Pharmacokinetics of antifungal drugs in severely ill patients

van Wanrooy, Marjolijn Johanna Petronella

CHAPTER 2

Simultaneous quantifiction of anidulafungin and caspofungin in plasma by an accurate and simple liquid chromatography tandem mass-spectrometric method

Marjolijn J.P. van Wanrooy[a]
Riaaz N. Santoe[a]
Kim C.M. van der Elst[a]
Carli M. Wilmer[a]
Kai van Hateren[a]
A. Mireille A. Wessels[a]
Ben Greijdanus[a]
Jan-Willem C. Alffenaar[a]
Donald R.A. Uges[a]

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[a] Department of Clinical and Hospital Pharmacy, University Medical Center Groningen

ABSTRACT

Introduction. Echinocandins are a valuable addition for the treatment of invasive fungal infections, as they are efficacious, demonstrate low toxicity and have limited drug-drug interactions. In specific clinical situations when altered pharmacokinetics can be expected or dosing guidelines are conflicting, it may be useful to measure concentrations. For this purpose, a liquid chromatography tandem mass-spectrometric method to measure anidulafungin and caspofungin in ethylenediaminetetraacetic acid plasma was developed.

Methods. The method was developed on a Thermo Fisher TSQ Quantum LC-MS/MS. For separation, a BetaBasic C4 (100 mm x 3.0 mm; 5µm) analytical column was used. Sample preparation consisted of protein precipitation directly in the autosampler vial. The internal standard aculeacin A is structurally related, not used in humans, and commercially available. The method was validated according to the guidelines for bioanalytical method validation of the Food and Drug Administration.

Results. The method was accurate (bias ranging from -3.0 to 1.9%) and precise (within run and between run coefficients of variation of 2.2 to 7.7% and 1.6 to 9.0 %, respectively). All calibration curves were linear over a range of 0.5 – 10.0 mg/L for anidulafungin and 0.1-20.0 mg/L for caspofungin, and if necessary, samples can be diluted 10 fold. The samples were stable for three freeze-thaw cycles, with a bias ranging from 0.6 to 11%. The maximum bias from the worst storage condition, 72 h at room temperature, was -14.7%. In patient samples, anidulafungin peak concentrations ranged from 2.8 to 8.6 mg/L (n=20) and trough concentrations ranged from 1.0 mg/L to 4.7 mg/L (n=79). The measured caspofungin concentrations ranged from 1.9 to 7.3 mg/L (n=20).

Conclusion. The method developed has a straightforward sample preparation and uses a structural analogue as the internal standard. This method has been applied successfully for the measurement of anidulafungin and caspofungin concentrations in patient samples, both for clinical practice as well as for research.

INTRODUCTION

Echinocandins are a valuable addition for the treatment of invasive fungal infections, as they are efficacious, demonstrate low toxicity and have limited drug-drug interactions (1). A good predictor of *in vitro* and *in vivo* efficacy of these antifungal drugs appears to be the area under the concentration-time curve (AUC) divided by the minimal inhibitory concentration (MIC) (2). The AUC/MIC ratio has an impact on the mortality of patients with invasive *Candida* infections, which was earlier shown for fluconazole (3). Although no clinical data are available to show a similar relationship for echinocandins, it may be useful to measure concentrations in specific clinical situations in which altered pharmacokinetics can be expected and dosing guidelines are conflicting. A typical situation in daily practice is dosing caspofungin in a patient weighing over 80 kg with hepatic impairment (4, 5). In this case a clinician has to choose between an increased dose based on body weight or a decreased dose based on the hepatic impairment. Another situation in which dosage guidelines are conflicting is when caspofungin and rifampicin are used concomitantly in a patient with hepatic impairment. The options are an increased dose based on the drug-drug interaction (6) or a decreased dose based on the hepatic impairment. In these cases, it would be informative and decision supportive to be able to measure plasma concentrations of echinocandins. Although liquid chromatography tandem mass-spectrometric (LC-MS/MS) methods have been described for the determination of anidulafungin (7) or caspofungin (8) alone and for the simultaneous quantification of echinocandins and azoles (9, 10), they have several limitations. Time-consuming sample preparation and diluting steps (8-10), use of online solid phase extraction (7), or expensive ultra-performance liquid chromatography (10), are major drawbacks for routine analysis. But most importantly, some methods (7, 10) do not use an internal standard that structurally resembles the echinocandins, such as a structural analogue or stable label. This should be discouraged because internal standards that are not structurally related are less able to compensate for unavoidable variances (11).

To overcome the problems with current methods of analysis our objective was to develop an accurate and simple LC-MS/MS method for the simultaneous quantification of anidulafungin and caspofungin, with straightforward sample preparation.

MATERIAL AND METHODS

Chemicals and reagents. Anidulafungin was provided by Pfizer (New York), and caspofungin was supplied by Merck Sharp & Dohme (Whitehouse Station, NJ). A structural analogue for the echinocandins, aculeacin A (Fig. 1), was obtained from Sigma-Aldrich (St. Louis, MO). Acetonitrile, trifluoroacetic anhydride and water for LC-MS were purchased from BioSolve (Valkenswaard, The Netherlands). Methanol Lichrosolv and formic acid were from Merck KGaA (Darmstadt, Germany). Acetic acid (100%), ammoniumacetate, and ammonium formate (98-100%) were from Acros Organics (Geel, Belgium). All reagents were of suitable analytical grade. Ultra pure water was obtained from a Milli-Q water purifying system (Millipore Corporation, Billerica, MA).

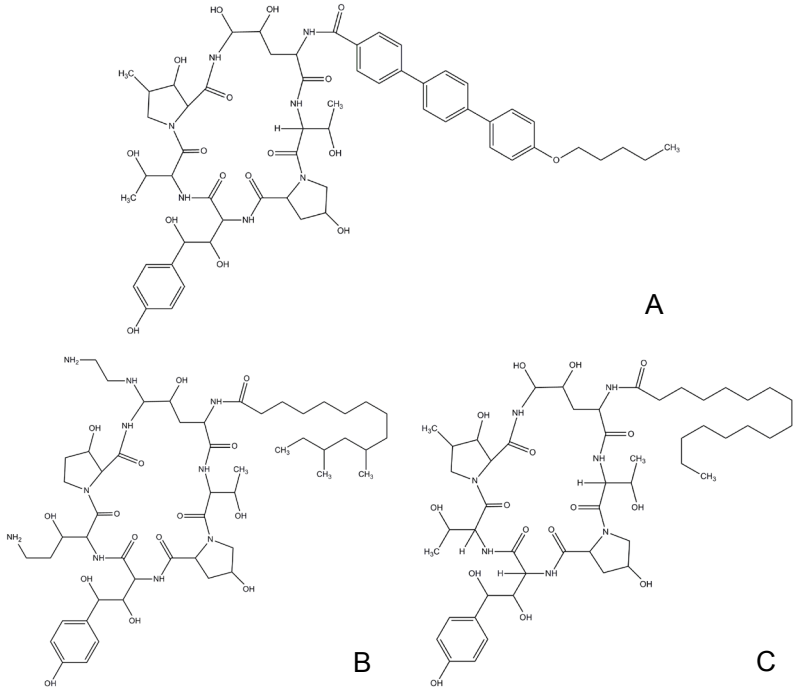

FIG 1 Chemical structures of anidulafungin (A), caspofungin (B), and the internal standard aculeacin A (C).

The precipitation reagent consisted of 0.5 mg/L internal standard in a solution containing 3 g/L formic acid and 180 mg/L ammonium formate in acetonitrile-methanol (10:3 [vol/vol]). High-performance liquid chromatography eluent A was an aqueous buffer (pH 3.5) containing ammonium acetate (5 g/L), acetic acid (35

mg/L), and trifluoroacetic anhydride (2 mL/L) in water. Water and acetonitrile were used as high-performance liquid chromatography eluents B and C, respectively.

Equipment. All experiments were performed on a triple-stage quadrupole LC-MS/MS (TSQ Quantum, Thermo Fisher, San Jose) with a Finnigan Surveyor MS pump and a Finnigan Surveyor autosampler (Thermo Fisher). The TSQ Quantum mass-selective detector was operated in selected reaction monitoring mode with positive electrospray ionization. Xcalibur software version 2.0 SR1 (Thermo Fisher) was used for peak height integration for all components. For separation, a BetaBasic C4 (100 by 3.0 mm; 5μm particle size; Thermo Fisher) analytical column was used.

Calibrators and quality control samples. Stock solutions were prepared in methanol-water (50:50 [vol/vol]). For the preparation of the calibration standards, 2 individual stock solutions were prepared, with 1000 mg/L anidulafungin or 1000 mg/L caspofungin. These stock solutions were diluted to obtain working stock solutions with either 100 mg/L anidulafungin or 100 mg/L caspofungin.

Separate stock solutions, containing 1000 mg/L anidulafungin or 1000 mg/L caspofungin, were made for the preparation of the quality control (QC) samples. Working stock solutions with either 100 mg/L anidulafungin or 100 mg/L caspofungin were obtained after dilution.

The calibration and QC samples were prepared by spiking pooled ethylenediaminetetraacetic acid (EDTA) plasma with the prepared stock solutions and working stock solutions. For the concentrations used, see Table 1. The calibration samples and QC samples were stored at -20 °C.

TABLE 1 Concentrations of calibration standards

Drug	Calibrator concentration (mg/L)	QC sample concentrations (mg/L)			
		LLOQ	LOW	MED	HIGH
Anidulafungin	0.5; 1; 1.25; 1.50; 2.5; 3.75; 5; 6.25; 7.5; 10	0.5	1.25	5.0	8.0
Caspofungin	0.1; 0.25; 0.5; 1; 2.5; 5; 7.5; 10; 15; 20	0.1	0.5	10.0	

Sample processing. In a 2 mL glass autosampler vial, 100 μL of EDTA plasma and 500 μL of precipitation reagent were vortexed for 1 minute on a Lab-tek multitube

vortexer (Christchurch, New Zealand). The vials were stored at -20 °C for 30 minutes to promote protein precipitation. Afterwards the vials were vortexed again for 1 minute. For the last preparation step, the vials were centrifuged at room temperature for 5 min at 10,164 x g (11,000 rpm) in a Hettich benchtop centrifuge (Mikro 22 centrifuge, Hettich, Bäch, Switserland).

From the clear upper layer of the samples, 5 µL was injected into the LC-MS/MS.

LC-MS/MS conditions. The eluent gradient was tuned in such a way that all compounds were baseline separated (Table 2). The flow rate was 250 µL/min and the column was kept at room temperature. The temperature in the autosampler was set at a temperature of 10 °C. The ion source spray voltage was set at 3500 V and the capillary temperature at 350°C. Nitrogen was used as sheath and auxiliary gas, pressures were set at 35 and 5 (arbitrary units), respectively. Argon was used as collision gas. The scan time was 0.050 s at a scan width of 0.5 *m/z*. The following *m/z* transitions of precursor ions to product ions were used: anidulafungin 1140.7 -> 343.2 (collision energy, 61 eV), caspofungin 547.4 -> 538.0 (collision energy, 11 eV), and aculeacin A 1036.7 -> 1018.7 (collision energy, 15 eV).

TABLE 2 Gradient elution program

Time (min)	Solvent A (%) [a]	Solvent B (%)	Solvent C (%)
0.0	5.0	75.0	20.0
1.0	5.0	35.0	60.0
2.0	5.0	35.0	60.0
3.2	5.0	20.0	75.0
3.5	5.0	5.0	90.0
4.0	5.0	0.0	95.0
9.0	5.0	0.0	95.0
10.0	5.0	75.0	20.0

[a] Solvent A, aqueous buffer with pH 3.5 (ammonium acetate 5 g/L, acetic acid 35 mg/L, and trifluoroacetic anhydride 2 mL/L in water); Solvent B: ultra pure water; Solvent C: acetonitrile

Calibration and calculation. The calibration curves were constructed using a linear

regression of the ratios of the observed peak heights of either anidulafungin or caspofungin and the internal standard aculeacin A against the spiked concentrations of the standards. The concentration of patient samples was determined based on a 1-point calibration. The highest calibrator concentration was used for the 1-point calibration. The results from the 1-point calibration were only valid if the bias of the control sample (plasma spiked with anidulafungin [1.0 mg/L] and caspofungin [2.0 mg/L]) was less than 15%.

Method validation. In accordance with the Guidance for Industry Bioanalytical Method Validation of the Food and Drug Administration (12), method validation included selectivity, linearity, accuracy, precision, sensitivity, recovery and stability. Precision was subdivided into within run and between run precision. On each of the 4 analytical days, a single calibration curve was obtained and the QC samples were analyzed in 5 replicates.

Selectivity and sensitivity. The selectivity of this method was evaluated by analyzing 6 lots of pooled EDTA plasma. Sensitivity was examined by comparing the response of 6 lots of pooled blank EDTA plasma with the response of lower limit of quantification (LLOQ) samples. The possible existence of ion suppression or ion enhancement was determined by analyzing 6 lots of pooled EDTA plasma during simultaneous post-column continuous infusion of a stock solution containing 15 mg/L anidulafungin, caspofungin, and internal standard by a syringe pump at a flow rate of 10 μL/min (13).

Accuracy and precision. The accuracy and precision of the method were determined by analyzing QC samples of 4 concentrations in 5-fold on 4 different days. The responses were analyzed using 1-way ANOVA for each single concentration level. The bias of the mean from the true value served as the measure of accuracy. The precision was calculated as the coefficient of variation of the concentrations within a single run (within run) and of the concentrations in the runs on the different days (between run).

Recovery. The recovery was determined at 3 concentrations (low, medium, and high) using 5 replicates of each by comparison of the mean peak height in plasma with the mean peak height of samples prepared in a nonbiological matrix (precipitation reagent without internal standard).

Stability. Stability tests included 3 freeze-thaw cycles, storage stability, and autosampler stability. For the freeze-thaw stability test, samples with low and high

concentration were stored at -20 °C. After 3 cycles of freeze-thaw, the concentration was compared with the mean concentration of the freshly prepared samples. In addition, the stability of anidulafungin and caspofungin in plasma was determined after 24, 48 and 72 hours of storage in the refrigerator at +4 °C and at room temperature. The stability of processed samples in the autosampler was also evaluated after residing 24, 48 and 72 hours in the autosampler.

Dilution integrity. On 4 consecutive days, a sample with a concentration of 25 mg/L for anidulafungin or caspofungin was diluted 10-fold, processed in 5 replicates and then analyzed.

Matrix comparison. The calibration curves of anidulafungin and caspofungin resulting from calibrators prepared in sodium heparin plasma and serum were compared and analysed by 1-way ANOVA with the calibration curve from the samples prepared in EDTA plasma.

Application of the method. This method has been used in our hospital, both for research and for clinical practice. For a clinical trial (NCT01047267) on the pharmacokinetics of anidulafungin in critically ill patients, samples from 20 patients were measured. The study protocol was approved by the local institutional ethics committee. Written informed consent was obtained from the patient or the legal representative of the patient. In addition, anidulafungin and caspofungin concentrations were measured in selected patients. For caspofungin, special attention was paid to pediatric patients and patients weighing more than 80 kg with elevated liver enzymes. Besides this, concentrations were measured in neutropenic patients treated with anidulafungin.

RESULTS

The retention times of anidulafungin, caspofungin and aculeacin A were 5.0, 4.3, and 5.6 minutes, respectively. Figure 2 shows ion chromatograms of a sample spiked at LLOQ and a blank sample.

Selectivity and sensitivity. There were no peaks observed in any of the pooled blank EDTA plasma samples at the retention time of the echinocandins or the internal standard. No ion suppression or ion enhancement was observed (see Figures, Supplemental Digital Content 1, presented after the references, which show representative ion chromatograms of the test).

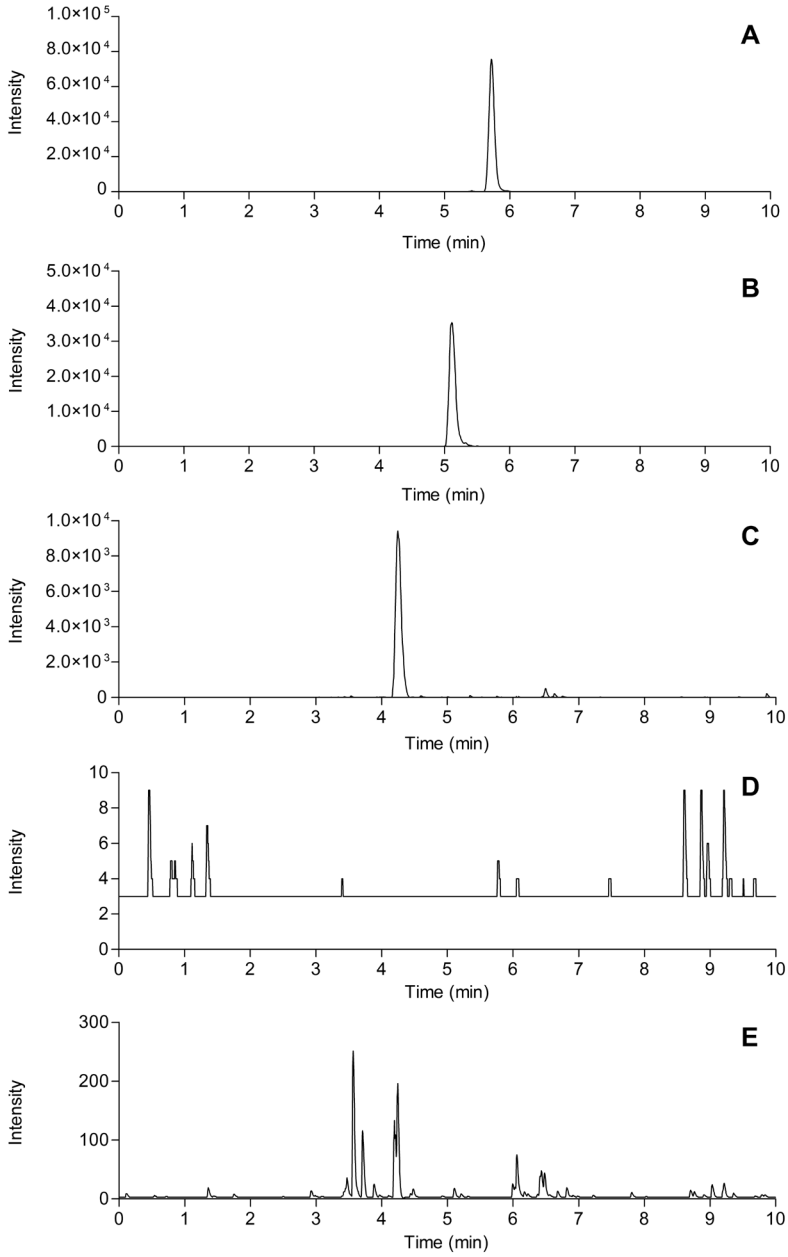

FIG 2 Ion chromatograms for aculeacin A (a), a LLOQ sample of anidulafungin (b) and caspofungin (c) and ion chromatograms of a blank EDTA plasma sample at the ion transitions of anidulafungin (d) and caspofungin (e).

Linearity, accuracy and precision. All calibration curves were linear over a range of 0.5 - 10.0 mg/L for anidulafungin and 0.1 - 20.0 mg/L for caspofungin. The mean equations were $y = 0.158x + 0.00343$ ($R^2 = 0.996$) for anidulafungin and $y = 0.877x + 0.00780$ ($R^2 = 0.999$) for caspofungin. For patient samples, a 1-point calibration was used because of the linearity of the calibration curves and the practically zero intercept.

The results of accuracy and precision tests are listed in Table 3

TABLE 3 Accuracy and precision

		Accuracy	Precision			
		Mean (mg/L)	Bias (%)	Within run (%)	Between run (%)	Overall (%)
Anidulafungin	LLOQ	0.509	1.9	7.7	9.0	11.9
	LOW	1.27	1.6	5.7	5.6	8.0
	MED	5.06	1.1	5.0	5.8	7.7
	HIGH	8.06	0.8	4.4	3.9	5.9
Caspofungin	LLOQ	0.0970	-3.0	6.0	8.3	10.2
	LOW	0.487	-2.6	5.6	2.9	6.3
	MED	10.1	0.8	2.2	1.8	2.8
	HIGH	16.0	0.2	4.7	1.6	4.9

Recovery. The recovery of anidulafungin and caspofungin ranged from 104.4% to 106.7% and from 91.4% to 114.7%, respectively. The internal standard, aculeacin A, had a recovery of 96.9%. All coefficients of variation were less than 7%.

Stability. The samples were stable for 3 freeze-thaw cycles, with a bias ranging from 0.6 to 11%. The maximum bias from the worst storage condition, room temperature, was -14.7%.

Dilution integrity. The mean concentration of the diluted plasma samples was 2.57 mg/L (undiluted: 25.0 mg/L) for anidulafungin with a bias of 3% and 2.49 mg/L (undiluted: 25.0 mg/L) for caspofungin with a bias of -1%.

Matrix comparison. There was no significant difference between the slope and the intercept of the calibration curves of anidulafungin and caspofungin in sodium heparin or serum in comparison with those of the calibration curve in EDTA plasma.

Application of the method. This method of analysis for anidulafungin and caspofungin has been used for more than 1 year in our hospital. For a clinical trial (NCT01047267) on the pharmacokinetics of anidulafungin in critically ill patients, samples from 20 patients were measured. The peak concentrations (n=20) ranged from 2.8 to 8.6 mg/L. For each patient, more than 1 trough concentration was measured. The anidulafungin trough concentrations (n=79) ranged from 1.0 mg/L to 4.7 mg/L. In addition, anidulafungin concentrations were measured in neutropenic patients. Figure 3A shows an ion chromatogram following protein precipitation of an anidulafungin trough concentration sample from a neutropenic patient.

Caspofungin concentrations were measured in 8 patients with elevated liver enzymes. In 2 infants, a peak (end of infusion) and trough concentration were measured. A 5-year-old boy, with a traumatic pancreas rupture, received 42 mg (50 mg/m^2) caspofungin after a loading dose of 60 mg (70 mg/m^2). Caspofungin peak and trough concentration were 10.8 and 1.9 mg/L, respectively. Another 8-year-old boy, with liver failure after liver transplantation, received 50 mg caspofungin after a loading dose of 70 mg. Caspofungin peak and trough concentration were 11.4 and 3.3 mg/L, respectively. The measured caspofungin concentrations (n=20) in adult patients ranged from 1.9 to 7.3 mg/L. Figure 3B shows an ion chromatogram following protein precipitation of a caspofungin trough concentration sample from an overweight patient (103 kg) with elevated liver enzymes.

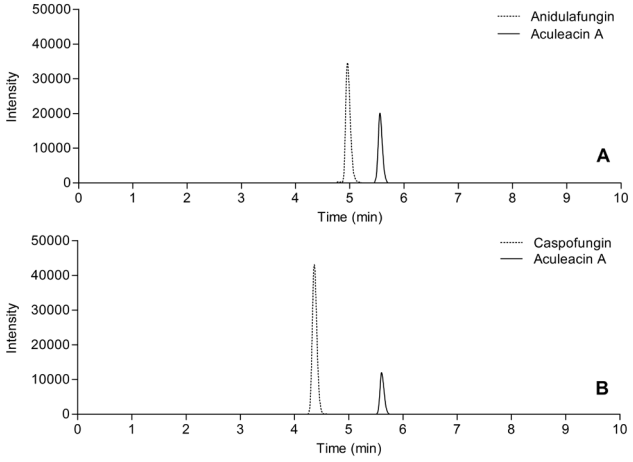

FIG 3 Ion chromatogram following extractions of a trough concentration (2.6 mg/L) from a patient receiving 100 mg anidulafungin (A) and of a trough concentration (1.9 mg/L) from a patient receiving 70 mg caspofungin (B).

DISCUSSION

We developed an accurate and simple LC-MS/MS method, which proved to be suitable for routine monitoring of anidulafungin and caspofungin. The method was validated according to the guidelines for bioanalytical method validation of the Food and Drug Administration (12).

One of the advantages of our method, compared with the methods currently available, is the internal standard, aculeacin A. When stable isotope-labeled internal standards are not available, a structural analogue is preferred as the internal standard (11). Aculeacin A, isolated from *Aspergillus aculeatus* (14), is structurally related to the echinocandins, not used in humans, and commercially available. Previously reported methods use ascomycin, an ethyl analogue of tacrolimus (7), or deuterated voriconazole (10) as internal standard. These internal standards have the disadvantage that they are not structurally related to the echinocandins and are, therefore, less able to compensate for the unavoidable variances in the method. The 2 methods that do use a structurally related internal standard (8, 9) chose a derivate of caspofungin as internal standard, which is not commercially available.

The method we used for sample preparation is rather straightforward. Although all previously published methods used protein precipitation in sample preparation, most used a more complex sample preparation. Farowski et al. used diluted plasma as matrix, which is obtained after centrifuging diluted blood layered onto a double-discontinuous Ficoll-Hypaque density gradient and another dilution step (9). The sample preparation method described by Decosterd et al. and Rochat et al. required an additional dilution step (8, 10) which may result in an increased risk of errors and an elevated assay variance.

The choice of the matrix does not seem to affect the analysis. No differences were observed in matrix comparisons between serum and plasma (heparin and EDTA). This observation is consistent with previously published results. However, the stability of samples seems to be influenced by the matrix. At room temperature, anidulafungin and caspofungin are more stable in EDTA plasma than in citrate plasma (10) or serum (results not shown).

The simple sample workup procedure contributed to the slightly increased LLOQ

of our method for analysis of anidulafungin compared to the LLOQ of previously described methods (7, 9, 10). No clinical implications are expected because the anidulafungin trough concentrations measured in this study, and previously reported anidulafungin trough concentrations (15-17), were above our LLOQ.

In conclusion, a simple and accurate method for determination of anidulafungin and caspofungin in plasma has been developed. This method has already been successfully applied to the measurement of anidulafungin and caspofungin concentrations in patient samples.

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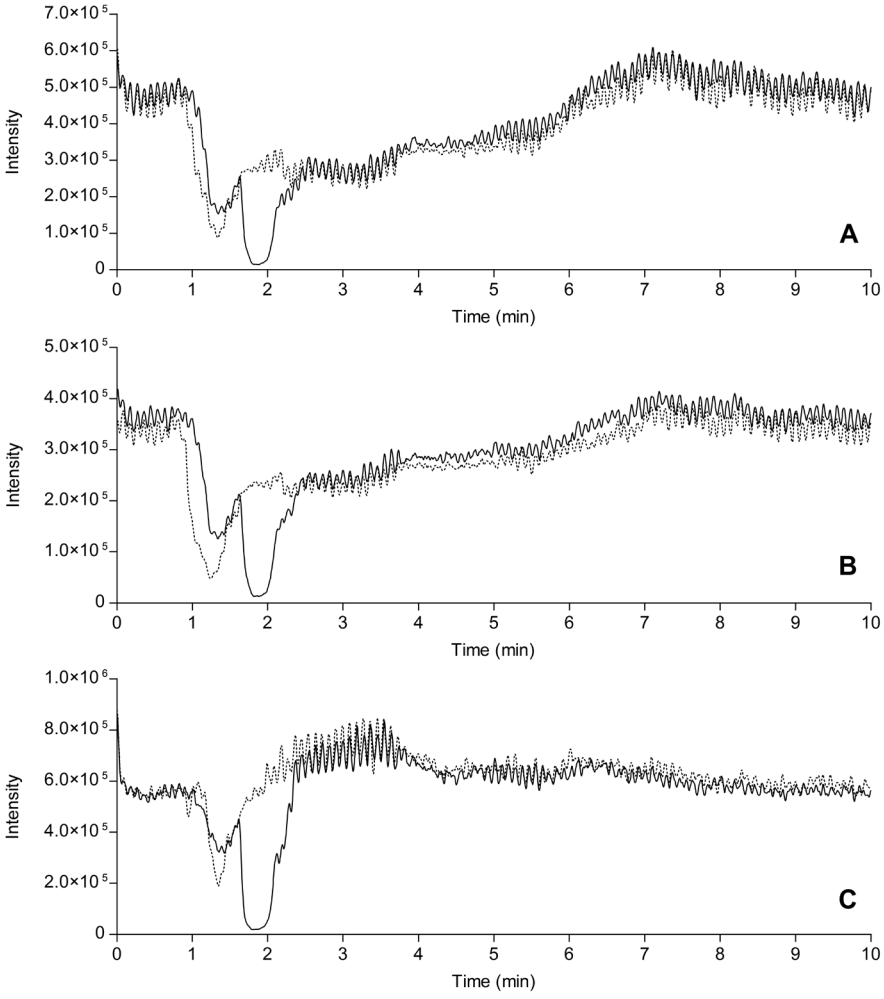

SUPPLEMENTAL DIGITAL CONTENT 1 Representative ion chromatograms of the ion suppression test. The dotted curve is an ion chromatogram after injection of water and the solid curve after injection of EDTA plasma during postcolumn infusion of aculeacin A (A), anidulafungin (B), and caspofungin (C).