterated caspofungin, anidulafungin and micafungin compounds were not available, reserpine was found to fit the requirements of an Internal Standard. Even if this compound is not structurally related to the echinocandins, it is widely used in mass spectrometry as a reference standard owing to its availability, ease of ionization under electrospray conditions and stability in solution. Its retention time is very close to those of echinocandins in the specific chromatographic assay (Fig. 1). Furthermore, investigations based on the recommendations of Matuszewski [13] demonstrated that the proposed extraction procedures minimize the matrix effect both for the three echinocandins and IS, or standardized it, if any was present. The extracted samples are not stable after resting 24 h in the autosampler at 10 °C, probably because of the echinocandins susceptibility to chemical degradation marked by cleavage of their cyclic core [26, 27]. A minimal plasma volume (100 μL) is needed for analysis, which is particularly suitable for the TDM of pediatric patients. In conclusion, the application of this simple and accurate method can be considered appropriate for the measurement of echinocandin concentrations to optimize treatment in both

adults and children, especially in critically ill patients or when the drugs are used for prophylaxis, to avoid breakthrough infections due to inappropriate drug levels.

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Compliance with Ethical Standards

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Conflict of interest The authors have no conflicts of interest to declare.

Ethical approval This research does not contain any studies with human participants or animals performed by any of the authors.

Informed consent For this type of study formal consent is not required.

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