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**RESEARCH ARTICLE**

# Development and Validation of a Rapid UV-HPLC Method for the Determination of Anidulafungin in Perfusion Solution

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**Abstract:** ***Background:*** Anidulafungin is an antifungal agent. The development of determination techniques can be a useful tool to realize stability and quality control studies.

***Methods:*** The determination was performed on an analytical Mediterranea SEA18 (15x0.4 cm, 5 µm) $C_{18}$ column at 35 ºC. The selected wavelength was 304 nm. The mobile phase was a mixture of 0.037 M sodium dihydrogen phosphate buffer, acetonitrile and methanol (40:50:10, v/v/v) at a flow rate of 2.0 mL $min^{-1}$. Dasatinib (12.5 µg $mL^{-1}$) was used as internal standard.

***Results:*** The assay enables the measurement of anidulafungin with a linear calibration curve ($r^2$= 0.999) over the concentration range 15–300 µg $mL^{-1}$. Accuracy, intra-day repeatability ($n$ = 5), and inter-day precision ($n$ = 3) were found to be satisfactory, being the accuracy 5.8% and precisions were intra-day and inter-day, 0.6% and 4.2%, respectively.

***Conclusions:*** A rapid, simple and sensitive high-performance liquid chromatography (HPLC) method with ultra violet detection has been developed for quantification of anidulafungin in perfusion solution. The retention time was clearly minor than the previous published HPLC determination methods of anidulafungin.

**Keywords:** Anidulafungin, antifungal agents, echinocandins, HPLC, validation studies.

## 1. INTRODUCTION

Anidulafungin is a new antifungal drug pertaining to the echinocandins class [1, 2]. Anidulafungin is a semi-synthetic echinocandin, a lipopeptide obtained from a fermentation product of Aspergilus nidulans [3]. This molecule exhibits its antifungal activity by inhibiting 1,3-β-D glucan synthase, an enzyme present in the fungal cells, and thereby the formation of the 1,3-β-D glucan, an essential component of the fungal cell wall, is inhibited.

Anidulafungin was approved by Food and Drugs Administration (FDA) in 2006, for the treatment of the fungal infection as invasive candidiasis [4], being this disease the most frequent among fungal infections. Also, this drug is also used the treatment of candidemia [5], esophageal candidiasis [6] or aspergillosis [7].

Anidulafungin is marketed as concentrated powder for reconstitution in vials of 100 mg and in the handling procedure it has to be reconstituted with 30 ml of water for injection. This reconstituted solution has to be solved in 100 ml of normal saline or 5% dextrose for infusion that is used in patients via parenteral. Actual recommended dose of anidulafungin is a fixed loading dose of 200 mg intravenous drug on the first treatment day, followed by 100 mg daily thereafter [8]. This drug is generally well tolerated with a low rate of adverse events in patients and also, studies evaluating the use of anidulafungin in combination with other commonly used drugs have not shown any significant drug-drug interactions [9].

The described chemical stability in the data sheet of anidulafungin [10] is 24 hours for the reconstituted solution with water at 25 ºC. In case of the perfusion solution the stability is two days when is stored at 25 ºC and three days when the storage temperature is -20 ºC. The absence of more stability studies is an important risk factor limiting the use of this drug.

For stability studies of the drug analytical methods including quantification of different molecules are necessary. . Quantification of anidulafungin can be done by using liquid chromatography tandem mass (LC-MS/MS) [11-16] or high

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performance liquid chromatography (HPLC) [8, 12, 17-22]. Comparing both methods [12] Martens-Lobenhoffer *et al.* have concluded that LC-MS/MS served only as identity confirmation of High Performance Liquid Chromatography Ultraviolet Detection (HPLC-UV) peaks. This is an important fact because HPLC is more common in most laboratories and it is less expensive than LC-MS/MS.

Martens-Lobenhoffer [12] employed an HPLC-UV system equipped with a Zorbax Eclipse XDB-C18 column (150 × 2.1 mm with 3.5 µm particle size) a gradient method consisted of 0.1% (w/w) ammonium acetate in water (with pH adjusted to 7.0) and acetonitrile. Under these conditions, anidulafungin has a retention time of 11.91 minutes and precision and accuracy were found to be 8.36 and 12.57, respectively. Tobudic and coworkers [19] have also used a gradient method to compare the anidulafungin stability in two peritoneal dialysis fluids. The HPLC-UV system was equipped with a Kinetex C18 column (2.6 µm core shell particles, 100 × 2.1 mm) and the mobile phase consisted of aqueous formic acid (1 mL $L^{-1}$, pH 3.0) and acetonitrile (10% to 60% ramp, 12 min) and the obtained value for precision was 0.56%, while the accuracy value was 0.79%.

Lohita *et al*. [17] described a validated isocratic method for the quantification of anidulafungin in bulk samples and parenteral dosage with a photodiode array detector (PDA). Employing a mobile phase of acetonitrile: water: 0.1% v/v trifluoroacetic acid (48:52:1) with pH 4.7 and using a column YMC ODS Pack AQ C18 (150 x 4.6 mm; 3 µm) the retention time was 8.86 minutes and the precision was lower i.e. 2% and the accuracy was 0.96%. Furthermore, these researchers have done some degradation studies in different stress conditions. Also, Sutherland *et al.* [18] employed an HPLC-UV system equipped with a 5 µm Zorbax SB-C8 column (250 x 4.6 mm) and used a mixture of 0.005M ammonium phosphate buffer: methanol (55:45) as a mobile phase. Under these conditions, anidulafungin has a retention time of 10.1 minutes and the values of precision and accuracy were 6.21% and 7.27%, respectively.

The previous obtained retention times [12, 17, 18] could be too high to be implanted in the routine clinical determination of anidulafungin concentration and to carry out with stability studies of this drug in perfusion solution.

The aim of the new determination method would be to enhance the cost-effectiveness of the quantification of this drug by improving accuracy and precision, and reducing the analysis time and the cost of the determination. Also, another reason to develop a validated method of anidulafungin determination is the recently published study considering the importance of adjusting the dose of this drug in obese subjects [20] and mainly, the development of a new chromatographic method that will help in designing studies to deeply know the stability of anidulafungin in perfusion solution because saving a resource in the pharmacy service of the health care will be useful.

## 2. MATERIALS

Anidulafungin (Lot #0009) was provided by Pfizer (New York, USA). Ecalta® (Lot #N31537) was acquired as commercial sample (Pfizer, Kent, UK). Excipients of Ecalta®, tartaric acid, fructose and polysorbate 80 were acquired from commercial samples from Sigma–Aldrich Química (Madrid, Spain). Dasatinib (Lot #BDS-107) was used as internal standard (IS) and it was acquired from LC Laboratories (Woburn, USA). HPLC-grade acetonitrile and water were obtained from Teknokroma (Barcelona, Spain) and HPLC-grade methanol was obtained from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) was acquired from Acofarma (Terrassa, Spain). Potassium dihydrogen phosphate was obtained from Panreac (Barcelona, Spain).

## 3. EXPERIMENTAL

### 3.1. Chromatographic Conditions

The equipment used was an HPLC system (Shimadzu, Kyoto, Japan) consisted of a quaternary pump (model LC-20AD), degasser (model DGU-20AS), autosampler (model SIL-20AC), thermostated column compartment (model CTO-10AS), and a UV-visible detector (model SPD-M20A). Data were acquired and processed with LCsolution® software from Shimadzu Corporation (Kyoto, Japan). Separation of the compounds was achieved by using a Mediterranean SEA18 $C_{18}$ column (5 µm; 150 x 4 mm) with a guard column packed with the same bonded phase Ultraguard SEA18 (10 x 3.2 mm). The chromatography separation was carried out by using a mobile phase [consisting of a mixture of potassium dihydrogen phosphate buffer solution (pH 4.3; 0.037 M), acetonitrile and methanol] in 40:50:10, v/v/v proportion, pumped at a constant flow rate of 2.0 mL $min^{-1}$. The column was maintained at 35 ºC, the injection volume was 10 µL and the eluents were monitored at a wavelength of 304 nm.

### 3.2. Preparation of Stock Solutions and Standards Solutions

#### *3.2.1. Stock and Working Solutions*

Stock solutions containing 10000 µg $mL^{-1}$ of anidulafungin and 5000 µg $mL^{-1}$ of dasatinib were prepared in DMSO and were stored for less than one month at 4 ºC in the dark. Each day, fresh anidulafungin working solutions were prepared by diluting the stock solutions with methanol to obtain concentrations of 200 and 2000 µg $mL^{-1}$. Likewise, dasatinib working solutions were further diluted with methanol to a final concentration of 500 µg $mL^{-1}$.

#### *3.2.2. Standard Solutions*

In order to validate the analytical method, calibrators and quality control (QC) samples were prepared. Calibrators were mobile phase samples containing known concentrations of anidulafungin. These calibrators were used to construct a calibration curve consisting of a blank sample (matrix sample processed without IS), a zero sample (matrix sample processed with IS), and 8 nonzero samples covering the expected range, including the lower limit of quantification (LLOQ). Anidulafungin calibrators were prepared by diluting working solutions with mobile phase each day to obtain concentrations of 15, 50, 70, 100, 150, 200, 250 and 300 µg $mL^{-1}$. QC samples were three of known concentrations of anidulafungin in mobile phase. These controls spanned the calibration curve, encompassing concentrations at 50, 100 and 250 µg $mL^{-1}$.

### 3.3. Validation of the Analytical Method

The method validation procedure was carried out following the guidelines for Bioanalytical Method Validation published by the FDA [23] and the European Medicines Agency (EMA) [24].

#### *3.3.1. Specificity and Selectivity*

The specificity of the method was evaluated with regard to interference, by presence of any other excipient, with the detection of the analyte and/or the IS. Six different mobile phases were prepared to evaluate the interference potential. Zero sample was analyzed to check the absence of interference with the analyte. The interference peak should be less than 5% of the peak area for both analyte and IS.

#### *3.3.2. Linearity*

Complete calibration curves (8 concentrations; 15, 50, 70, 100, 150, 200, 250 and 300 μg $mL^{-1}$) were analysed on three separate days. Calibrations curves were generated by plotting the peak height ratio of anidulafungin to that of the IS. 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 QCs.

Calibration parameters are obtained from the equation $y= a +b*C;$ where $a$ is the y-intercept of the line; $b$ is the slope of the line; $C$ is the anidulafungin concentration (μg $mL^{-1}$) and $r$ is the correlation coefficient of the calibration curve. Calibration range linearity from 15 to 300 μg $mL^{-1}$ was concluded if $r$ was greater than 0.99 for all calibration curves.

#### *3.3.3. Limit of Detection and Lower Limit of Quantification*

The assay sensitivity was evaluated by determining the limit of detection (LOD) and the LLOQ. LOD was defined as anidulafungin concentration required to give a signal equal to the blank plus 3 times standard deviation of the blank (S/N = 3), whereas LLOQ was the lowest anidulafungin concentration required to give a signal equal to the blank plus 10 times standard deviation of the blank (S/N = 10), and acceptable accuracy and precision data.

#### *3.3.4. Precision and Accuracy*

Accuracy and precision were assessed by determining anidulafungin concentration at LLOQ and in QC samples at 50, 100 and 250 μg $mL^{-1}$, and measuring five replicates per concentration on three different days. Precision, expressed as the relative standard deviation (RSD), was calculated as the standard deviation for intra-day and inter-day runs divided by the average for those runs. Accuracy was determined by the mean relative error (MRE) from the theoretical concentrations, calculated as the absolute value of 100 minus the average estimated concentration divided by theoretical concentration. For each concentration, both RSD and MRE should be lower than 15% except for LLOQ, where they should not deviate by more than 20%.

#### *3.3.5. Stability*

Anidulafungin and dasatinib stabilities in QC samples at 50, 100 and 250 μg $mL^{-1}$ were evaluated in autosampler at room temperature for 2, 4, 6 and 24 hours. It was selected 24 hours because it is the maximum time that samples could remain in the autosampler to be processed.

### 3.4. Applicability of Analytical Method

This assay was used to quantify the anidulafungin concentration of three Ecalta® solutions in three different days. Each vial of Ecalta 100 mg® is reconstituted under aseptic conditions with 30 ml of water for injection to achieve a concentration of 3330 μg $mL^{-1}$. The reconstitution time was 5 minutes. Each day, as from the anidulafungin solution 3330 μg $mL^{-1}$, another three solutions were prepared in mobile phase at concentrations of 266, 166 and 67 μg $mL^{-1}$. Internal standard dasatinib was also added to achieve a final concentration of 12.5 μg $mL^{-1}$. Each solution was analyzed by quintupled. For assessing the applicability of the method precision and accuracy intra-day and inter-day of each of the dilutions prepared, have been obtained.

## 4. RESULTS

### 4.1. Validation Method

#### *4.1.1. Specificity and Selectivity*

Chromatograms obtained with blank sample, QC of anidulafungin (50 μg $mL^{-1}$) and IS, and diluted Ecalta® sample (166 μg $mL^{-1}$) spiked with IS are presented to corroborate the absence of interferences at retention time of analyte and IS (Fig. **1**). Retention times for anidulafungin and dasatinib were 3.7 and 0.9 minutes, respectively. The assay was found to be specific and selective because no interference was observed with other excipients of Ecalta® presentation like tartaric acid, fructose and polysorbate 80 (Fig. **1**). The rest of the Ecalta® excipients sodium hydroxide and hydrochloric acid do not produce signal in UV spectra and only mannitol can produce it after a derivatization procedure [25].

In this study, the potential matrix effect was evaluated by six different mobile phases samples and with a zero sample. Also, two different columns were tested. The best separation mechanism was obtained with the column Mediterranean SEA18 (15x0.4, 5 μm) $C_{18}$ column. Also, five wavelengths were used to obtain the best peak shape and LOD. Best conditions were obtained with 304 nm. Finally, the best conditions were achieved with a mobile phase being a mixture of 0.037 M sodium dihydrogen phosphate buffer, acetonitrile and methanol (40:50:10, v/v/v) at a flow rate of 2.0 ml $min^{-1}$. The interference peak should be less than 5% of the peak area for both analyte and IS. Based on this, it can be concluded that the matrix did not appear to interfere significantly with the integrity of our analytical method.

#### *4.1.2. Linearity*

For linearity assessment, the Levene statistic test showed a significant difference ($p<0,05$) between the variance increased more than proportionally to the concentration, the best weighting factor was 1/(peak area ratio) [26]. Calibration curve parameters of the first day were $a$ = 0.0436 and $b$ = 0.53 with an $r$ value of 0.998. Second day parameters were $a$ = 43.6 and $b$ = -3777 with an $r$ value of 0.999 and finally, third day parameters were $a$ = 48.9 and $b$ = 0.0035 with an $r$ value of 0.999.

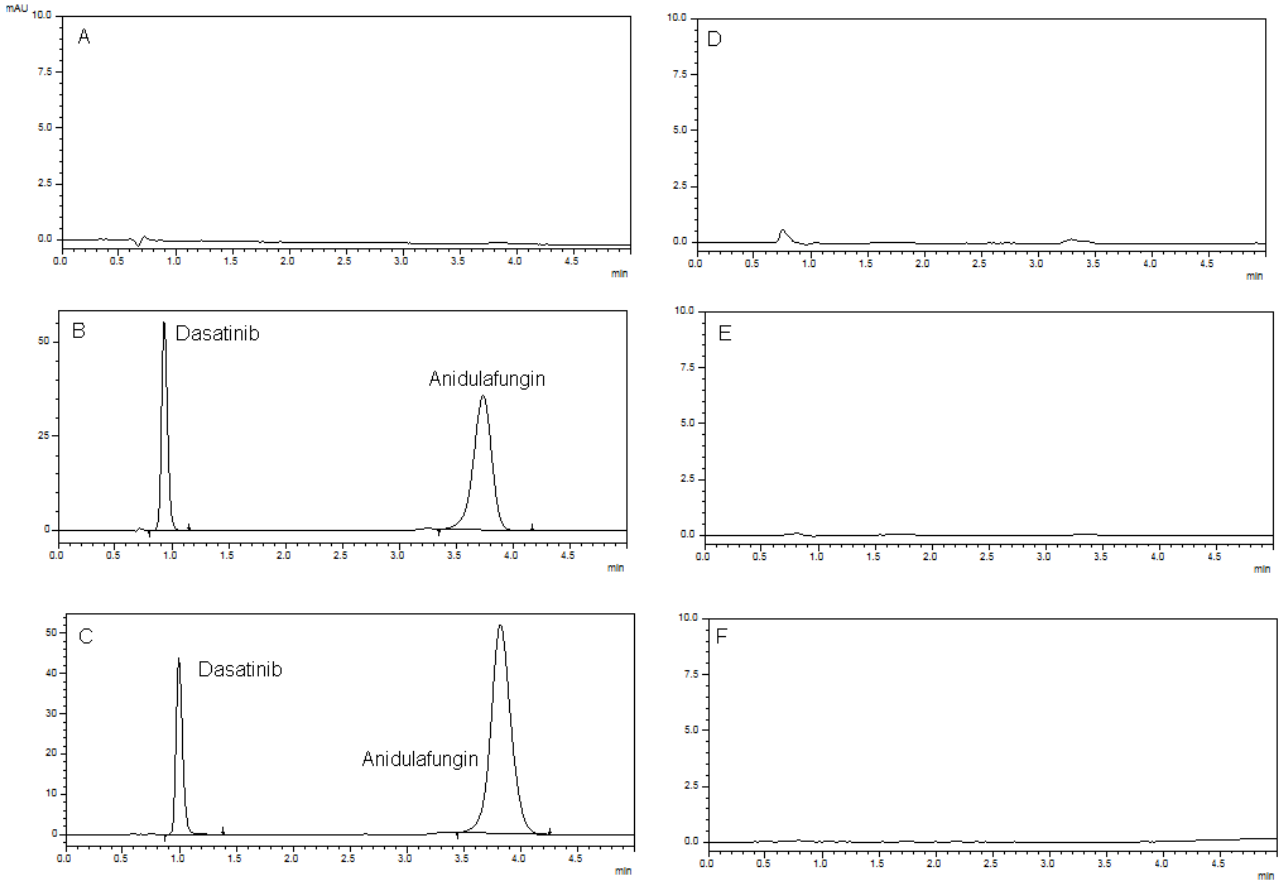


**Fig. (1).** Representative chromatograms: (**A**) blank sample, (**B**) anidulafungin QC (50 µg $mL^{-1}$), (**C**) Ecalta® sample (166 µg $mL^{-1}$), (**D**) blank sample after addition of tartaric acid, (**E**) blank sample after addition of fructose and (**F**) blank sample after addition of polysorbate 80.

#### *4.1.3. Limit of Detection and Lower Limit of Quantification*

The LLOQ was the lowest concentration of de calibration curves (15 µg $mL^{-1}$), and the LOD was determined to be 0.15 µg $mL^{-1}$ of anidulafungin. In addition, 100% of the evaluated samples for the LLOQ showed that MRE and RSD values were lower than 10.1% (Tables **1a** and **1b**). For all other concentrations tested, all samples showed a deviation from the nominal value of less than 15% (data no shown). These data confirm that the assay meets the acceptance criteria in relation to the linearity specified by FDA [23] and EMA [24].

#### *4.1.4. Precision and Accuracy*

In Table **2**, intra-day and inter-day precision and accuracy data are summarized. For 3 QC (50, 100 and 250 µg $mL^{-1}$), intra-day and inter-day accuracy ranged from -4.0 to 5.8 and from -0.7 to 0.7, respectively. Precision for the intra-day and inter-day results was lower than 0.6 and 4.2, respectively.

These values of precision and accuracy showed that the assay is reliable and reproducible relative to the requirements of regulatory agencies [23, 24].

#### *4.1.5. Stability*

The stability of anidulafungin and dasatinib was assessed with respect to requirements of therapeutics drug monitoring. These analytes were stable in mobile phase up to 24 hours in autosampler without any significant degradation. The nominal concentration values in each of the QC were between 97% and 99% of the theoretical value of concentration. In this study, the stability of anidulafungin and dasatinib in DMSO and methanol has not been evaluated because it was already described in a previous work [18, 27-29].

### 4.2. Applicability of Analytical Method

The here-described method has been used to quantify the concentration of anidulafungin in three Ecalta® presentation reconstituted in water for administration to the patient. In Fig. (**1a**), chromatogram of a sample of Ecalta® is displayed. Each sample was inspected visually to check for precipitation or color change and then the measurement was done by HPLC.

As of the observed concentrations in each of the dilutions, and multiplying by the dilution factor applied it was obtained anidulafungin concentration in the Ecalta® solution for each day of study. The concentration of Ecalta® determined on the first day was 3252±128 µg $mL^{-1}$, on the second day was 3177±17 µg $mL^{-1}$ and on the third day 3189±123 µg $mL^{-1}$ with RSD (%) of 3.9, 0.5 and 3.8, respectively

## 5. DISCUSSION

This assay was to develop and validate the measurement of anidulafungin and was successfully used in a routine practice to quantify the drug concentration in the solutions prepared for patient administration.

**Table 1a. Intra-day precision and accuracy.**

| Theoretical concentration ($\mu g\ mL^{-1}$) | Intra-day precision and accuracy | | |
|---|---|---|---|
| | Mean observed concentration (SD) ($\mu g\ mL^{-1}$)* | Precision (RSD, %) | Accuracy (MRE, %) |
| 15 | 15.9 (0.1) | 0.5 | -5.8 |
| 50 | 52.0 (0.3) | 0.6 | -4.0 |
| 100 | 94.2 (0.2) | 0.2 | 5.8 |
| 250 | 252.5 (1.2) | 0.5 | -1.0 |

*Results expressed as mean (SD) from 5 replicates.

**Table 1b. Inter-day precision and accuracy.**

| Theoretical concentration ($\mu g\ mL^{-1}$) | Inter-day precision and accuracy | | |
|---|---|---|---|
| | Mean observed concentration (SD) ($\mu g\ mL^{-1}$)* | Precision (RSD, %) | Accuracy (MRE, %) |
| 15 | 14.4 (1.1) | 7.7 | 4.1 |
| 50 | 50.3 (1.3) | 2.7 | -0.7 |
| 100 | 99.4 (4.1) | 4.2 | 0.6 |
| 250 | 248.4 (3.4) | 1.4 | 0.7 |

*Results expressed as mean (SD) from 5 replicates.

**Table 2. Intra- and inter-day precision and accuracy of each of the dilutions prepared.**

| Theoretical concentration ($\mu g\ mL^{-1}$) | Intra-day precision | | Inter-day precision | |
|---|---|---|---|---|
| | Mean observed concentration (SD) ($\mu g\ mL^{-1}$)* | Precision (RSD, %) | Mean observed concentration (SD) ($\mu g\ mL^{-1}$)* | Precision (RSD, %) |
| 67 | 61 (0.2) | 0.4 | 64 (2.8) | 4.3 |
| 166 | 158 (0.4) | 0.2 | 161 (3.5) | 2.2 |
| 266 | 255 (1.5) | 0.6 | 254 (1.3) | 0.5 |

*Results expressed as mean (SD) from 5 replicates.

During the assay optimization phase, different stationary and mobile phase compositions, such as the mixture of potassium dihydrogen phosphate buffer or ammonium acetate buffer and acetonitrile and methanol, were evaluated for their capability to separate anidulafungin and dasatinib. The mixture of potassium dihydrogen phosphate buffer solution (pH 4.3; 0.037 M), acetonitrile and methanol in 40:50:10, v/v/v proportion, produced optimal separation with an excellent retention time and very sharp and symmetrical peak shapes for both anidulafungin and dasatinib. This contrasts with other studies that suggest that an acid pH of the mobile phase partially de-ionized molecule anidulafungin increase retention times and distort the peak resolution [16]. Total run time for each sample analysis was 5.0 minutes, which was similar to a previous study [13] and about 33% lower than in other published trials [17, 18, 21].

The choice of the IS was a critical aspect of the method development because it influences both accuracy and precision that are particularly important aspects in HPLC-UV analytical methods. Dasatinib is a molecule tyrosine kinase inhibitor which is readily measurable signal by UV detection. Furthermore, at these analysis conditions, dasatinib has a short retention time which does not interfere with the analyte. Also, the cost of dasatinib was three times less than the cost of the previously used IS (micafungin) in anidulafungin determination [12, 18].

Analyte concentration was selected on the basis of the order of magnitude of the analyte signal for the purpose to minimize the error in calculating ratios responses.

During the process of the assay development and validation, it is necessary to ensure the stability of the solutions.

This study has shown that samples of anidulafungin and dasatinib in mobile phase preserved in the autosampler are stable for at least 24 hours. However, the stability study of the analyte and IS in stock and working solutions was not carried out because this study had already been done by other authors. In this regard, the preservative properties of DMSO in anidulafungin solutions were described [27]. Stability of the methanol working solutions of anidulafungin was studied by Lohita *et al.* [17] which showed a stability of at least 24 hours. Also, a three-month stability of dasatinib (IS) solutions in DMSO stored between 2 and 8 ºC was reported by van Erp *et al*. [29]. Stability of the methanol working solution of dasatinib at room temperature was studied by Furlong *et al*. [29].

Linearity, precision and accuracy of this newest method fall in appropriate ranges given in the FDA and EMA Guidelines [23, 24] to assure the quality of the determination of anidulafungin. The LLOQ was higher than previously reported with an HPLC-UV method [12, 18, 21]. However, the assay LLOQ (15 $\mu g\ mL^{-1}$) is deemed adequate to monitor anidulafungin concentration in quality controls of dosage form and other analysis as stability of drug solutions.

Compared with Martens-Lobenhoffer *et al.* [12] and Sutherland *et al.* [18] with accuracies values of 10.44% and 7.27%, respectively, the obtained value with our method is lower (5.8%). The accuracy by Tobudic *et al.* [19] is not going to be considered here because the experimental procedure is not clearly described, not even the retention time. Lohita *et al*. [17] exposed an accuracy of 1%, but the authors do not use the internal standard in this work, as recommended.

In case of intra-run precision, Sutherland *et al*. [18] have a value of 3.70 and for inter-run a value of 6.21%. The intra-run precision for Martens-Lobenhoffer *et al.* [12] is 4.3% and for inter-run is 8.36%. In both cases for our method the obtained values are lower being 0.6% and 4.2% for intra- and inter-run precisions, respectively. Only Lohita *et al* [17] exposed a lower precision values, defending precisions lower than 2%.

Until now, all previously published analytical methods have been validated for the quantification of anidulafungin in human plasma. The present study has shown the validation of a rapid analytical method for quantification of anidulafungin in the drug solution through HPLC-UV. This method is easily implantable in routine practice for quality control due to its short run time of five minutes. Also, this method can be used to carry out temporal stability and degradation studies of anidulafungin in perfusion solution.

## CONCLUSION

In conclusion, the HPLC-UV method to determine anidulafungin described here is sensitive, selective, reproducible and rapid with lower retention time, and can be used easily for stability and degradation studies of anidulafungin. The fact that it is a quick method - using UV detection - makes it possible to easily implement it in a routine practice and allows studies of stability in a short time of period.

## CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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