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Liquid chromatography-mass spectrometry method for quantification of caspofungin in clinical plasma samples

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Caspofungin [(CASPO) MK-0991] is the first broad-spectrum anti-fungal agent of the echinocandin class approved for clinical use. Measurement of CASPO levels in blood might help monitor therapy in patients who are critically ill, in particular, if high-dose regimens or combinations of CASPO with other anti-fungals are used. The objective of this study was to develop a fast method for the measurement of CASPO levels in clinical blood samples using liquid chromatography coupled to a triple-quadrupole mass spectrometer. Stock solutions were prepared in plasma to avoid CASPO adsorption to glass and plastic surfaces during processing. CASPO and the internal standard (IS) were extracted from 100 µl of plasma using acetonitrile protein precipitation. The supernatant was diluted and directly injected into an analytical column (C8; 2.1 × 30 mm). The total run time was 15 min. CASPO was ionized by electrospray in the positive mode. CASPO and IS $[M + 2H]^{2+}$ parent ions (*m/z* 547.3 and 547.8, respectively) and specific product ions (*m/z* 137.1 and 62.2, respectively) were used for the ion transitions. No carry over or cross-talk was observed on the column. The mean method recovery was 90 ± 3%. Neither blood from different individuals (*n* = 6) nor the presence of concomitant drugs (*n* = 33) in plasma samples interfered with CASPO quantification. Quantification over time of the CASPO levels in plasma and whole blood was investigated at different pre-analysis storage conditions. The calibration curve included the clinically relevant CASPO concentration range from 0.04 to 20 µg/ml. Mean intra- and inter-day accuracy was 96.1 ± 2.2% and 102.5 ± 2.4%, respectively. Mean intra- and inter-day precision was 7.9 ± 3.2% and 6.3 ± 1.8%, respectively. This simple and robust liquid chromatography-tandem mass spectrometry (LC-MS/MS) method may easily be implemented for monitoring CASPO therapy. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: liquid chromatography; tandem mass spectrometry; anti-fungal drug; plasma; caspofungin

INTRODUCTION

Caspofungin [CASPO (MK-0991)] is the first anti-fungal agent of the echinocandin family approved for clinical use.[1] Broad-spectrum anti-fungal activity, a unique mechanism of action resulting in the lack of cross-resistance with other classes of anti-fungals, and a favourable safety profile are the main characteristics of this new compound. CASPO is licensed for the treatment of mucosal or invasive candidiasis, for empirical anti-fungal therapy in neutropenic cancer patients with persistent fever, and for salvage therapy of aspergillosis in patients not responding or intolerant to conventional anti-fungal drugs.[2–6] Experimental and preliminary clinical data suggest that high-dose CASPO therapy (e.g. 70–150 mg/day in humans) or a combination of CASPO with amphotericin B or azoles may improve the outcome in patients with severe systemic mycoses.[7–11]

The pharmacokinetics of CASPO has been studied mainly in healthy adult subjects and is being intensively investigated in children.[12,13] Rapid alteration of the volume of distribution, multiple organ dysfunctions, and drug–drug interactions may result in inter-individual variations of CASPO levels in blood in patients who are critically ill with life-threatening infections.[14–16] Measurement of CASPO levels in blood might contribute to improvements in clinical management in these settings as well as in patients treated with high-dose regimens or combinations of CASPO with other anti-fungal agents.

LC-MS/MS is a powerful analytical tool for the quantification of chemical compounds. The reported LC-MS/MS methods for measurement of CASPO concentrations in plasma require solid-phase extraction or two-dimensional LC (2D-LC).[17,18] An LC method with fluorescence detection after solid-phase extraction has also been reported.[19] The objective of this study was to develop and validate an LC-MS/MS method using a fast extraction procedure from

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a small plasma volume followed by direct injection into an analytical column (1D-LC). In addition, coverage of the relevant *in vivo* CASPO concentration range (0.2–15 μg/ml, MIC_{90} of fungal pathogens 0.5 μg/ml), stability at different pre-analysis processing conditions, and quantification in presence of co-medications were included in this development.

EXPERIMENTAL

Chemicals and reagents

CASPO (Fig. 1, $C_{52}H_{88}N_{10}O_{15}$; monoisotopic mass: 1092.64) and the internal standard (IS) (Fig. 1, $C_{52}H_{89}N_9O_{16}$; monoisotopic mass: 1093.64) were kindly supplied by Merck Research Laboratories as acetate (molecular weight 1213.42, potency 906 μg/mg) and ditrifluoroacetate salts, respectively (Rahway, NJ, USA).

Acetonitrile (MeCN) used for protein precipitation and chromatography was of LiChrosolv grade (Merck, Dietikon, Switzerland). Formic acid (100%) was purchased from Fluka (Buchs, Switzerland). Ultra-pure H_2O was obtained by ultrafiltration using a Milli-Q UF-Plus apparatus (Millipore Corp., Burlington, MA, USA). All other chemicals were of analytical grade.

LC-MS/MS system and conditions

The LC system, including a de-gasser, a binary pump, and a thermostated autosampler, was an Agilent HP1100 system (Agilent Technologies, Palo Alto, CA, USA). The chromatographic system was coupled to a TSQ mass spectrometer from Thermo Corporation (San Jose, CA, USA), a TSQ Quantum discovery, equipped with electrospray ionization (ESI).

Chromatography was performed on a 2.1 × 30 mm Symmetry Shield 3.5-μm RP8 analytical column (Waters, Milford, MA, USA), which was placed in an oven CH5-A100 (Bon Technologies SA, Lausanne, Switzerland) set at 30 °C. Mobile phase A consisted of 10 mM ammonium acetate in H_2O containing 1% acetic acid, and mobile phase B consisted of 1% acetic acid in MeCN.

The mobile phase was delivered at 0.4 ml/min using the following gradient: at start of run 75/25% of A/B, at 5.5 min 30/70% of A/B, and at 5.6 min 2/98% of A/B. From 5.6 to 10 min, the column was washed at 0.5 ml/min with 2/98% of A/B. At 10.1 min, 75/25% of A/B was delivered at 0.5 ml/min. Finally, the initial conditions were restored at 14 min during 1 min. The run time was 15 min. The injection volume was 20 μl.

The LC-MS/MS was operated in the positive mode. The ESI conditions were as follows: capillary temperature 350 °C; ESI spray voltage 4 kV; the source collision induced dissociation (CID) voltage 10 V; tubes lens voltage 125 V; and the sheath and auxiliary gas (nitrogen) flow rates 40 and 20 arbitrary units, respectively.

The first (Q1) and third (Q3) quadrupoles were set at ≤1 amu mass resolution with a full width half-maximum of 0.6 and 0.7, respectively. The gas (argon) pressure in the collision cell (Q2) was 1 mTorr (0.13 Pa). The scan time and scan width were 0.07 s and 0.5 amu, respectively. The non-smoothed chromatographic peaks (including the lower limit of quantification (LLOQ) samples) were the result of at least 10 MS scans. In order to tune the instrument for the best detection of CASPO and its IS, direct infusions of the compounds, diluted in acetonitrile and 1% acetic acid in H_2O (50 : 50, v : v) at 1 μg/ml, were performed and full ion scans or product scans were recorded. For CASPO quantification, the compounds were detected via selected reaction monitoring (SRM) transitions in the centroid mode employing the transition of $[M + 2H]^{2+}$ precursor ions to product ions. The selected m/z transitions were 547.3 → 137.1 and

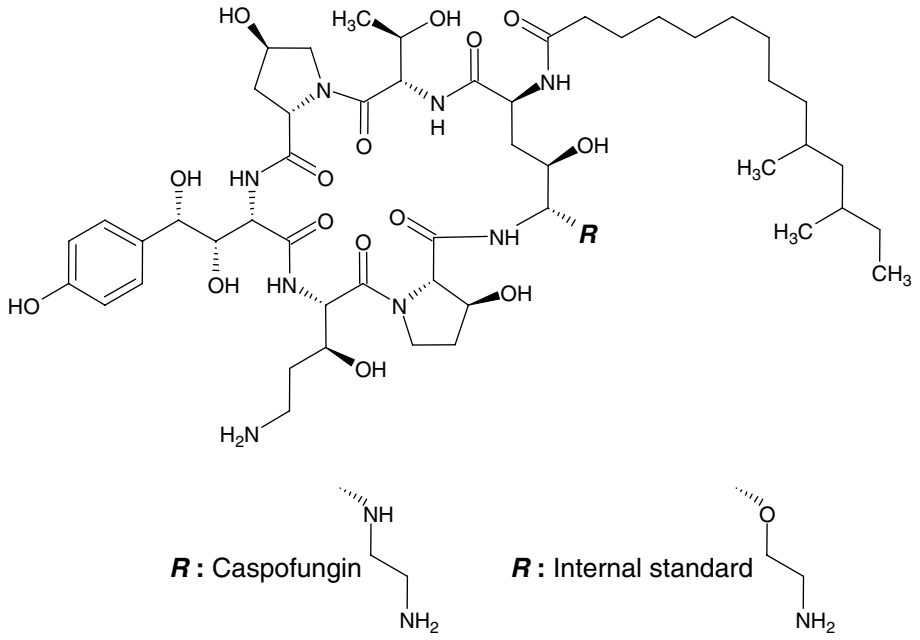

Figure 1. Chemical structure of caspofungin (CASPO; $C_{52}H_{88}N_{10}O_{15}$, monoisototopic mass = 1092.6 Da) and internal standard (IS; $C_{52}H_{87}N_{11}O_{14}$, monoisototopic mass = 1093.6 Da).

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547.8 → 62.2 with collision energies of 41 and 20 eV for CASPO and IS, respectively. Because of the specificity of the product ions, no cross-talks were observed between transitions. Transitions using the $[M + H]^+$ parent ions were also recorded but not considered in this validation owing to lower peak areas.

Chromatographic data acquisition, peak integration, and quantification were performed using the Xcalibur software package from Thermo Co. For CASPO quantification, the chromatographic peaks were smoothed using the boxcar algorithm (set at 9).

Working solutions, calibration standards, and quality controls

Stock solutions of CASPO (0.5 mg/ml) and the IS (1 mg/ml) were prepared in a pooled lot of citrated plasmas from healthy volunteers and H_2O, respectively. The stock solution of CASPO was further diluted in the pooled plasma to prepare eight calibration standards (CS: 0.04, 0.11, 0.49, 0.78, 1.2, 4.9, 7.8 and 20.0 μg/ml) and three quality controls (QCs: 0.18, 1.91 and 12.5 μg/ml). The CS and QC samples were stored at −80 °C in glass flasks. The stock solution of the IS was further diluted in H_2O and added at 1 μg/ml in plasma.

Sample preparation

Citrated human plasma was used as the biological matrix. CS, QC, and the clinical plasma samples were spiked with 10 μl of the IS (final sample concentration 1 μg/ml). One hundred microlitres of the samples to be analysed was disposed in 1.5 ml Eppendorf tubes and de-proteinized by the addition of 100 μl of MeCN at 4 °C followed by centrifugation for 10 min at 20 000 *g* and 4 °C using a Hettich benchtop centrifuge (Benchtop Universal 16R centrifuge, Bäch, Switzerland). One hundred microlitres of the supernatant was transferred into new vials, diluted 2 times with the mobile phase A, and again centrifuged at the same conditions. Finally, 100 μl of the supernatant was transferred into injection vials for analysis (injection volume 20 μl).

In order to increase the sensitivity of the method (see Section 'Lower limit of quantification, intra-, and inter-day validation'), a modified extraction procedure was studied. Five hundred microlitres of plasma and 1000 μl of MeCN were mixed and centrifuged as described above. The supernatant (900 μl) was evaporated under a nitrogen stream and the dried residue reconstituted in 60 μl of mobile phase A and B (75 : 25%) prior to injection of 10 μl into the system.

Method validation procedure

Specificity of the ion transitions

Monoisotopic masses of CASPO and the IS have a difference of 1 mass unit and their parent ions have only a difference of 0.5 mass unit. Both compounds share a similar chemical structure (Fig. 1) and should produce similar fragmentations. As unspecific transitions (parent to product m/z ions) might show a cross-talk effect, specific product ions were selected and the specificity of the transitions was evaluated in LC-MS/MS runs.

Recoveries, matrix effect, and interference due to simultaneous presence of a second drug

Extraction yield (extraction recovery), matrix effect (ion suppression), and overall method recovery were evaluated at the following CASPO concentrations: 0.15 μg/ml, 1.5 μg/ml, and 15.0 μg/ml. As recommended by other authors,[20–23] three procedures (A, B, and C) were performed: (A) CASPO and the IS were spiked in the HPLC mobile phase, diluted (see Section 'Sample preparation'), and directly injected into the LC-MS/MS system; (B) CASPO and the IS were spiked in de-proteinized plasma samples with MeCN (see Section 'Sample preparation'), processed, and injected into the LC-MS/MS system; (C) CASPO and the IS were spiked in plasma samples, the complete extraction procedure was carried through (see Section 'Sample preparation'), and the samples were injected into the LC-MS/MS system. The mean chromatographic peak areas obtained using the procedures A, B, and C were compared. The ratios C/B, B/A, and C/A determined the yield of extraction, the matrix effect, and the overall method recovery, respectively.

The matrix effect was also studied in duplicate in plasma samples containing different types of anticoagulants (potassium ethylenediaminetetraacetic acid (EDTA) or lithium heparin), and serum (no anticoagulant) collected from a healthy donor using 5-ml polypropylene tubes (Monovettes, Sarstedt, Nümbrecht, Germany). After centrifugation, the samples were spiked with the three QC concentrations. Accuracy and precision of measurements of CASPO levels were determined by the procedure described in Section 'Lower limit of quantification, intra- and inter-day validation'.

In addition, the relative plasma-to-plasma matrix effect was studied in plasmas from different individuals according to recent (2006) recommendations:[24] (1) Samples from six different donors were spiked with the three QC concentrations and the accuracy and precision of measurements of the CASPO levels were determined by the procedure described in Section 'Lower limit of quantification, intra- and inter-day validation'. (2) Five calibration curves (0.04–20 μg/ml) were prepared in citrated plasmas drawn from five different healthy volunteers, and standard curves were constructed and compared according to Matuszewski's recommendations (2006).[24]

Finally, the interference on CASPO quantification from drugs frequently used in patients suffering from invasive fungal infections was evaluated. Thirty-three compounds were studied: (1) antibiotics including amoxicillin/clavulanic acid, piperacillin/tazobactam, ceftriaxone, ceftazidime, cefepime, imipenem, meropenem, ciprofloxacin, levofloxacin, clarithromycin, metronidazole, amikacin, vancomycin, teicoplanin, trimethoprim, clindamycin, rifampicin; (2) anti-fungal agents including fluconazole, itraconazole, voriconazole, amphotericin B, flucytosine; (3) anti-retroviral agents including indinavir, amprenavir, saquinavir, ritonavir, efavirenz, nelfinavir, lopinavir, atazanavir, nevirapine; and (4) immunosuppressive agents including cyclosporine and tacrolimus.

Plasma samples were spiked with CASPO and one of the above drugs, both at 1 μg/ml (a concentration that is observed *in vivo*). The CASPO quantification in the absence

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and presence of a second drug was compared (mean of four and mean of two determinations, respectively).

Lower limit of quantification

The LLOQ is defined as the lowest concentration for which an accuracy between 80% and 120% and a precision with a coefficient of variation of ± 20% is obtained over six measurements.

Calibration curve calculation

The calibration curve included eight CS in duplicate over the following concentration range: 0.04 to 20 μg/ml (LLOQ, and upper limit of quantification (ULOQ), respectively). Six calibration curves obtained over a period of 4 months were taken into account for the determination of the best fit. The best fit among linear and quadratic equations was determined using various weighting factors of the inverse concentration (e.g. $1/x$ and $1/x^2$). The equation showing the lowest and most constant percentage total bias from nominal CS values was considered as the best-fit model. The IS method was used for plasma level quantification: CASPO/IS ratios were plotted against the spiked concentrations. Back-calculated concentrations of the CS had to be within 85–115% of spiked (nominal) concentrations. Eight CS were measured in duplicate: a maximum of 4 measurements out of 16 could be excluded when the deviation from the nominal value was higher than 15%.[25,26]

CASPO quantification over time at different storage conditions

CASPO quantification over time was studied at different temperatures and in different bio-matrices. The IS was added to the tested samples at the time of protein extraction. Four different storage conditions (A to D) were studied as follows: (A) The QC samples were stored at room temperature, 4 °C, and −80 °C, over a period of 3 days, 3 days, and 6 months, respectively; (B) Citrated whole blood from a patient receiving CASPO therapy was stored at room temperature and 4 °C over a period of 5 days and centrifuged just before extraction; (C) Citrated plasma obtained from a patient receiving CASPO therapy was stored over a period of 6 months at −80 °C; (D) QC samples were measured after 1, 2, and 3 freeze–thaw cycles. The CASPO quantification over time was calculated by comparing the CASPO plasma levels measured using freshly prepared CS at a given time point with those initially measured at the time of sample preparation.

Intra- and inter-day validation

Accuracy (measured value/nominal value × 100) and precision (coefficient of variation: standard deviation of measured values/mean measured values × 100) were determined for the QC samples (0.18, 1.91 and 12.5 μg/ml) spiked in a single lot of citrated human plasma. For the intra-day assay (within day), six replicates of each QC sample were processed in the same experiment. For the inter-day assay (between days), each QC sample was processed in duplicate on six different days over a period of 2 months. According to international standards, an accuracy within the range 85–115% of the nominal values and a precision with a coefficient of variation of ± 15% were required.[25,26]

Clinical observations

Three patients treated with standard dosages of CASPO (i.e. 70 mg loading dose, followed by 50 mg/day i.v.) for suspected or documented fungal infections were studied after written informed consent. The study protocol had been approved by the institutional Ethical Committee. In order to obtain time–concentration curves, blood was drawn at 2, 4, 8, and 24 h post-dosing on the second and seventh day of therapy, processed, and analysed as described above.

RESULTS AND DISCUSSION

Optimization of analytical and MS conditions (LC-MS chromatograms)

During the tuning of CASPO and the IS, the ESI-MS system revealed the presence of $[M + H]^+$ as well as $[M + 2H]^{2+}$ parent (precursor) ions (Fig. 2). The chromatographic peak areas were 2 to 4 times higher for transitions using $[M + 2H]^{2+}$ than for $[M + H]^+$ parent ions. Therefore, SRM using the $[M + 2H]^{2+}$ parent ions was employed for method validation. For the determination of the transitions, we had to take into account the fact that there is only a difference of 0.5 mass unit between the precursor ions with m/z 547.3 and 547.8 for CASPO and IS, respectively (Fig. 2). With collision energies of 41 and 20 eV for CASPO and the IS, respectively, we found specific product ions without cross-talk effect between transitions (Fig. 3(A, B)). The product ion formulae revealed the specific fragmentation pathways of CASPO and the IS (the chemical structure of the fragment ions is shown in Fig. 3).[27] The absence of the cross-talk effect between the CASPO transition (SRM: m/z 547.3 → 137.1) and the IS transition (SRM: m/z 547.8 → 62.2) was confirmed during a LC-MS/MS run performed after injection of a plasma extract spiked with CASPO and the IS (Fig. 4(A, B)). The specificity of the product ions of CASPO and the IS may be an advantage of this new method in comparison to some of the published LC-MS/MS methods that acquire ion transition with a similar product ion (m/z at 1033.6) for both CASPO (m/z at 1093.7) and the IS (m/z at 1094.7).[17]

Figure 5 depicts the LC-MS/MS runs after injections of extracted plasma samples spiked with CASPO at 0.04 μg/ml (LLOQ; Fig. 5(A)) or 20 μg/ml (ULOQ; Fig. 5(B)). An H_2O sample and an extracted blank plasma, both injected after the ULOQ samples (Fig. 5(C)), show the absence of carry over. The run on a patient's plasma extract is shown in Fig. 5(D).

Adsorption of CASPO on plastic and glassware

During the determination of extraction recovery, it was observed that the low levels of the QC samples (e.g. 0.18 μg/ml) of CASPO diluted in the mobile phase (procedure A in 'Recoveries, matrix effect, and interference of simultaneous presence of a second drug' in the experimental section) produced lower chromatographic peak areas (a decrease of about 30%) than in the extracted plasma samples (procedure C). In contrast, when the CASPO stock solution was dissolved in plasma prior to subsequent dilution in the

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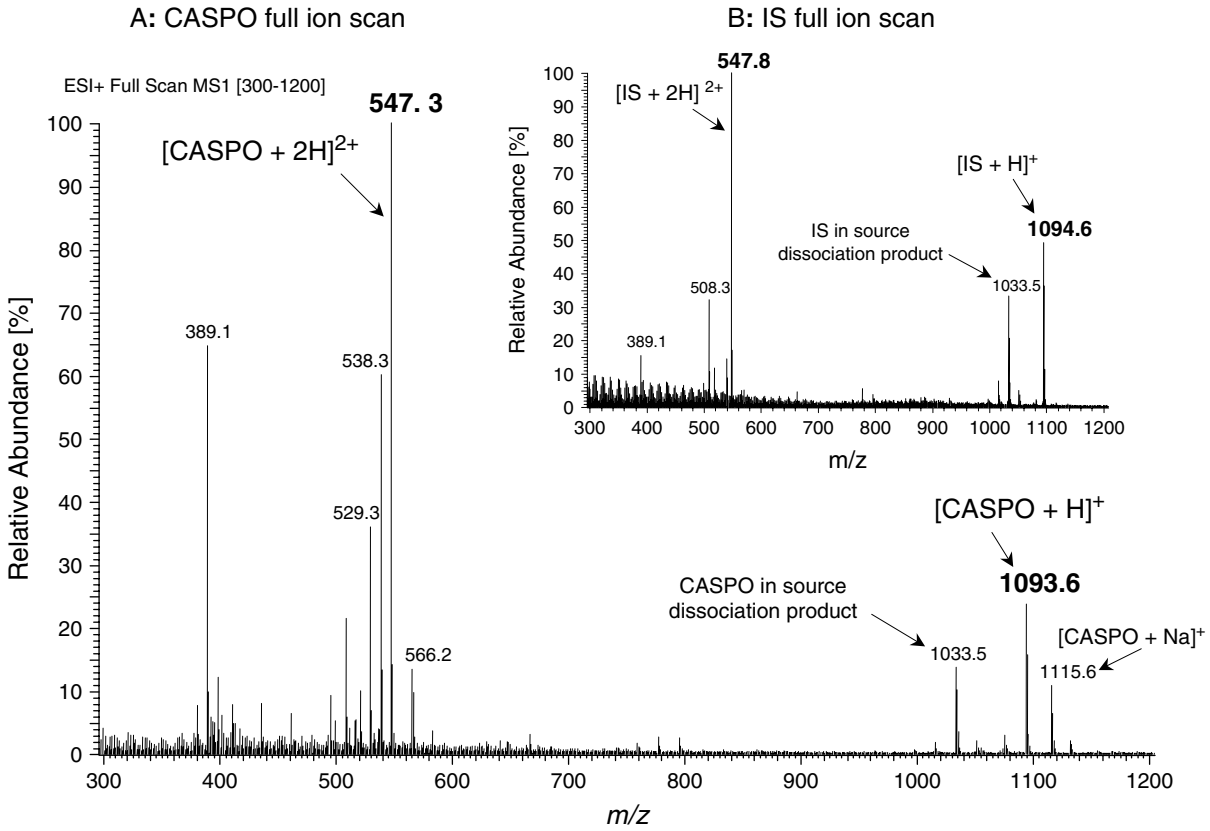

Figure 2. Full positive ion scan of CASPO (A) and IS (B, inset). The compounds were diluted at 1 μg/ml in acetonitrile and in 1% acetic acid in H_2O (50 : 50, v : v), respectively, and infused (10 μl/ min) into the triple-quadrupole MS set-up with the ESI source. $(M + 2H)^{2+}$ parent ions had 2 to 4 times higher signal intensities than $(M + H)^{+}$. This finding was confirmed in the LC-MS/MS analytical runs.

mobile phase (final plasma volume proportional ≤1%), the peak areas were higher than those of the extracted plasma. This observation suggested the adsorption of CASPO on plastic and glassware, which could be prevented by the addition of small volumes of plasma.

Recoveries, matrix effect, and interference of the simultaneous presence of a second drug

The extraction yield, matrix effect, and overall recovery of the method were independent of the CASPO levels (Table 1). The mean extraction yield of CASPO was 79 ± 5%. The extraction yield of the IS was in the same range: 81.5 ± 12.5%. The mean matrix effect was 113 ± 7%, suggesting that plasma extraction residues may moderately enhance CASPO detection. The mean overall method recovery of CASPO was 90% ± 3%. Measurements in plasma samples containing potassium EDTA, lithium heparin or no anticoagulant (serum) from the same donor were accurate: 88 ± 3.6%, 95 ± 3%, and 93 ± 1%, respectively (mean ± standard deviation of low, medium, and high QCs).

Quantification of the QC samples prepared in citrated plasma from six healthy volunteers was accurate (98.6%, 97.9% and 96.3% of nominal values for low, medium, and high QC, respectively) and precise (coefficients of variation 12.7%, 8.3%, and 8.1% for low, medium, and high QCs, respectively). Five CS curves prepared in plasma from five different individuals were compared: the variability of the

Table 1. Extraction yield, matrix effect, and overall method recovery of caspofungin (CASPO) and the internal standard (IS) in human plasma. Three procedures (A, B and C) are compared: dilution of compounds in mobile phase (A), compounds added to extracted plasma (B), and compounds added to plasma prior to extraction (C). Extraction yield, matrix effect, and the overall method recovery were calculated from the ratios of the chromatographic peak areas. Consistent results were obtained at low (0.15 μg/ml), medium (1.5 μg/ml), and high (15 μg/ml) CASPO concentrations

			Extraction yield	Matrix effect	Method recovery
	μg/ml		(C/B in %)	(B/A in %)	(C/A in %)
Caspo	0.15	$n = 6$			
		Mean	81.5	106.9	87.1
		CV (%)	6.4	7.7	6.2
	1.5	$n = 6$			
		Mean	83.0	112.2	93.1
		CV (%)	6.0	5.0	6.5
	15.0	$n = 6$			
		Mean	73.7	120.8	89.0
		CV (%)	4.4	4.7	4.6
IS		$n = 18$			
	0.1	Mean	81.5	115.2	93.9
		CV (%)	12.5	13.0	11.2

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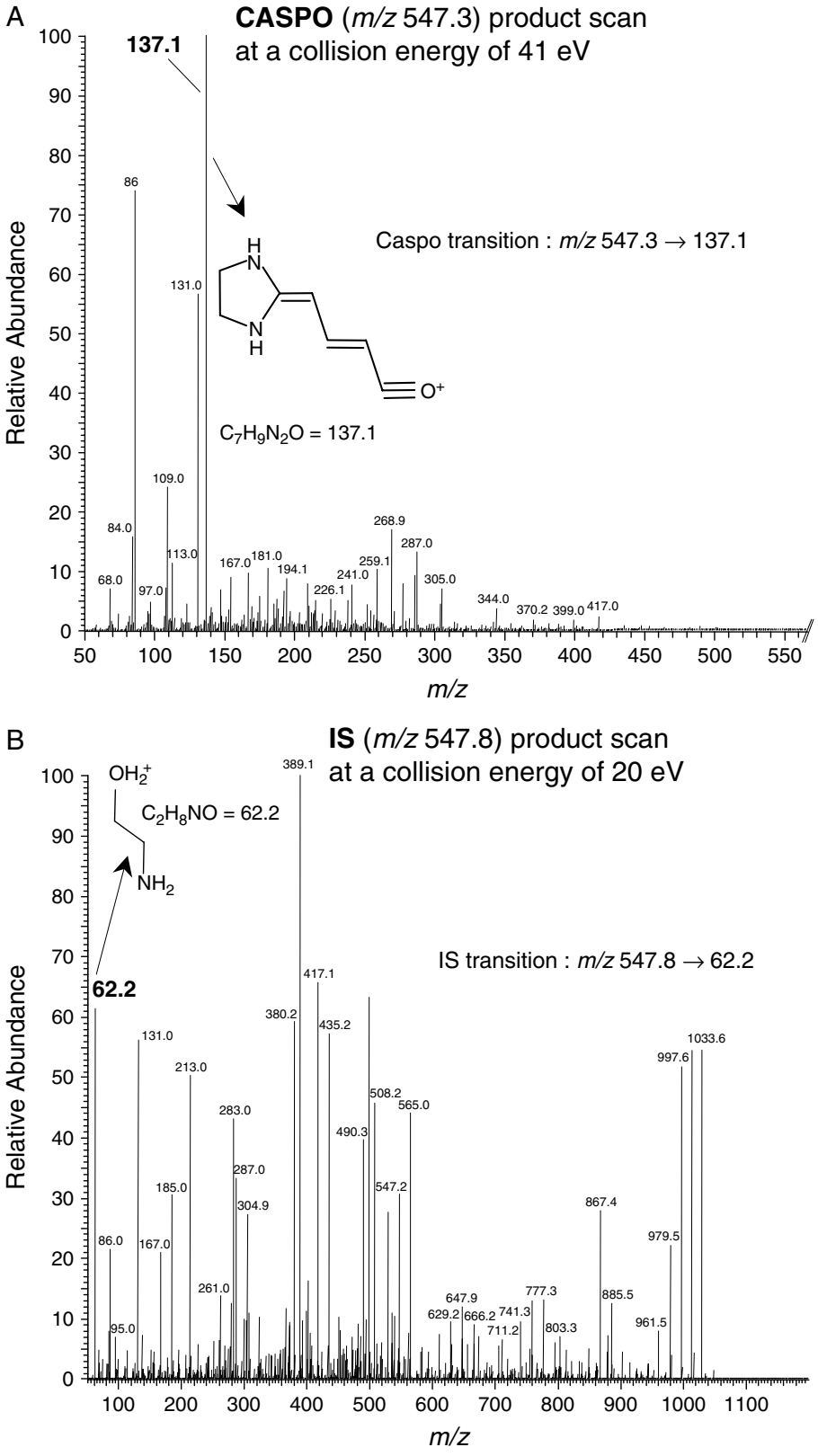

Figure 3. Selection of parent to product ions for the transition of CASPO and the IS: (A) The product scan of CASPO (collision energy = 41 eV) with the selected product ion (m/z = 137.1) for the CASPO transition (product ions above m/z 500 were below 5% relative abundance and are not displayed); (B) The product scan of the IS (collision energy = 20 eV) with the selected product ions (m/z = 62.2) for the IS transition. Probable chemical structures of the product ions are given. The specific m/z transitions (SRM mode) were finally selected at m/z 547.3 → 137.1 and 547.8 → 62.2 with collision energies of 41 and 20 eV for CASPO and the IS, respectively.

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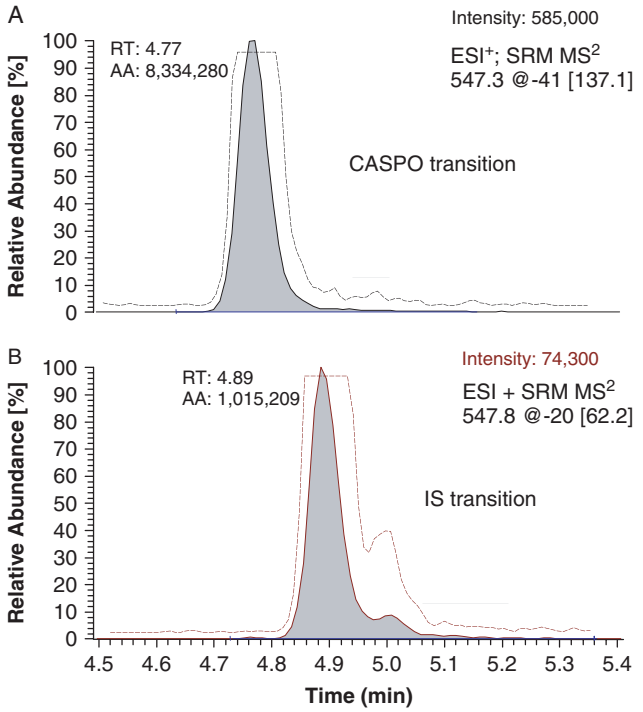

Figure 4. LC-MS/MS chromatograms of ion transitions for CASPO (A) and IS (B). CASPO and the IS were extracted from plasma (at a concentration of 20 and 1 μg/ml, respectively) and injected into the LC-MS/MS system. The specificity of the transitions is confirmed by the lack of cross-talk on each chromatogram. The chromatograms were not smoothed. Dashed lines correspond to the chromatograms zoomed at 10% of the relative abundance. RT is the retention time in min and AA is the peak area in arbitrary units.

slopes fulfilled the acceptance criteria recommended by Matuszewski[24] (data not shown). These results indicate that the analytical procedure is unlikely to be modified by intra- or inter-individual variations in the bio-matrix (absence of significant relative plasma-to-plasma matrix effect).

The simultaneous presence of a second drug, including anti-infective or immunosuppressive agents frequently used in patients with invasive mycoses, did not interfere with CASPO quantification (1 μg/ml). Mean CASPO levels in plasma measured in samples containing a second drug (n = 32) were 94.8 ± 3.5% of those measured in plasma containing CASPO alone. As single exception, a reproducible and significant decrease of CASPO quantification was observed in presence of itraconazole: −14.1 ± 6.3% (ten determinations performed on three different days). However, this decrease remained within the recommended 15% limit of deviation from the nominal value.

Lower and upper limit of quantification

The determinations of LLOQ, accuracy, and precision of intra- and inter-day assays performed using a single lot of human plasma are shown in Table 2. The LLOQ, 0.04 μg/ml, was obtained with the extraction of 100 μl of plasma, which is equivalent to an amount of 0.2 ng detected on column. This LLOQ is appropriate for clinical purposes, i.e. below the *in vitro* minimally inhibitory concentrations of CASPO for most fungal pathogens (MIC_{90} 0.5 μg/ml) and the mean through CASPO plasma levels in humans receiving standard dosages.[12,28,29] The ULOQ was 20 μg/ml.

Table 2. Lower limit of quantification (LLOQ), intra-day and inter-day validation using quality controls (nominal concentrations: 0.18, 1.91 and 12.5 μg/ml). Accuracy = measured values/nominal values × 100. Precision is expressed as the coefficient of variation = SD of measured value/mean measured value × 100

LLOQ	Nominal concentration (μg/ml)	0.040	–	–
(n = 6)	Accuracy (%)	108.8	–	–
	Precision (%)	11.6	–	–
QC Intra-day	Nominal concentration (μg/ml)	0.18	1.91	12.50
(n = 6)	Accuracy (%)	95.4	105.7	98.5
	Precision (%)	5.7	6.3	11.6
QC Inter-day	Nominal concentration (μg/ml)	0.18	1.91	12.5
(n = 6)	Accuracy (%)	101.7	100.6	105.3
	Precision (%)	6.7	7.8	4.3

Using the modified extraction procedure of 500 μl of plasma (see Section 'Sample preparation'), the LLOQ was further reduced to 0.005 μg/ml (0.5 ng detected on column; precision 17.8% and accuracy 4.2% among five measurements). The ULOQ was 5 μg/ml. This ultra-sensitive extraction method may therefore be an alternative when a sample volume larger than 500 μl is available. The standard extraction procedure with an LLOQ of 0.04 and a ULOQ of 20 μg/ml in a 100 μl sample volume remains, however, the most appropriate option for clinical requirements.

Calculation of the calibration curves

Although calibration curves appeared linear over the range 0.04–20 μg/ml, a quadratic equation weighted by the inverse squared concentration ($1/x^2$) resulted in the best fit. The mean bias of 83 measurements from 6 calibration curves was 5.1 ± 4.2% (Fig. 6).

CASPO quantification over time under different storage conditions

Three freeze–thaw cycles or storage of the plasma samples at −80 °C for 6 months did not result in significant changes of the CASPO plasma concentrations in the QC samples: values were within 85–115% of the initial values (data not shown). Similarly, the plasma level of CASPO from a patient treated with CASPO remained within 85% and 115% of the initial value (14 μg/ml) when stored for 6 months at −80 °C, suggesting that CASPO metabolites were not generated from or converted to the parent drug under these conditions (data not shown). When the QC samples were stored at 4 °C, CASPO levels remained within 85–115% of the initial values during 3 days. In contrast, the values decreased significantly (i.e. <85% of initial values) when samples were stored at room temperature for >48 h (data not shown). In whole blood from a patient treated with CASPO, plasma levels (14 μg/ml) remained within 85% and

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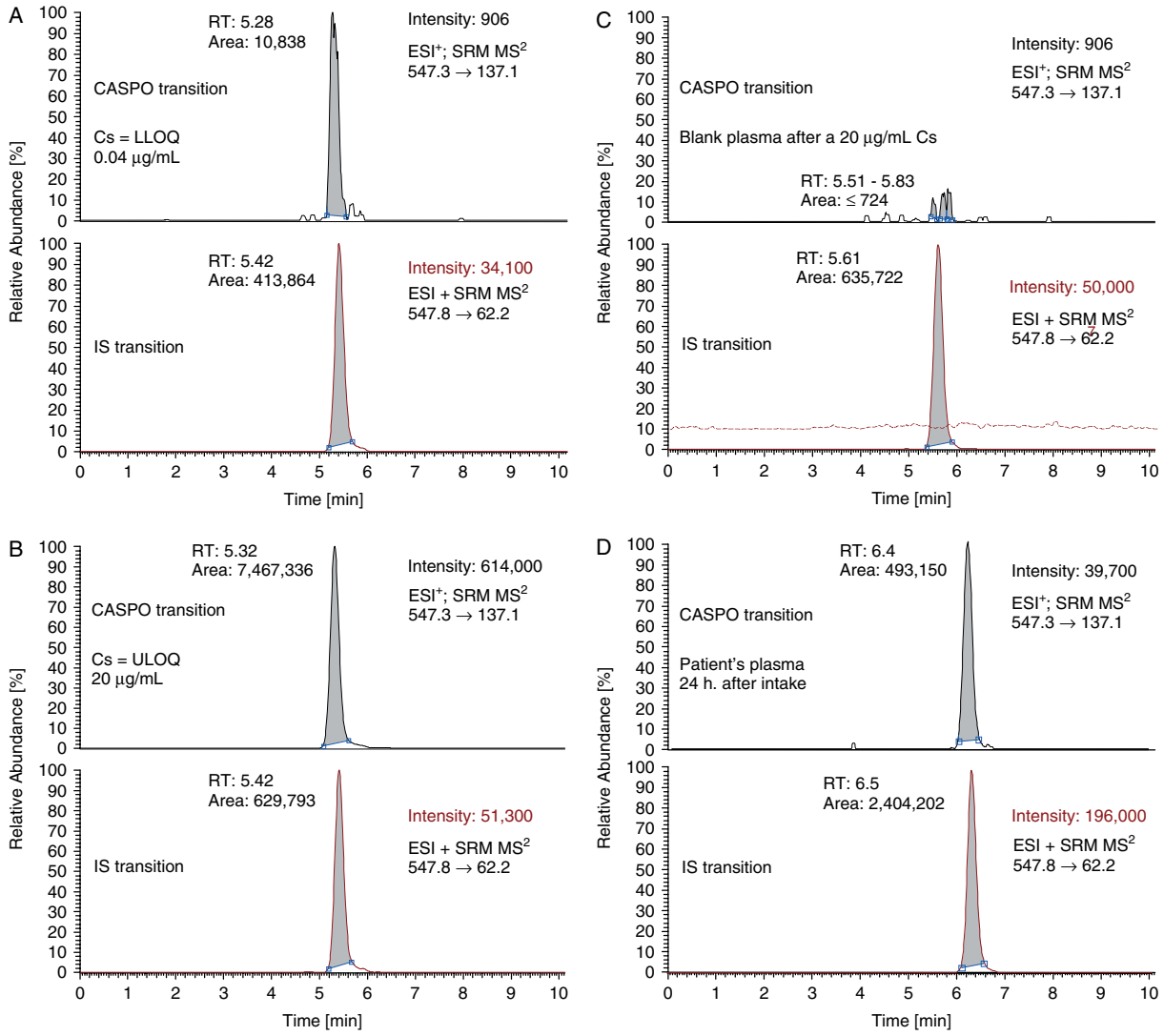

Figure 5. LC-MS/MS chromatograms of the ion transitions for CASPO and the IS: (A) Injection (20 µl) of a calibration sample (CS) spiked with 0.04 µg/ml (= LLOQ); (B) Injection of a CS spiked with 20 µg/ml (= ULOQ); (C) Injection of a blank plasma extract following the injection of an ULOQ CASPO sample (chromatogram with identical signal intensity as in panel A). The dash line on the IS transition depicts the signal of H_2O injected after the ULOQ samples; (D) Injection of a plasma extract from a patient receiving CASPO therapy. RT is the retention time in min; AA is the peak area in arbitrary units.

115% of the initial value for storage of 5 days at 4 °C, but decreased significantly (<85% of initial value) when stored at room temperature for >3 days (data not shown). Under these different storage conditions, no increase of the CASPO quantification was observed, which suggests that there was no back-transformation of hypothetical unstable metabolites to the parent drug. These observations are helpful for clinical investigators and physicians to establish procedures of pre-analysis handling of clinical blood samples.

Intra- and inter-day validation

Overall mean intra- and inter-day accuracy was 96.1 ± 2.2% and 102.5 ± 2.4%, respectively; the overall mean intra- and inter-day precision was 7.9 ± 3.2% and 6.3 ± 1.8%, respectively (Table 2).

Clinical observations

CASPO levels in blood were measured in three adult patients 2 and 7 days after starting therapy with 50 mg/day after a 70-mg loading dose. Two had received myeloablative chemotherapy and were neutropenic and one had no underlying condition. Indications for CASPO therapy were proven invasive aspergillosis, probable invasive candidiasis, and possible invasive mycosis. The patient suffering of proven invasive aspergillosis received concomitant therapy with voriconazole. The time–concentration curves of CASPO are shown in Fig. 7 ((A): day-2; (B): day-7).

CONCLUSIONS

A fast LC-MS/MS method was developed for quantification of CASPO levels. The small volume of plasma required (100 µl) is an advantage when sample volume is an issue (e.g. in serial measurements, children, or experimental animal models).

The main advantages of this LC-MS/MS method, when compared to methods reported in the literature, are: (1) gentle, simple, rapid, and inexpensive pre-analysis

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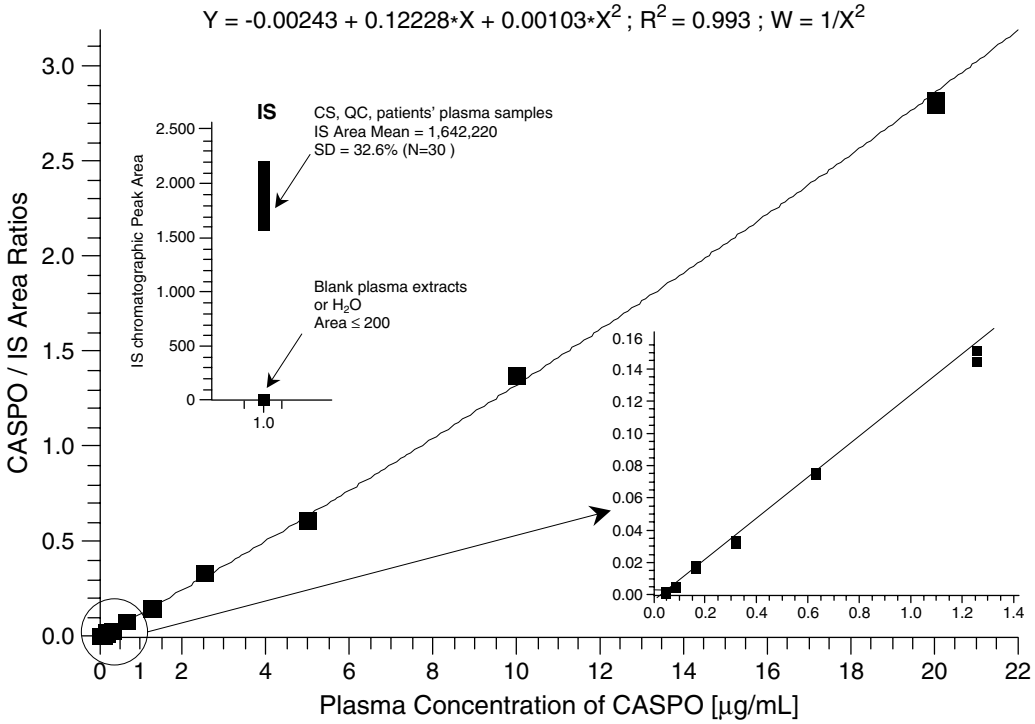

Figure 6. Calibration curve in plasma: CASPO concentrations are plotted against ratios of CASPO/IS chromatographic peak areas. Although the curve is almost linear, a better fit is obtained by quadratic regression and weighting by the inverse squared concentration ($1/x^2$). The low CASPO concentration range is zoomed and depicted on the right. The variability of the IS chromatographic peak areas of the CS, QC and patients' plasma samples is shown: standard deviation is 33% ($n = 30$).

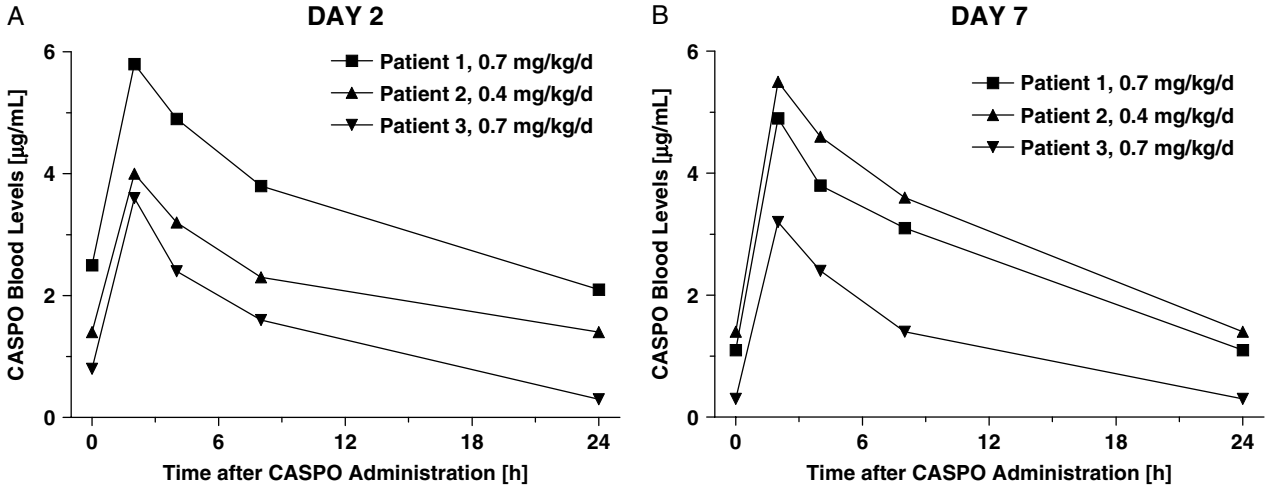

Figure 7. Time–concentration curves of CASPO on day-2 (A) and on day-7 (B) after starting therapy with standard doses (70-mg loading on day-1, followed by 50 mg/day i.v.) in three patients with suspected or documented invasive mycoses.

processing using protein precipitation by acetonitrile; (2) excellent recovery; (3) absence of matrix effect; (4) ion transition specificity; (5) absence of carry over; (6) short analytical time; and (7) validation over a large dynamic range (calibrators from 0.04 to 20 µg/ml) including the whole spectrum of clinically relevant CASPO levels and minimally inhibitory concentrations of fungal pathogens. Such a dynamic range avoids repeated measurements after sample dilution for out-of-range values (a frequent situation when using methods with an upper limit of detection below 2 µg/ml). Moreover, the LLOQ can be further reduced (0.005 µg/ml) for special requirements using a modified extraction procedure with an additional concentration step.

Adsorption on plastic or glassware of aqueous CASPO solutions, stability over time at different pre-analysis processing conditions, absence of evidence for misquantification due to interference by back-transformation of labile CASPO metabolites, and lack of interference of frequently used concomitant drugs on CASPO quantification and of different types of anticoagulants used for blood sampling are additional meaningful information for laboratory and clinical practice. This simple, accurate, precise, and robust analytical

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method may be useful for monitoring CASPO therapy in patients with life-threatening mycoses.

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