SUPPLEMENTAL MATERIALS

METHODOLOGICAL DETAILS

Technique of heel puncture

Heel stick is a routine blood sampling procedure in neonatal care units. To collect the blood into a capillary tube, the heel is pierced and then squeezed. The surface that has to be pierced is shown in **Figure S1**. Applying a heat pack to the skin increases the skin surface temperature, and the increase in the skin surface temperature causes proximal blood vessels to dilate. Vasodilatation usually reduces the squeezing pressure on the heel of neonates, because drawing blood becomes easier. Therefore before the puncture we always apply a heat pack to the heel: We put water at 40 °C in a thermal bag and apply the thermal bag against the puncture point for five minutes. The heel stick was performed immediately after removing the thermal bag with an automatic incision device (Gentleheel®). Both the left and the right heels were used.

Figure S1. Illustration of the region of heel stick (shaded) and Gentleheel$^{(R)}$ lancing device

Analyte Stability Testing

Stability tests have been performed for the standard plasma micafungin solutions, at concentrations of 5 µg / mL and 20 µg / mL. The standard solutions were stored at 4°C and analyzed using the High Performance Liquid Chromatography (HPLC) method for the dosage of

micafungin, at the following time intervals: Time 0; Time 2h; Time 5h; Time 8h; Time 12h; Time 24h. Results of the stability tests are listed in the **Table S1**.

Table S1. Results of the stability tests at two concentrations at + 4°C: the percentage decline in concentration over time is highlighted. A concentration drop of up to 10% from the initial concentration is considered acceptable.

Micafungin	
Solution 5µg/mL at +4°C	% Remaining
Time 0	100%
Time 2h	99.2%
Time 5h	96.3%
Time 8h	91.0%
Time 12h	79.4%
Tempo 24h	67.8%
Solution 20µg/mL at +4°C	% Remaining
Time 0	100%
Time 2h	98.8%
Time 5h	96.5%
Time 8h	90.9%
Time 12h	83.2%
Time 24h	64.5%

Stability tests were also performed for the aliquoted micafungin and anidulafungin (internal standard) stock solutions. Micafungin stock solution was prepared dissolving 10.68 mg of micafungin in 20 ml of a mixture of water and metanol (50:50) (v/v). Anidulafungin stock solution was prepared dissolving 20 mg of anidulafungin in 20 ml of a mixture of water and metanol (50:50). Results of the stability tests are listed in **Table S2**. For the analysis, micafungin, and anidulafungin as internal standard (20 mcg/ml), were extracted using protein precipitation and chromatographic separation on a reversed phase column. The effluents were

monitored at the two UV-wavelengths of 273 nm and 306 nm, which represent the absorption maxima of micafungin and anidulafungin.

Table S2. Results of the stability tests: the percentage decline in concentration over time is highlighted. A decrease in concentration of up to 15% from the initial concentration is considered acceptable.

Stock solution 0.5 mg/mL at -80°C	Micafungin %Remaining	Anidulafungin %Remaining
Time 0	100%	100%
Time 30 days	98,7%	98,9%
Time 60 days	98,5%	96,4%
Time 90 days	92,6%	90,3%
Time 365 days	90,5%	86,2%
Time 730 days	85,1%	74,5%

VALIDATION OF THE QUANTITATIVE HPLC-DAD METHOD FOR DETERMINATION OF PLASMA CONCETRATIONS OF MICAFUNGIN

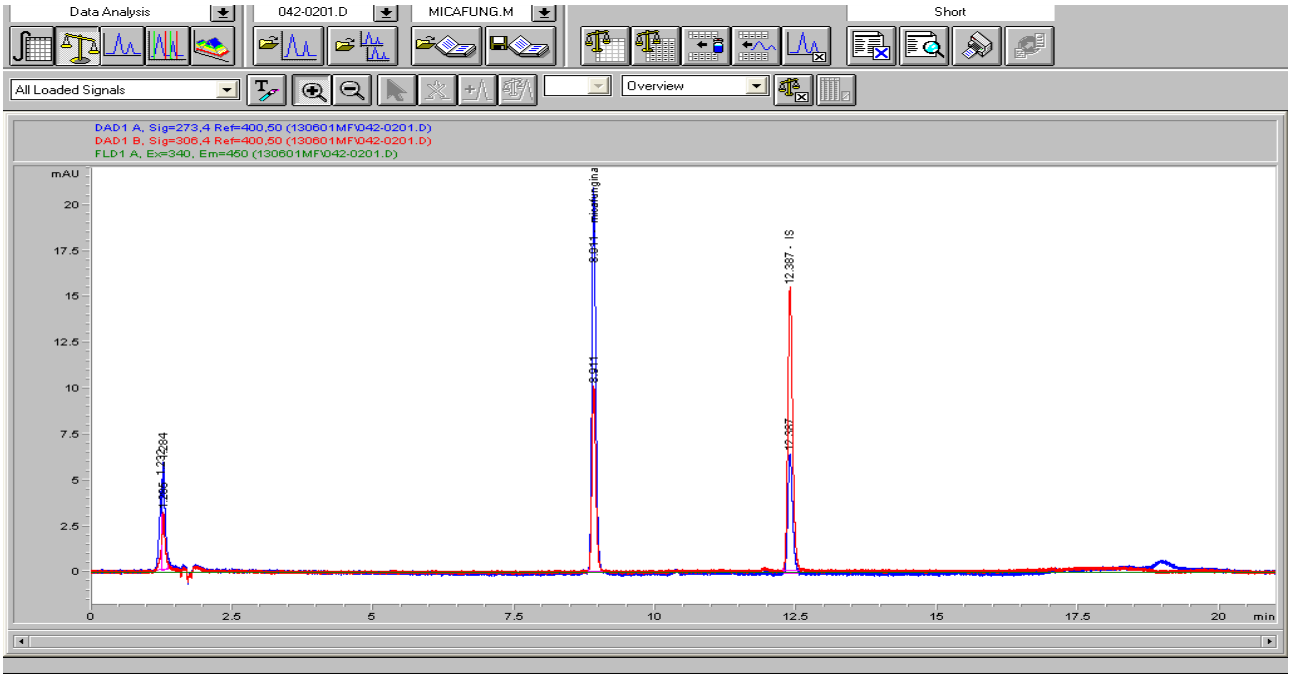
The quantitative HPLC-DAD method was validated to determine selectivity, calibration range and linearity, precision, accuracy, lower limit of detection (LLOD), lower limit of quantification (LLOQ), extraction recovery.

Selectivity

The selectivity of the method was evaluated by processing and analyzing six independent pools of drug-free plasma. Potential interferences from endogenous and environmental constituents were evaluated. The peak intensity of interfering compounds did not exceed 20% of LLOQ. The

method was found to have high selectivity (**Figure S2**) for micafungin, since no interfering peaks from endogenous or environmental compounds were detected at the expected retention time for micafungin (8.911 min) in any six independent drug-free plasma extract evaluated.

Figure S2. Representative chromatogram for micafungin in plasma sample.

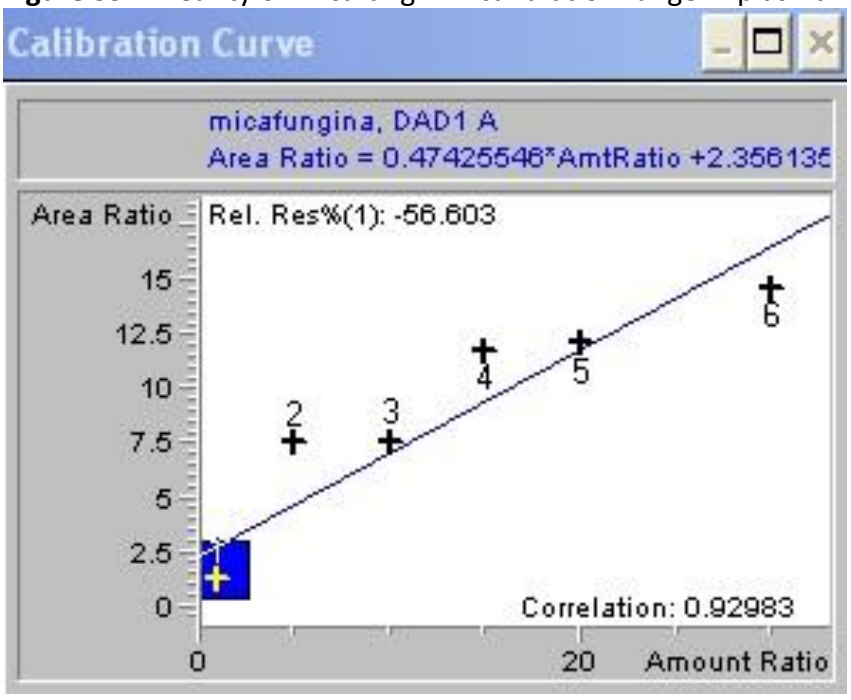


Calibration Curve

Six-point calibration curve of micafungin was constructed by plotting the peak-area ratio of micafungin to the internal standard (anidulafungin) vs the nominal concentration of micafungin using an equal weighted least-squares linear regression. The concentrations of the calibration standards of micafungin were then back-calculated and a relative error (relative error %: [(back-calculated concentration – nominal concentration)/nominal concentration] x 100) within +/-

15% was considered satisfactory (LLOQ within +/- 20%). Since back-calculated concentrations with a relative error of <15% of the nominal drug concentrations (+/-20% for the lowest point of the standard curve) were considered satisfactory, the calibration curve of micafungin consisted of six calibration standard. The linearity of calibration curve was determined by plotting the peak-are ratio of Micafungin to internal standard vs the nominal concentration of Micafungin. The calibration curve was obtained by weighted (1/x2) linear regression analysis. To evaluate the linearity of HPLC method plasma calibration curve was determinate in six curves on three separate days. The calibration curves for Micafungin in human plasma were linear over the range 1-30 µg/mL. The mean correlation coefficients was r^2=0.929.

Figure S3: Linearity of Micafungin in calibration range in plasma



Precision and Accuracy

In order to evaluate intra- assay precision, calibration standards low, medium and high were extracted and run ten times each on three consecutive day. To evaluate inter-assay precision,

calibration standards were extracted and run six time each on six consecutive days. Intra-assay precision was determined by calculating the coefficient of variation (CV%: [standard deviation/mean concentration]*100) of ten individual standards within one assay. Inter-assay precision was obtained by calculating the total CV% of all replicates of each standards.

To evaluate the accuracy of the method, spiked plasma at two concentration levels (QC low and QC high) were analyzed on three separate days (n=2 at each levels each days). Accuracy was estimated by the relative error (relative error %: [(back-calculated concentration – spiked concentration)/ spiked concentration] *100). Intra-day and inter-day mean back-calculated concentration, variation of calibration standards of Micafungin in plasma are listed in **Table S3**.

Table S3. Precision and accuracy data from quality control samples at two concentration levels. Acceptable inter-day precision has been confirmed by the QC samples that have demonstrated CVs below 15% in daily use.

Nominal Concentration (mg/L)	Intra-day (n=10)		Inter-day (n=10)	
	Mean +/- SD	CV (%)	Mean +/-SD	CV (%)
1	1.3 +/- 0.1	7.6	1.2 +/- 0.1	8.3
5			5.5 +/- 0.2	3.6
10	10.4 +/- 0.2	1.9	10.8 +/- 0.5	4.8
15			15.2 +/- 0.3	1.9
20			20.2 +/- 0.5	2.4
30	30.2 +/- 0.3	0.9	30.6 +/- 0.4	1.3

Limit of detection (LLOD) and limit of quantification (LLOQ)

LLOQ was determined based on the criterion that the analyte response at LLOQ is five times

baseline noise, CV% is <20% and relative error < +/-20%. LLOD of micafungin was defined by concentration with a signal-to-noise ratio of three and was determined analyzing the lowest calibration standard diluted 1:2. The LLOQ for micafungin was well below the recommended normal range. LLOQ of micafungin was 1 mg/L. LLOD of micafungin was found to be 0.5 mg/L.

Extraction recovery, matrix effect

Extraction recovery of calibration standards was determined by comparing the response of three extracted QC levels (QC low, medium and high) with that of direct injection of extracted blank plasma spiked with the same nominal concentrations of Micafungin as in the QC samples. To further investigate matrix effects, water was spiked with internal standard and micafungin at low, medium and high level of concentration. The percentage extraction recovery (mean +/- SD) of Micafungin from water spiked with 1 mg/L, 10 mg/L, 30 mg/L was 112 +/-3%, 107 +/-2% and 102 +/- 2% respectively.

Carry-over

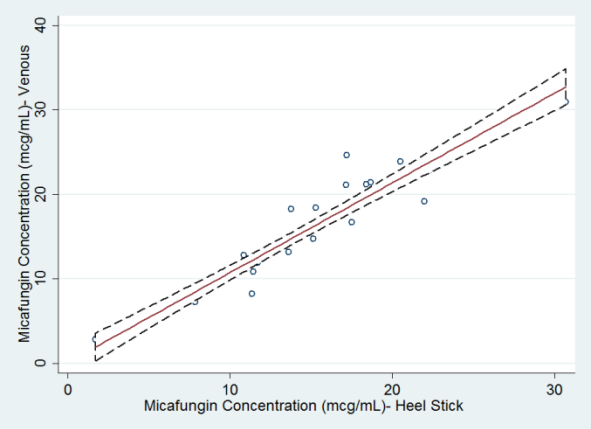
Tests for carry-over were performed by injecting a blank plasma sample after the highest concentration in the calibration curve. To rule out any carry-over effect, the peak area of micafungin in the blank sample had to be <20% of the peak area of the LLOQ sample.

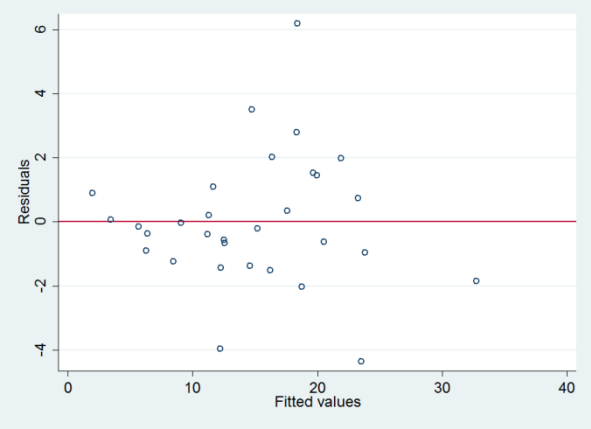
No carry –over was observed for any of the compounds based on the criterion that the signal observed in a blank injected directly after the highest calibrator is <20% of the signal of the lowest calibrator.

SUPPLEMENTAL MATERIALS

RESULTS

Figure S4. Scatter-linear (95% confidence interval) fit plot and residual versus fitted plot for the regression of micafungin concentrations by heel stick samples and venous samples






```
. regress micafungin_plasma micafungin_heel
```

Source	SS	df	MS	Number of obs	=	32
				F(1, 30)	=	328.79
Model	1389.65556	1	1389.65556	Prob > F	=	0.0000
Residual	126.798306	30	4.2266102	R-squared	=	0.9164
				Adj R-squared	=	0.9136
Total	1516.45386	31	48.9178665	Root MSE	=	2.0559

micafungin_pl~a	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
micafungin_heel	1.062223	.0585812	18.13	0.000	.9425841	1.181862
_cons	.1348393	.8900465	0.15	0.881	-1.682878	1.952557