Development of an HPLC Method for the Determination of Anidulafungin in Human Plasma and Saline

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Abstract

An ultraviolet high-performance liquid chromatography (HPLC) method was developed to analyze anidulafungin in human plasma and saline. A reversed-phase column was used with a UV detector set at 310 nm. The mobile phase consisted of methanol and ammonium phosphate buffer at a flow rate of 1 mL/min. Micafungin was used as the internal standard. Both standard curves were linear over a range of 1 to 10 µg/mL. The intra-assay relative standard deviations (RSD) for plasma and saline matrices were 1.60–1.81% and 1.96–3.70%, respectively. The inter-assay RSD for plasma and saline matrices were 2.41–7.25% and 1.31–3.16%, respectively. This method successfully recapitulated anidulafungin plasma concentrations previously analyzed by HPLC–tandem mass spectrometry with precision and accuracy of 6.9% and 1.59%, respectively.

Introduction

Anidulafungin (Eraxis®, Pfizer Inc., New York, NY) is a semi synthetic lipopetide (Figure 1) in the echinocandin class of antifungal drugs, which inhibit 1,3-β-D-glucan synthase, an enzyme essential to fungal cell wall synthesis. Anidulafungin is indicated for the treatment of esophageal candidiasis, candidemia, and other invasive *Candida* infections including intra-abdominal abscess and peritonitis (1). Like other echinocandins, anidulafungin displays potent in vitro activity against *Candida* and *Aspergillus* species, including azole resistant strains (2, 3). As a result of this microbiological activity, once daily dosing, and favorable safety profiles, echinocandins are now favored by the Infectious Diseases Society of America for patients with moderate to severe *Candida* infections (4).

Anidulafungin concentrations in human plasma have previously only been characterized by high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS–MS) (5). To date, no ultraviolet (UV) HPLC analytical methods for the quantification of anidulafungin in human plasma or saline have been reported. We modified an existing solid-phase HPLC assay derived for animal plasma to a liquid–liquid extraction (6). The purpose of this present study was to develop a simple, reproducible, and selective HPLC method for use with plasma and saline samples for application in human pharmacokinetic studies as well as product stability assessments.

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Experimental

Chemicals

Anidulafungin (Lot #0002) standard powder was provided by Pfizer Inc. (Groton, CT). Commercially available micafungin (Mycamine®, Astellas Pharma US, Deerfield, IL) for injection was obtained from the Department of Pharmacy at Hartford Hospital and used as the internal standard. Ammonium phosphate monobasic was purchased from Sigma (St. Louis, MO). HPLC-grade acetonitrile (Mallinckrodt Baker Inc., Phillipsburg NJ) and HPLC-grade methanol (Mallinckrodt) were used without further purification. Deionized water was obtained from a Milli-Q analytical deionization system (Bedford, MA).

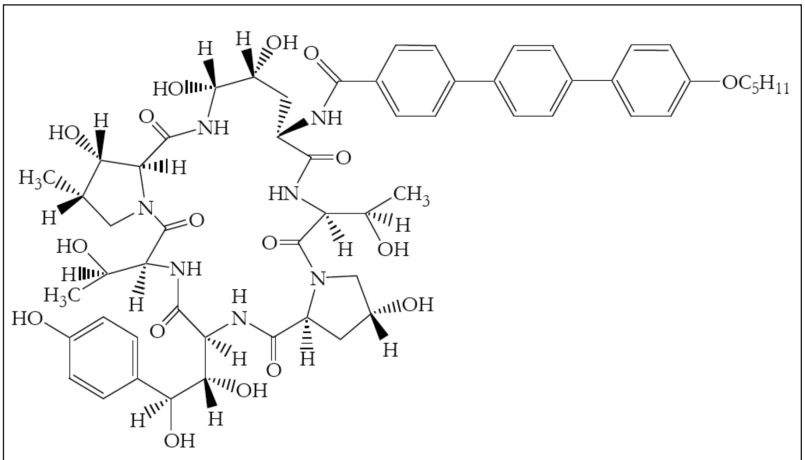

Figure 1. Chemical structure of anidulafungin.

Instrumentation and chromatographic conditions

An HPLC system consisting of a Waters 515 pump (Waters Associates, Milford, MA) and 717 plus autosampler (Waters) was equipped with a 5 µm Zorbax SB-C8 column (4.6 × 250 mm, Agilent Technologies, Santa Clara, CA) coupled to a Bondapak C18 Guard-pak precolumn (Waters). The autosampler was cooled to 10°C. The column was maintained at room temperature. A programmable UV detector set at 310 nm (Model 526; ESA Inc., Chelmsford, MA) was used to detect the analytes. EZChrom Elite chromatography data system (Scientific Software Inc., Pleasanton, CA) was used to quantify peak heights.

The mobile phase consisted of a mixture of 55% 0.005M ammonium phosphate buffer and 45% methanol. The flow rate was 1 mL/min. The running time for one sample was 13 min. All chromatographic procedures were performed at room temperature.

Standard solutions and controls

Anidulafungin standard powder was prepared in a volumetric flask using methanol to dissolve and dilute the stock standard. The internal standard, micafungin 20 µg/mL, was prepared in 0.9% sodium chloride according to manufacturer's instructions.

Drug free human plasma was purchased from Bioreclamation Inc. (Hicksville, NY). Anidulafungin was spiked into human plasma to make 7 standard solutions (1, 3, 4, 5, 7, 8, and 10 µg/mL) and three quality control samples (2, 6, and 9 µg/mL). Anidulafungin was spiked into 50% saline with 50% methanol to make 7 standard solutions (1, 3, 4, 6, 7, 8, and 10 µg/mL) and three quality controls (2, 5, and 9 µg/mL). Methanol was a necessary addition to the saline samples only to prevent loss of drug due to nonspecific binding. Aliquots of the standards and internal standard were stored at –80°C until analysis.

Sample extraction

A 100 µL sample of standard, quality control, or unknown sample along with a 25 µL aliquot of internal standard was pipetted into a polyethylene tube. The plasma samples were extracted with the addition of 350 µL of methanol. The sample was vortexed for 30 s and centrifuged for 10 min at 3600 rpm. The sample was placed into a WISP vial (Waters) for injection.

Assay validation

Calibration curves were generated by plotting the peak height ratio of anidulafungin to that of the internal standard. Weighted (1/concentration) least square regression analyses were applied to generate the linear regression equation. This equation was used to calculate the concentrations of the quality controls and unknown samples. Linearity of the standard curve was assessed with the correlation coefficient by plotting the peak height ratio of anidulafungin and the internal standard versus the theoretical concentrations.

The calibration curve consisted of seven standards. Three quality controls with low, middle, and high concentrations were used to evaluate precision and accuracy. Precision, expressed as the relative standard deviation (RSD), was calculated as the standard deviation for inter-day and intra-day runs divided by the average for those runs. Accuracy was determined by the relative error from the theoretical concentrations, calculated as the absolute value of 100 minus the average estimated concentration divided by the theoretical concentration. The lower limit of quantification (LLQ) for the assay was evaluated on five samples with 1 µg/mL of anidulafungin.

The room temperature, autosampler, and freeze-and-thaw stability of anidulafungin were determined in triplicate on each quality control sample. The stability of the internal standard was assessed at room temperature. Values of percent recoveries of anidulafungin were calculated by comparing the peak height ratio of anidulafungin and the internal standard in plasma to that of nonextracted aqueous solutions.

Comparison to HPLC–MS–MS

To confirm the reliability of this assay for plasma, samples from a human pharmacokinetic and bronchopulmonary penetration study previously analyzed by HPLC–MS–MS (7) were reanalyzed with this current method. In brief, 20 healthy adult volunteer participants were administered intravenous voriconazole and anidulafungin in combination daily until steady-state was reached. On the day of bronchoscopy, blood samples were collected from each participant at 0, 1.4, 2, 4, 8, 12, and 24 h after the last dose. Blood samples were centrifuged (1000 × g at 4°C for 10 min) immediately after collection. Separated plasma was stored at –80°C until analysis by HPLC–MS–MS as previously described and reported (7). One to two samples per volunteer (n = 35 total) representing the peak, and mid-point or trough concentrations were selected for reanalysis via our HPLC method. Accuracy and precision were calculated as described earlier.

Results and Discussion

Chromatography

While developing the assay for saline, several saline standard curves were prepared with various concentrations of methanol. It was observed that increasing the methanol concentration increased the peak height for both anidulafungin and micafungin. As a result, the concentration of methanol in each of the saline standards was kept consistent at 50%. Lower concentrations of methanol produced non linear standard curves.

Six sources of human plasma were tested for interference using the HPLC method described. Figure 2 represents a typical

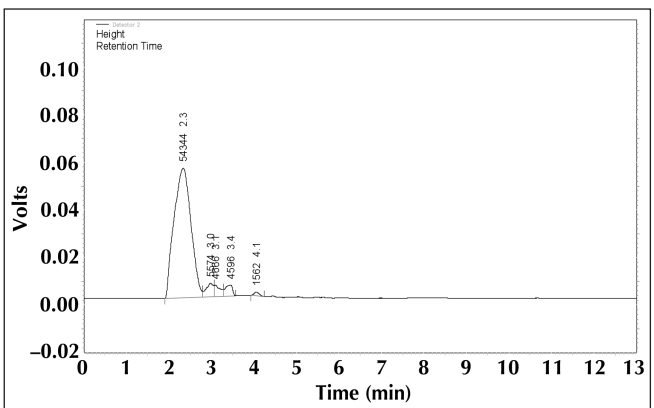

Figure 2. Chromatogram of blank human plasma.

chromatogram with blank human plasma. The chromatogram shows no interfering peaks with anidulafungin or the internal standard. The retention time of the internal standard and anidulafungin were 4.7 and 10.1 min, respectively.

Linearity, precision, and accuracy

Linearity, as demonstrated by the correlation coefficient (r) for each calibration curves, was ≥ 0.995 for both plasma (n = 7) and saline (n = 6) assays. The plasma slope was 0.3230 ± 3.67% (mean ± SD) and the intercept was 0.0078 ± 3.95% (mean ± SD). The saline slope was 0.2628 ± 3.96% (mean ± SD) and the intercept was 0.1064 ± 8.1% (mean ± SD). Summary results for the inter- and intra-day precision and accuracy in both the human and saline matrixes are provided in Tables I and II.

Lower limit of quantification

The LLQ of 1 µg/mL anidulafungin was chosen as the concentration for the lowest standard sample in line with concentrations typically observed in humans (Figure 3). The precision and accuracy of LLQ (n = 5) was 3.02% and 6.70%, respectively.

Table I. Precision and Accuracy of Anidulafungin in Human Plasma*

	Theoretical concentration (µg/mL)		
	Low (2)	Medium (6)	High (9)
Inter-run			
Mean	1.86	5.86	8.44
SD	0.033	0.237	0.124
RSD (%)	1.79	4.05	1.47
Relative error (%)	7.25	2.41	6.19
Intra-run			
Mean	1.86	5.97	8.54
SD	0.033	0.96	0.155
RSD (%)	1.79	1.60	1.81
Relative error (%)	6.85	0.50	5.15

* SD = standard deviation; RSD = relative standard deviation (i.e., precision); relative error = accuracy.

Table II. Precision and Accuracy of Anidulafungin in Saline*

	Theoretical concentration (µg/mL)		
	Low (2)	Medium (5)	High (9)
Inter-run			
Mean	1.96	5.16	8.88
SD	0.111	0.297	0.552
RSD (%)	5.67	5.75	6.21
Relative error (%)	2.16	3.16	1.31
Intra-run			
Mean	2.04	5.30	8.35
SD	0.075	0.169	0.163
RSD (%)	3.70	3.19	1.96
Relative error (%)	1.86	5.94	7.27

* SD = standard deviation; RSD = relative standard deviation (i.e., precision); relative error = accuracy

Recovery

Recovery experiments were performed in triplicate by comparing the analytical results for extracted plasma samples at the quality control concentrations with unextracted controls that represent 100% recovery. The recovery of the 2, 6, and 9 µg/mL samples was 88.8% ± 4.7%, 86.0% ± 1.1%, and 84.2% ± 0.4%, respectively.

Stability

Anidulafungin saline and plasma quality controls were stable at room temperature at 22°C for 6 h with ≤ 10% degradation. Micafungin internal standard solution was stable at room temperature at 22°C for 6 h with ≤ 2% degradation. The freeze-and-thaw stability was performed by completely thawing the quality controls at room temperature and refreezing at –80°C for 24 h. The freeze-and-thaw cycles were repeated two additional times and then analyzed on the third cycle. Anidulafungin saline and plasma quality controls were stable for three freeze-and-thaw cycles with < 10% degradation. The saline and plasma quality control samples after extraction in the 10°C autosampler were stable for 24 h with ≤ 10% degradation.

Comparison to HPLC–MS–MS

A total of 35 samples were assayed with a concentration ranging from 2.40 to 7.89 µg/mL. Average recovery of these samples was 101.6% ± 7.0%. Precision and accuracy were 6.90% and 1.59%, respectively.

Conclusions

Herein, we developed an efficient and reliable HPLC method to determine anidulafungin concentrations in human plasma and saline, as might be required for human pharmacokinetic studies and studies of in vitro stability. Precision and accuracy of the QC samples were with in acceptable limits. The acceptance criteria in our laboratory are taken from the FDA guidelines (8). This UV method of detection accurately replicated results

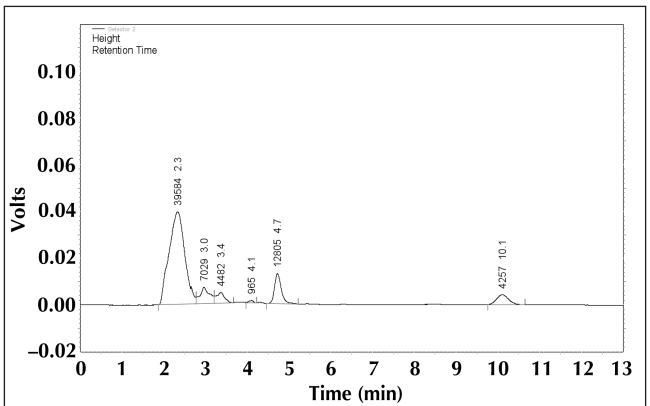

Figure 3. Chromatogram of LLQ in human plasma. The retention time of the internal standard and anidulafungin were 4.7 and 10.1 min, respectively.

obtained by HPLC–MS–MS. This assay has greater accessibility for many laboratories and provides a cost effective alternative to HPLC–MS–MS.

Conflict of interest statement

The authors do not have a commercial or other association that might pose a conflict of interest. All authors are employees of the Center for Anti-Infective Research and Development, Hartford Hospital (Hartford, CT).

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