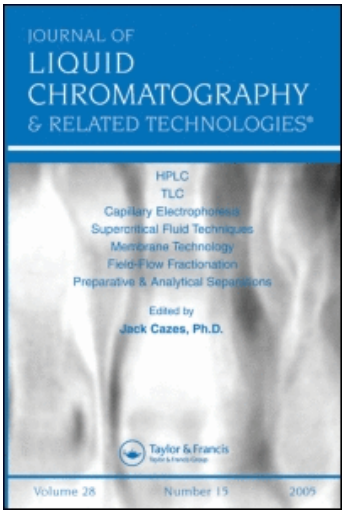

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A Semi-Automated Procedure for the Determination of Caspofungin in Human Plasma Using Solid-Phase Extraction and HPLC with Fluorescence Detection Using Secondary Ionic Interactions to Obtain a Highly Purified Extract

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Abstract: A semi-automated assay for the determination of caspofungin in human plasma is presented. High assay throughput was achieved through the use of a robotic sample processor and 96 well format solid phase extraction (SPE). Drug and internal standard (an isostere) were extracted from plasma using a silica based, C_8 stationary phase. The extraction yielded a highly purified extract, as retention was mediated by a combination of reverse phase and secondary ionic interactions. Conditioned SPE plates (50 mg sorbent/well) were loaded with buffered (pH 4.9) plasma containing drug and internal standard. The wells were washed with water and neat methanol prior to elution with a reagent optimized for both recovery and selectivity (0.25 M ammonium hydroxide/0.05% trifluoroacetic acid in methanol). Excess residual water in the SPE wells during the methanol wash was found to cause variable drug recovery and was eliminated by centrifugation of the SPE plate. After evaporation of the SPE eluent, plasma extracts were dissolved in mobile phase and analyzed using a Keystone Betasil C_{18} analytical column (4.6 × 50 mm, 3 μm) with fluorescence detection (excitation 220 nm, emission 304 nm). The mobile phase was composed of a 38 : 62 (v : v) mixture of acetonitrile and 0.1% trifluoroacetic acid (adjusted to pH 3 with triethylamine) and was pumped at a flow rate of 1.5 mL/minute.

Seven-point calibration curves over the concentration range 125–10,000 ng/mL

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yielded a linear response (drug concentration *vs* drug/internal standard peak height ratio) using a weighed $(1/x)$ linear regression model. Based on the replicate analyses ($n = 5$) of spiked plasma standards, intra-day assay precision was better than 5.7% coefficient of variation (CV) and intra-day accuracy was within 1.7% of nominal at all points of the standard curve. Inter-day precision, as assessed by daily analysis of high, mid, and low concentration quality control samples ($n = 6$), was better than 5.3% CV. Inter-day accuracy was within 10.7% of nominal value.

Keywords: Caspofungin, Solid-phase extraction, HPLC, Fluorescence detection, Secondary ionic interactions

INTRODUCTION

Caspofungin (Cancidas™, MK-0991) is a semi-synthetic cyclic hexapeptide, currently being used as a new therapeutic agent for the treatment of serious human fungal infections caused by organisms such as *Aspergillus* and *Candida*. Previously, Amphotericin B was the only available broad-spectrum fungicidal agent for treatment of serious infections, but its use was limited due to inherent toxicity.[1] Caspofungin is water soluble, basic, and contains a phenolic hydroxyl group and an acyl side chain. It is structurally related to the echinocandin class of antimicrobial agents and has a molecular weight of 1093 Da. The drug is thought to work by inhibiting the synthesis of (1,3)-D-glycan, an essential and major cell wall component in pathogens such as *Aspergillus*, *Candida*, and *Pneumocystis carinii*.[2–5]

Methods for the determination of Caspofungin in human plasma were required to support ongoing human clinical pharmacokinetic studies. Previously, we described a manual method for the determination of Caspofungin in plasma that used an individual diol SPE column to isolate the analyte, followed by HPLC with fluorescence detection.[6] A method utilizing the same sample preparation scheme coupled with HPLC-MS/MS analysis has also been published.[7]

A major drawback of the published methods is low sample throughput. Both methods use individual diol SPE columns for analyte isolation. Since sample preparation is labor intensive, the number of samples that can be processed per day is limited. In order to support large clinical studies, the development of a less labor intensive, high throughput procedure was desired. Since solid phase extraction (SPE) in the 96-well format has, in recent years, become a method of choice in many laboratories to prepare biological fluid samples for analysis,[8] we focused our efforts on the use of this technology, combined with laboratory robotics, to develop an improved human plasma assay for Caspofungin.

Here, we describe a high throughput method for the determination of Caspofungin in human plasma using semi automated sample preparation with a 96 position, reverse phase SPE plate. The SPE procedure utilizes a com-

bination of reverse phase and secondary ionic interactions between the analyte and SPE sorbent to obtain an extract free of interference from endogenous compounds.

EXPERIMENTAL

Material

Caspofungin and internal standard (Figure 1) were synthesized at Merck Research Laboratory (Rahway, NJ, USA) as diacetate and dihydrochloride salts, respectively. HPLC grade acetonitrile (ACN) and methanol were purchased from Fisher (Fair Lawn, NJ, USA). Sequenal grade triethylamine (TEA) and trifluroacetic acid (TFA) were supplied by Pierce (Rockford, USA). Drug-free control human plasma was supplied by Biological Specialty (Colmar, PA, USA). All other reagents were ACS grade.

Bond Elut Matrix slurry packed 96-well SPE plates (C_8, 50 mg/well) were purchased from Varian (Harbor City, CA, USA). Five hundred mg, 3 mL diol columns were purchased from J. T. Baker (Phillipsburg NJ, USA) and 25 mg/well Strata Screen C (mixed phase strong cation exchange/reverse phase) plates were obtained from Phenominex (Torrance, CA, USA). Square well polypropylene 96-position plates (2.4 mL/well) used to mix plasma samples with buffer and internal standard were purchased from SUN International (Wilmington, NC, USA). Round well 96-position plates (1 mL/well) used to evaporate the SPE eluates and matching prescored silicone/PTFE cover mats were obtained from Agilent (Wilmington, DE, USA).

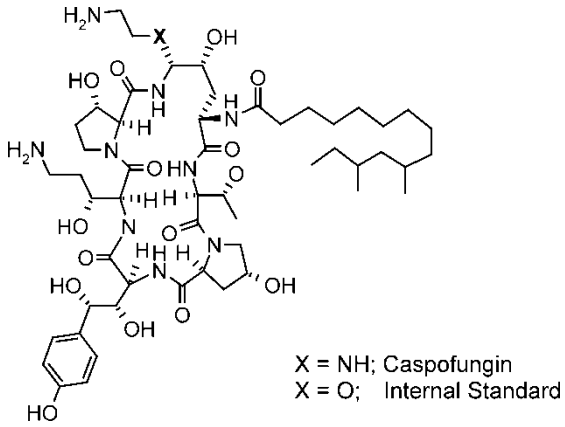

Figure 1. Chemical structure of Caspofungin and internal standard.

Instrumentation

The HPLC system consisted of an Agilent 1100 series binary pump, column compartment, well-plate autosampler, and fluorescence detector (Wilmington, DE, USA). The detector output was connected to a PE Nelson (Cupertino, CA, USA) Totalchrom data system via a PE Nelson NCI900 analog-to-digital interface.

A Packard MultiProbe II ^{HTEX} robotic sample processor (Downers Grover, IL, USA) equipped with eight 1 mL syringes, Versatip probes, and a solid phase extraction manifold was used to perform the extraction procedure. The layout of the robot deck is shown in Figure 2. A Sigma 4K15 centrifuge equipped with a 96-well plate rotor was obtained from Qiagen (Chatsworth, CA, USA). SPE plate eluents were evaporated using a SPE Dry-96^{TM} sample concentrator from Jones Chromatography (Lakewood, CO, USA).

Chromatographic Conditions

A Keystone Betasil C18 analytical column (50 × 4.6 mm, 3 μm) was used for the separation. The column was operated at a flow rate of 1.5 mL/minute and a temperature of 35°C. The mobile phase consisted of the following two components; A—0.1% TFA (adjusted to pH 3 with TEA) and B—acetonitrile. Mobile phase composition was as follows during the analysis; 0–3 minutes—38% B, 3.0–3.5 minutes—100% B, and 3.5–5 minutes—38% B. The sample injection volume was 65 μL. The fluorescence detector excitation and emission wavelengths were set at 220 and 304 nm, respectively.

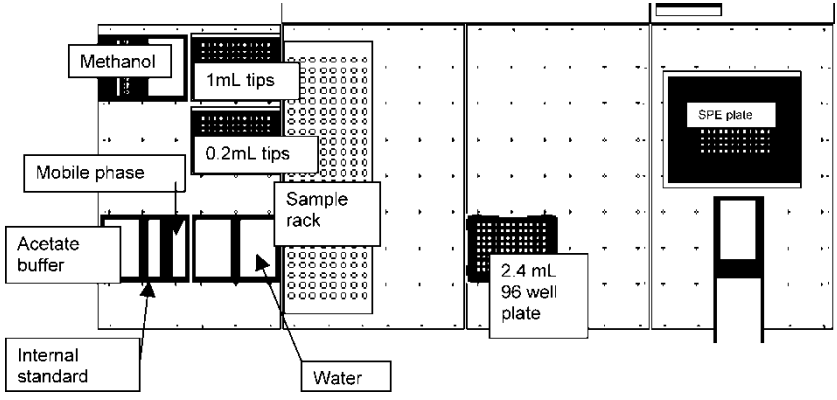

Figure 2. Multiprobe deck layout.

Preparation of Standards

A stock standard of Caspofungin (1 mg/mL free base) was made up in 62 : 38 0.1% TFA (adjusted to pH 3 with TEA):acetonitrile ("sample solvent"). This standard was further diluted in the same solvent to give a series of working standards. The working standard concentrations were 1000, 500, 250, 100, 50, 25, and 12.5 μg/mL. Stock and working standards were stored at −70°C. A stock solution of internal standard (1 mg/mL free base) was prepared using dimethyl sulfoxide (DMSO) as solvent and stored at room temperature. The stock internal standard solution was diluted with sample solvent to give a 2 μg/mL working solution.

Plasma standard samples were prepared by spiking 10 μL of each working standard into 1 mL of control human plasma. The resulting plasma standards were used to quantitate samples containing Caspofungin over the concentration range of 125–10,000 ng/mL.

Quality control (QC) samples were prepared using stock and working standards other than those used for the preparation of plasma standards. QC samples containing Caspofungin at concentrations of 7500 ng/mL (high QC), 1250 ng/mL (medium QC), and 250 ng/mL (low QC) were prepared. The QC samples were stored in 0.6 mL aliquots at −70°C.

Comparison of Diol, C_8, and Mixed Phase SPE Sorbents

One hundred μL control plasma containing 10 μg/mL Caspofungin was mixed with 0.4 mL potassium acetate buffer (1 M, pH 4.9) and applied to conditioned, diol, C_8, and mixed phase (Screen C, strong cation exchange/reverse phase) SPE columns/wells. After the columns/wells were washed with water and neat methanol and centrifuged (1000–1500 × g), they were eluted with various concentrations of ammonium hydroxide (0.025 to 0.5 M) or TFA (0.005 to 0.1%) in methanol. The eluents were evaporated using a stream of nitrogen and assayed by HPLC-fluorescence using conditions described in Chromatographic section.

Plasma Extraction Procedure

Solid phase extraction was performed using an eight channel Packard Multiprobe II sample processor. The eight liquid handling probes (Versatips) were equipped to use disposable or fixed tips without hardware change. Conductive, disposable pipette tips were used to pipette raw plasma and plasma mixed with buffer and internal standard (0.2 and 1.0 mL tips, respectively). All other liquid transfers used fixed tips. The C_8 SPE plate was conditioned using 250 μL of methanol and 250 μL water per well. After the conditioning was completed, the sample processor pipetted 0.1 mL plasma, 50 μL internal

standard working solution, and 400 μL 0.2 M potassium acetate buffer (pH 4.9) into a clean 96 position, square well plate. After thorough mixing *via* multiple aspirate/dispense cycles, the resulting solution was transferred to the conditioned SPE plate. After 15–20 minutes under slight vacuum (vacuum on, manifold bleed valve open), all wells were washed with 0.5 mL water (vacuum on, manifold bleed valve closed.) and the plate was centrifuged at 1500 × g for 2 minutes to remove residual water. Upon completion of an additional wash with 0.5 mL methanol, the SPE plate was ready for elution using a reagent containing 0.05% TFA and 0.25 M ammonium hydroxide in methanol. The elution was performed by placing the SPE plate on top of a clean, 96 position, 2 mL round well plate and adding 0.5 mL elution reagent to each well. After 10 minutes, the stacked plates were centrifuged at 1500 × g for 2 minutes. The top (SPE) plate was discarded and the eluents in the bottom plate dried at a temperature of 35°C under a stream of nitrogen. Lastly, 250 μL sample solvent (see Preparation of Standards) was added to each well, the plate was sealed with a prescored silicone/PTFE mat, and vortexed.

Data Acquisition and Analysis

Peak heights were calculated using TotalChrom (Perkin-Elmer) software and exported to a Watson LIMS system where the daily standard curves of peak height ratios (drug/internal standard) vs. standard concentration were constructed. Concentrations of unknown samples were calculated from the equation $y = mx + b$ as determined by the weighted $(1/x)$ linear regression analysis of the standard line.

RESULTS AND DISCUSSION

Comparison of SPE Sorbents

In developing a high throughput assay, our initial efforts focused on identifying a 96 well format SPE plate suitable for extracting Caspofungin from human plasma. A previous manual assay employed individual 500 mg diol SPE columns for this purpose. Our experience with these columns suggested that the drug was retained by both reverse phase and secondary cation exchange mechanisms. The basis for this conclusion was the observation that Caspofungin, a basic compound containing both primary and secondary amines, could not be eluted from diol columns by neat methanol. To further investigate the retention mechanisms and help identify a substitute SPE phase, diol, C_8, and mixed phase (strong cation exchange/reverse phase) sorbents were compared using conditions described in the experimental section. Control human plasma containing the drug was applied to conditioned

diol, C_8, and mixed phase SPE columns/wells. After being washed with water and methanol, they were eluted with ammonium hydroxide in methanol or TFA in methanol and the recovery was determined. The results are shown in Figures 3 and 4.

Whether using acidic (TFA) or basic (ammonium hydroxide) reagents, the drug was more readily eluted from the C_8 wells than from the diol or mixed phase columns. It was surprising that the diol sorbent was far more retentive than the C_8, as the two were thought to operate via reverse phase and secondary ionic interactions. Possible explanations were the higher mass of the diol column sorbent (500 mg diol *vs* 50 mg C_8), higher silanol activity in the diol silica, and/or polar interactions (H bonding, dipole interactions, etc) with the diol phase.

The mixed phase sorbent behaved as expected. The drug was not eluted using the TFA reagent because the stationary phase contained a strong cation exchanger ($R\text{-}SO_3^-$). Relatively high concentrations of ammonium hydroxide in methanol were needed to overcome the high ion exchange capacity of the mixed phase column and the high affinity of the drug for the strong cation exchange sites.

Optimization of C_8 SPE Elution Solvent

Overall, the above results suggested that the C_8 SPE plate was the optimal one for a 96 well format Caspofungin assay. However, it was observed that when blank plasma was extracted using this plate with ammonium hydroxide

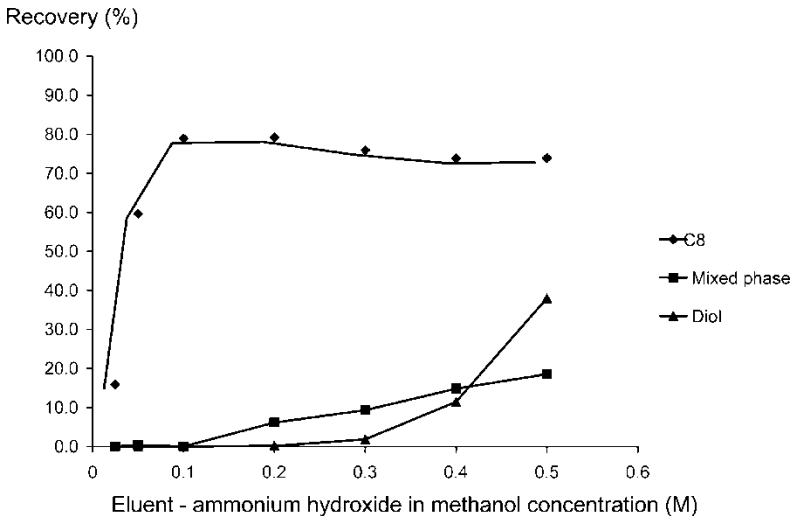

Figure 3. The effect of SPE eluent strength on Caspofungin recovery using Varian C_8, Strata mixed phase, and Baker diol SPE columns.

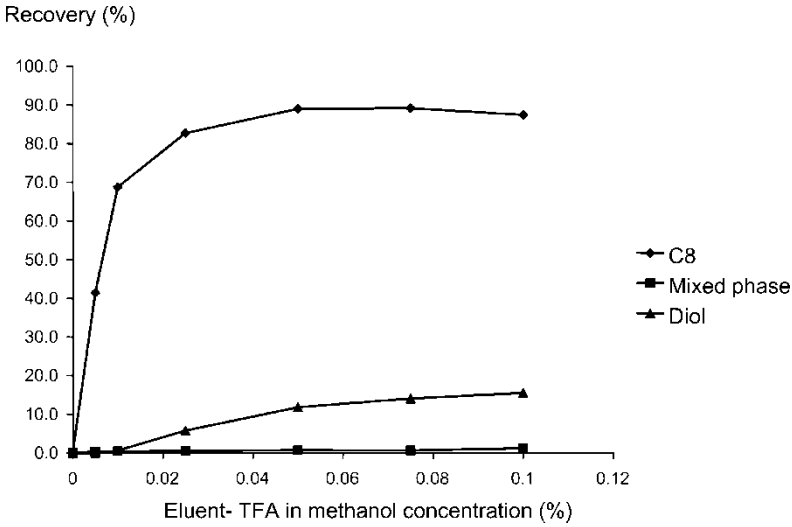

Figure 4. The effect of SPE eluent strength on Caspofungin recovery using Varian C_8, Strata mixed phase, and Baker diol SPE columns.

(0.1–0.5 M) in methanol as the eluent, a small tailing peak was present in the chromatograms which eluted about 0.4 minutes before Caspofungin. We found that the use of an elution reagent containing both ammonium hydroxide (0.25 M) and TFA (0.05%) in methanol eliminated the peak without compromising the recovery of the drug. This eluent composition was, in fact, the same one used for the manual assays and was selected as the optimal reagent for the C_8 plate.

Optimization of C_8 SPE Procedure

During initial attempts to validate the method, variable recoveries were sometimes observed and possible causes investigated. Preliminarily experiments suggested that the problem was related to drug breakthrough during the methanol wash step. Subsequent experiments demonstrated that the breakthrough was indeed caused by excess residual water in the SPE wells prior to the methanol wash. Centrifugation of the plate ($1500\times g$) before the methanol wash prevented any significant loss of drug during this step (Table 1). It is likely that the mixing of excess residual water with incoming methanol caused excessive amounts of the drug to elute before it was able to "lock on" to the weak cation exchange sites in the stationary phase. Removal of water from the plate by centrifugation was an effective and convenient way to prevent this problem. No interfering peaks were observed in chromatograms of blank plasma extracted using this technique.

Table 1. The effect of centrifugation on drug breakthrough in SPE methanol wash

	Recovery, % (no centrifugation before methanol wash)			Recovery, % (centrifugation before methanol wash)		
Replicate #	Methanol wash	Eluent	Total (methanol + eluent)	Methanol wash	Eluent	Total (methanol + eluent)
1	8.3	76.8	85.0	0.6	88.9	89.5
2	24.0	61.2	85.2	1.2	77.6	78.8
3	33.7	55.4	89.1	0.5	83.4	83.9
4	3.5	81.7	85.2	0.6	84.0	84.6
5	4.4	85.3	89.6	0.8	83.9	84.7
6	4.4	85.3	89.7	0.9	81.4	82.4
7	4.3	86.2	90.4	0.5	78.7	79.2
8	3.3	85.7	89.1	0.5	77.9	78.4
Mean	10.7	77.2	87.9	0.7	82.6	83.3
SD[a]	11.6	12.2	2.3	0.3	3.9	3.8
%CV[b]	107.9	15.8	2.7	34.3	4.7	4.6

[a]Standard deviation.
[b]Percent coefficient of variation.

Chromatographic Conditions

In our previously described SPE-HPLC method,[6] the total run time using a Zorbax C_8 analytical column (4.6 × 150 mm, 5 μm) was 10.5 minutes. In order to improve sample throughput a shorter run time was desirable. Therefore, alternative HPLC columns were evaluated to separate Caspofungin and internal standard from co-extracted endogenous components. Best results were obtained using a Keystone Betasil C_{18} analytical column (4.6 × 50 mm, 3 μm); the run time was reduced to five minutes using this column. The use of the 3 μm packing material provided higher column efficiency and reduced analysis time. A column washout procedure was employed during each run in which the acetonitrile content of the mobile phase was briefly increased to 100%. This eliminated late eluting peaks and insured reproducible retention times after multiple injections.

Assay Automation

The most laborious and time consuming steps of the SPE assay were automated using a Packard MultiProbe liquid handling system. Conductive disposable tips (0.2 mL) accurately pipetted plasma directly from clinical sample tubes to 96 well plates using the instrument's liquid sensing capability. One mL disposable tips mixed plasma with buffer and internal standard and loaded the SPE wells in a single operation. The centrifugation and SPE elution steps were done manually.

Assay Selectivity

Figure 5 shows chromatograms of extracted control plasma (A), plasma spiked with Caspofungin and internal standard (B), and plasma from a patient 1 hour after receiving a 50 mg/M^2/day intravenous dose of Caspofungin (C). A comparison of Figure 5A and Figure 5B illustrates that no endogenous peaks co-eluted with either the analyte or internal standard. The selectivity of the assay was further confirmed by analysis of pre-dose plasma sample from subjects involved in clinical trials; all of the samples were free of interfering peaks.

Extraction Recovery

Extraction recovery was evaluated for Caspofungin and internal standard using standards prepared in human plasma spiked at the following analyte concentrations: 125 ng/mL, 1000 ng/mL, and 10,000 ng/mL. The internal standard was spiked at a nominal concentration of 1000 ng/mL. Extraction recovery of the analyte and internal standard was determined by comparing

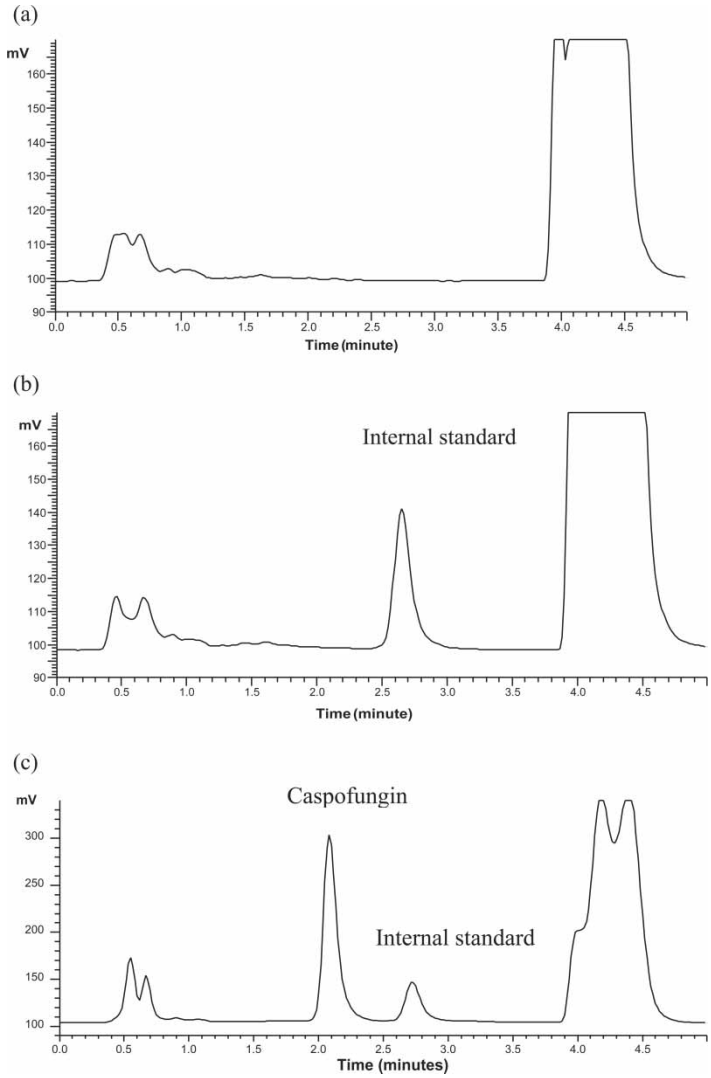

Figure 5. Representative chromatograms of Caspofungin and internal standard in human plasma. (a) Blank control human plasma (b) Control human plasma spiked with 1000 ng/mL internal standard. (c) Post-dose sample (diluted five fold with control plasma) containing 23.9 μg/mL Caspofungin and 1000 ng/mL internal standard.

the absolute peak heights of drug or internal standard, in human plasma extracted as described above, to control plasma extracted in the same manner and then spiked post-extraction with drug and internal standard. The mean recovery was approximately 75.6% and 72.1% for Caspofungin and internal standard, respectively. Recovery was found not to vary signifi-

cantly over the concentration range examined, indicating that the analyte and internal standard were extracted consistently over the quantitation range of the assay (Table 2).

Assay Precision and Accuracy

Replicate standards (n = 5 at each concentration, where each curve was prepared in a different lot of plasma) at 125, 250, 500, 1000, 2500, 5000, and 10,000 ng/mL in plasma prepared from the working solutions were analyzed to assess the within-day variability of the assay. The results are shown in Table 3. Accuracy was within 1.7% of nominal and precision was 5.7% coefficient of variation (CV%) or better for all standards.

Limit of Quantitation

The limit of quantification of the assay, defined as the lowest concentration that yields a within-day precision of less than 15% and a within-day accuracy of ±15% of nominal concentration, was 125 ng/mL in human plasma using 100 μL of plasma.

Quality Control Samples

Quality control samples (QCs), were prepared and frozen (−70°C). It was found that Caspofungin in plasma was stable through three freeze-thaw cycles (Table 4). The QCs were analyzed daily along with clinical plasma samples to assess the inter-day variability of the assay. Inter-day precision, assessed over a 7 month period (n = 6), was 5.3% CV or better. Accuracy

Table 2. Extraction recovery of caspofungin and internal standard[a] from human plasma

Nominal analyte concentration (ng/mL)	Recovery[b] mean (n = 5) drug/internal standard
125	70.1/69.9
1000	77.2/73.7
10000	79.5/72.7

[a]Internal standard concentration was 1000 ng/mL.

[b]Calculated by dividing pre-extraction spike peak height by post-extraction spike peak height.

Table 3. Within-day precision and accuracy data for the determination of Caspofungin in five different lots of human plasma

Nominal concentration (ng/mL)	Mean[a] concentration (ng/mL)	Precision[b] CV (%)	Accuracy[c] (%)
125	126.9	5.7	101.6
250	250.6	4.2	100.3
500	503.1	2.1	100.6
1000	983.1	3.6	98.3
2500	2488.4	2.5	99.5
5000	4950.2	2.6	99.0
10000	10072.7	2.9	100.7

[a]Mean concentrations calculated from the weighted linear least-squares regression curve constructed using all five replicate values at each concentration.

[b]Expressed as coefficient of variation (CV).

[c]Expressed as [(mean calculated concentration)/(nominal concentration) × 100].

was between 106.9 and 110.7% of nominal (Table 5). Based on long-term analysis of quality control samples (data not shown), Caspofungin was found to be stable in human plasma stored at −70°C for at least 3 years.

CONCLUSION

A high throughput semi-automated assay has been developed for the determination of Caspofungin in human plasma. Application of 96-well SPE technology,

Table 4. Stability of caspofungin in human plasma quality control samples through three freeze/thaw cycles (F/T)

	Caspofungin		
Nominal concentration (ng/mL)	Control found concentration mean[a] (ng/mL, n = 5)	3 F/T Cycles found concentraton mean[a] (ng/mL, n = 5)	Difference from control[b] (%)
7500	7536.4 (1.7)	7586.0 (0.2)	0.7
1250	1304.9 (1.0)	1308.9 (0.9)	0.3
250	269.5 (2.3)	266.9 (3.1)	−1.0

[a]Numbers in parentheses are coefficients of variation (CV%).

[b]Calculated as [(mean found concentration in 3 F/T − mean found concentration in control)/mean found concentration in 3 F/T] × 100.

Table 5. Inter-day precision and accuracy data for the determination of caspofungin in plasma quality control samples

	QC concentrations (ng/mL)		
Nominal	7500	1250	250
Mean[a]	8014.5	1384.4	269.3
SD[b]	350.4	64.4	14.2
% CV[c]	4.4	4.7	5.3
Accuracy %	106.9	110.7	107.7

[a]Mean of 6 inter-day quality control samples tested over 7 months period.
[b]Standard deviation.
[c]Percent coefficient of variation.

together with improved chromatography, has led to significant improvements in sample throughput. The assay was found to be precise, accurate, and suitable for the analysis of plasma sample collected during large scale clinical studies.

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