Determination of antifungal caspofungin in RPMI-1640 cell culture medium by column-switching HPLC-FLD

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Abstract

The actual scenario in the fight against fungal infections forces researchers to carry through with resistance studies to improve the therapies. These studies, which are performed in cell culture media, need accurate and sensitive analytical methodologies. That is why, in this work, an analytical method for caspofungin (CSF) concentration determination in RPMI-1640 cell culture medium with on-line sample treatment was developed and validated. CSF concentration was determined by HPLC-FLD using a column-switching procedure. The chromatographic analysis was carried out in less than 10 min using a C8 column (4 × 4 mm, 5 µm) as extraction stationary phase and a HSS T3 column (4.6 × 100 mm, 5µm) as the analytical column. The used mobile phases were mixtures of phase A: pH 2 (adjusted with TFA) aqueous phase and phase B: ACN. For the extraction, the composition was (95:5, A:B v/v) and for the analysis (60:40, A:B v/v), both done in isocratic elution mode. These chromatographic conditions allowed reaching a limit of quantification of 10 µg/L, using 100 µL of sample with an injected volume of 40 µL. The proposed method was successfully validated in terms of selectivity, carryover, linear concentration range, accuracy and precision according to the criteria established by the Food and Drug Administration. Available amount of CSF in RPMI-1640 solution was found critical. CSF concentrations remained stable up to 2 h at room temperature. The developed method was applied for the direct analysis of CSF concentrations from in vitro experiments in presence of *C. glabrata* (CAGL18). The results highlight the decrease of cell proliferation even if the CSF amount decreases too, which asks question about the real value of the efficient concentration for CSF antifungal activity.

Keywords: caspofungin, echinocandin, cell culture, *Candida* spp., HPLC, column-switching

1 Introduction

Since the last decades, the incidence of fungal diseases has drastically increased. Severity of fungal diseases range from mild mucocutaneous infections to potentially life-threatening invasive infections. Serious invasive fungal diseases occurs often as a consequence of underlying health problems such as AIDS, cancer, organ transplantation and corticosteroid therapies (1).

Candidiasis is one of the most frequent invasive fungal infection and an important cause of morbidity and mortality worldwide (2). Among the 200 species described for *Candida* about 15 can cause human diseases and 5 of them are more frequent. In a retrospective study of candidemia at Nantes Hospital between 2004 and 2010, *C. albicans* was the predominant species (51.8%) followed by *C. parapsilosis* (14.5%), *C. glabrata* (9.8*%), C. tropicalis* (9.8%) and *C. krusei* (4.1%) (3). Candidiasis are under growing interest due to the increase of incidence (particularly among immunocompromised patients), the emergence of new species (i.e. *Candida auris*) (4) and the rise of resistant and multi-resistant isolates to antifungal treatments (5).

The current armamentarium of antifungal drugs is limited to four families: azoles, polyenes, echinocandins and pyrimidines (6). Over the years, the emergence of acquired resistance to azoles has forced the specialists to begin handling echinocandins as first-line therapy. According to Infectious Diseases Society of America (IDSA) (7, 8), European congress of Clinical Microbiology & Infectious Diseases (ESCMID) (9) and European Conference on Infections in Leukaemia (ECIL) (10) guidelines, this therapeutic strategy was implemented for invasive and disseminated candidiasis and for candidemia in neutropenic and non-neutropenic patients.

The echinocandin class of antifungal drugs is composed by caspofungin (CSF), micafungin, anidulafungin (11, 12) and rezafungin, a drug in phase 3 of clinical trial (13). Echinocandins inhibit β-(1,3)-D-glucan synthesis by binding non competitively to FKS p subunit of β-(1,3)-D-glucan synthase, a heteromeric glycosyltransferase enzyme complex present in the fungal cell membrane. Echinocandins are fungicidal against the majority of pathogenic *Candida* spp., even those resistant to azole compounds. Resistance to echinocandins can develop due to exposure to agents of this class and appears as point mutations on highly conserved regions of FKS1 and FKS2 genes, which encodes for subunit of the glucan synthase enzyme (14-16). Even though the actual difficulties in the treatment of fungal infections are partly due to the resistance phenomenon, resistance to echinocandin-class drugs remains low in most of *Candida* species except in *Candida glabrata* where an increase has been observed (17). For example, at Duke Hospital between 2001 and 2010, the number of *Candida glabrata* isolates resistant to echinocandins

increased from 4.9% to 12.3% and among that 14.1% were resistant to fluconazole (3, 18). In France, *in vitro* micafungin resistance among *C. glabrata* reached 3.9% (19). Emergence of multidrug resistant (MDR) *C. glabrata* isolates is of concern with 36% of MDR *C. glabrata* isolates in the USA (15, 20, 21).

Due to the increasing medical importance of invasive candidiasis (2) and based on the reported therapeutic failures in particular with caspofungin (18), drug resistance studies are needed (3). It is necessary to have adequate *in vitro* study approaches, which allow evaluating and comparing the antifungal efficacy of drugs. In laboratory of clinical microbiology, for example, echinocandin resistance is assessed by measuring MICs and comparing results to reference breakpoints or epidemiological cut-off value (ECOFF) established by European Committee on Antimicrobial Susceptibility Testing (EUCAST) or Clinical & Laboratory Standards Institute (CLSI). Unfortunately, because of significant interlaboratory variability in caspofungin MICs, EUCAST and CLSI do not propose interpretive criteria for this drug. Furthermore, echinocandin MICs have not been shown to correlate consistently with outcomes among patients with invasive candidiasis who are treated with these agents (22).

In vitro studies present important difficulties related to the need of appropriate analytical methodologies that allow the sensitive quantification of the drugs in culture media. Cell culture media is a complex matrix composed by amino acids, salts and vitamins among others that usually requires from a sample treatment before performing the analysis. Determination of CSF has been carried out in biofluids using different sample treatments such as solid phase extraction, protein precipitation and column-switching prior to liquid chromatography coupled to mass spectrometry (MS), photodiode-array (PDA) or fluorescence (FLD) detectors (23). Nevertheless, to our knowledge, no method for the determination of CSF in cell culture media has been developed. In this aspect, column-switching procedure can be useful for *in vitro* studies considering the amount of samples and the small volume that should be handled. This kind of sample treatment has been already used for CSF determination in serum by Egle et al. (24), but the LLOQ obtained (200 µg/L) might not be low enough for its application to several *in vitro* studies (25-26).

Therefore, the aim of this work was the development and validation of an on-line sample treatment liquid chromatography method for the quantitative determination of caspofungin in RPMI-1640 medium. The column-switching-HPLC-FLD method proposed here requires only 100 µL of sample and was successfully applied for the quantification of CSF in samples obtained from *in vitro* studies of *C. glabrata* cell growth under treatment by CSF.

2 Materials and Methods

2.1 Reagents and solutions

Caspofungin acetate was purchased from Finetech (Wuhan, Hubei). Cell culture media used was RPMI 1640 with L-glutamine with MOPS buffer obtained from Capricorn Scientific (Ebsdorfergrund, Germany), Lonza (Aubergenville Cedex, France) and RPMI-1640 medium with L-glutamine and without sodium bicarbonate (Sigma, Saint Quentin Fallavier, France) with addition of 1% glucose and 0.0825 mol/L MOPS (Sigma, Saint Quentin Fallavier, France) with pH adjusted to 7.0.

[3-(2-Aminoethylamino)propyl]trimethoxysilane (AATMS) was bought from Sigma-Aldrich (St. Louis, USA). Sodium hydroxide (≥99%) was purchased from Merk (Darmstadt, Germany). Acetonitrile (LC-MS grade) and trifluoroacetic acid (for LC-MS) were supplied by VWR chemicals. Ultrapure analytical water was obtained from an ELGA PURELAB classic system from Veolia Water STI (Saucats, France).

2.2 Instruments and analytical conditions

Dionex Ultimate 3000 chromatograph System (Thermo Scientific, Waltham, Massachusetts, USA) coupled to a 2475 multi wavelength fluorescence detector (Waters, Mildford, USA) was used for the analysis. Fluorescence detection was performed at 278 and 299 nm for excitation and emission wavelengths, respectively (23). For the switching system, a Rheodyne 6 port valve (IDEX Health & Science, Erlangen, Germany) and a Spectra SYSTEM P1000 Isocratic (pump 1, **Figure 1**) were used (Thermo Scientific). System control, data collection and data processing were accomplished using Chromeleon software.

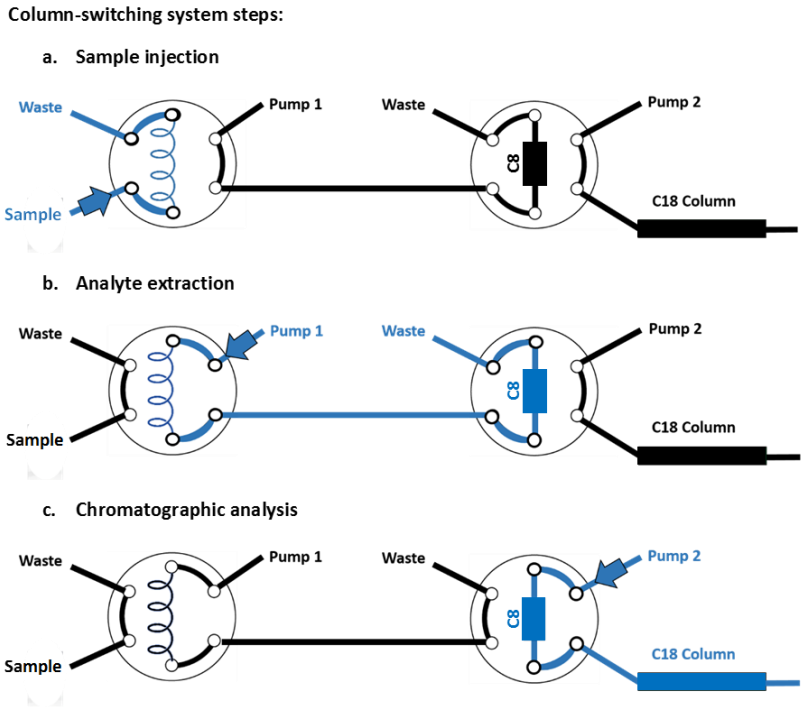

Figure 1: Column-switching system scheme with the different positions (a, b and c). In blue the connection where the analyte can be found in each moment

The column-switching for the on-line extraction was performed with a LiChroCART C8 column (4 x 4mm, 5 µm) (Merck, Darmstadt, Germany). The mobile phase consisted of an aqueous mobile phase of pH 2 adjusted with TFA (A) and acetonitrile as organic modifier (B) at 95:5 (A:B, v/v) proportion. A flow rate of 1 mL/min during 1.25 min was used in the extraction step. After, the analyte was eluted to the analytical column, HSS T3 (4.6 x 100 mm, 2.5 µm), from Waters, which was heated using a 100-040-220P column oven (Croco-cil, Cluzeau, France) to 40 °C. The mobile phase condition was at 60:40 (A:B, v/v) mixture at a flow rate of 0.8 mL/min. 40 µL of sample volume was injected.

2.3 Glassware treatment

Due to the sorption of caspofungin to glass surfaces (23, 27) all the glass material was treated with AATMS silanization agent following the procedure established in the work of Fukazawa *et al*. (28) for minimization of the peptide adsorption. In short, the material used was treated filling it with a solution of 1% AATMS in 1% of acetic acid. After few minutes, the liquid was removed and the material was dried at 50 °C for 4 hours.

2.4 Standard solution and spiked cell culture samples

Individual stock solutions were prepared each validation day by dissolving commercial CSF diacetate powder in Milli Q H_2O at a concentration of 800 mg/L of CSF. An intermediate solution of 50 mg/L CSF and the calibration standards, with concentrations from 10 to 1000 µg/L of CSF, were freshly prepared each day of analysis in RPMI-1640. The quality control (QC) samples were prepared by dilution to four levels of concentration: 750, 500, 30 and 10 (LLOQ) µg/L of CSF in RPMI-1640.

The different solutions in MOPS used were freshly prepared each experimentation day from the stock solution; 20 µg/L CSF for the switching optimization and QCs concentration for the recovery.

2.5 Column-switching optimization

Column-switching procedure is based on an on-line extraction of the sample (**Figure 1b**) before eluting the analyte to the analytical column where the chromatographic separation is performed (**Figure 1c**).

In preliminary trials, results showed a high variability in CSF chromatographic peak area when using a 20% of ACN in the mobile phase of the extraction step, probably due to an excessive elution strength of the mobile phase mixture. Based on this, the range studied in the optimization was limited from 5% until 15% ACN. The switching time (from 0.5 min to 2 min) was optimized. A 20 µg/L CSF diacetate solution in RPMI-1640 was used for this study. Chomatographic peak areas and their variation (RSD %) were taken into account to decide the optimum conditions.

To test the loading capacity of the extraction column, two columns with different dimensions were studied: 4 x 4 mm and 4.6 x 25 mm (Lichrospher, Interchim, Montluçon, France). A 750 µg/L CSF in RPMI-1640 sample was analysed and five replicates were injected in each extraction column. Chromatographic peak areas were compared to study if there were significant differences. Additionally, the recovery of the on-line sample treatment was calculated at the different QC levels by analysing samples in MOPS buffer at pH 7 with and without column switching. The recovery was calculated by the ratio of the chromatographic peak areas obtained in the different conditions.

2.6 Validation of the column-switching HPLC-FLD method

The method was validated in terms of selectivity, carryover, linear concentration range, stability, accuracy and precision following the criteria listed in the last guideline on bioanalytical method validation of the Food and Drug Administration (FDA) (29).

The RPMI-1640 cell culture media is a matrix prepared in laboratory, not a biological matrix as blood or urine, with a small variability and scarce sources. In consequence, authors decided that three sources were enough to ensure the reliability of the selectivity of this methodology. Selectivity of the method was assessed comparing different RPMI-1640 cell culture media in order to evaluate if there were interferences. A selective method should not have interference of more than 20% the response of the lower limit of quantification (LLOQ).

The absence of carryover was checked injecting RPMI-1640 blank samples after the injection of the high quality control (HQC) sample (750 µg/L CSF). The response in the blank was compared with the response in the LLOQ. For acceptance, the signal obtained in blank samples should be lower than 20% the response of CSF at the LLOQ.

The selected range for the calibration was from 0.01 mg/L to 1 mg/L in order to be able to use this method in different contexts. This range was based on CSF pharmacokinetic data (25-27). In order to study the effect of the matrix, calibration curves in MOPS buffer and in RPMI were built and compared. For validation, the calibration curve was built in RPMI using 6 calibration levels (10, 50, 90, 300, 600 and 1000 µg/L CSF) repeated at three different days. The curve was adjusted using a regression weighted by a factor of $1/x^2$. As established in the guidelines, non-zero calibrators should be ± 15% of the nominal concentration except at LLOQ where the calibrator should be ± 20% of the nominal concentrations in each validation run. LLOQ was defined as the lowest nonzero standard on the calibration curve. The sensitivity of the method was evaluated regarding the intended use and ensuring the reliable quantification in terms of accuracy and precision.

Accuracy and precision were evaluated at LLOQ (10 µg/L) and three QCs at 30 (LQC), 500 (MQC) and 750 µg/L CSF. Each validation day, five replicates of LLOQ and QCs were injected. Acceptance of accuracy and precision was expressed in terms of relative error (RE) of the nominal concentration and relative standard deviation (RSD), respectively. The acceptance criterion for accuracy was that the mean value should be within 15% of the nominal value (20% for the LLOQ) and precision was that % RSD should be ± 15% (20% for LLOQ).

The long-term stability of the analyte was studied in the stock solution (800 mg/L) in H_2O. LQC and HQC samples' (in RPMI) stability was checked at room temperature (autosampler conditions). Concentrations obtained in the different conditions were compared with the nominal concentrations of the fresh ones. The mean concentration in each case should be ± 15% of the nominal concentration to be considered acceptable.

2.7 Culture assay and analysis of CSF in cell culture medium

One clinical isolate of *C. glabrata* (CAGL18) was selected into IICiMed collection to perform the study of cell growth under caspofungin treatment. CAGL18 is a susceptible to caspofungin with a MIC of 0.5 -1 µg/mL, determined by the broth microdilution method with RPMI 1640 medium according to the procedures of the Clinical and Laboratory Standard Institute described in document M27-A2 (30). The isolate was stored at -80 ^{o}C for long-term storage and grown on Sabouraud dextrose agar at 37 ^{o}C for 24h prior to experiments.

10 mL of yeast suspension containing 1 Mac Farland were prepared in RPMI medium (with L-glutamine and without sodium bicarbonate, 1% glucose, 0.0825 mol/L MOPS and with pH = 7.0) from 24 h cultures on Sabouraud dextrose agar at 37 ^{o}C. 2 mL of yeast suspension were inoculated into 18 mL of RPMI medium in a 50 mL erlenmeyer pre-treated with AATMS. When necessary 50 µL of fresh caspofungin stock solution (50000 µg/L in DMSO) were added to a final concentration of 125 µg/L. Erlenmeyers were incubated at 35 ^{o}C under agitation (110-150 rpm) in a non CO_2 incubator. Samples were collected from the erlenmeyers from 0 to 4 hours each hour and after at 6, 8 and 24 h. 50 µL were counted on Malassez cells. Growth curves were obtained and displayed as log (number cells) = f (time of culture). In each sampling time (0, 1, 2, 3, 4, 6, 8, and 24 h), 100 µL of sample were transferred to vials for the direct injection of 40 µL in the chromatographic system. The experiments without *C. glabrata*, constitutes a stability study of CSF in the cell culture medium.

3 Results and discussion

3.1 Column-switching optimization

When optimizing the column-switching, the organic modifier percentage and the switching time were studied simultaneously. The results shown (**Figure**) that a higher CSF peak area was obtained when using 5 % of ACN. This proportion was fixed as the optimum for the analysis. Regarding the switching time, no significant difference was seen in the chromatographic peak area when increasing it. However, a minimum of 1 min was required to remove a compound eluting just after the injection peak.Taking this in account, a switching time of 1.25 min (the equivalent to 20 times the volume of the extraction column) was fixed for the routine analysis.

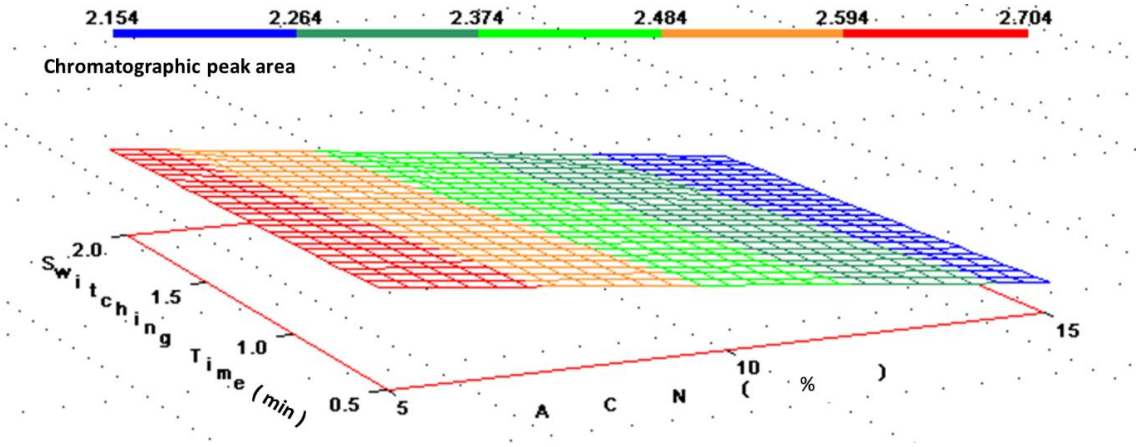

Figure 2: Response surface of CSF chromatographic peak area. Switching time range from 0.5 to 2 min and ACN % range from 5% to 15%

Due to the previously mentioned matrix complexity of cell culture, two different extraction column dimensions were checked for the on-line analyte extraction with the aim of studying a potential saturation of the column, which may lead to a loss of CSF. No significant differences were observed ($p > 0.05$) when comparing the responses of different extraction columns. Consequently, the loading capacity of the smaller column (4 × 4 mm) was found sufficient for the retention of caspofungin at high concentration.

The recovery of CSF using the sample treatment by column switching was calculated (**Table 1**). These results demonstrate that this on-line approach allows a sample treatment with a high recovery for CSF.

Table 1: Recovery results (± standard deviation) of the on-line sample treatment (4 x4 mm extraction column, n=5)

Sample	HQC	MQC	LQC	LLOQ
Recovery (%) ± s	109 ± 3	108 ± 4	107 ± 5	108 ± 13

3.2 Validation of the column switching HPLC-FLD method

3.2.1 Selectivity

Different RPMI-1640 cell culture media solutions were compared. Thanks to the on-line sample preparation, no interference from the different constituents of the RPMI was observed at CSF retention time with the different cell culture solutions analysed. **Figure 2** shows the RPMI analysis in this method.

3.2.2 Carryover

No chromatographic peak was observed in the blanks analysed after injecting the HQC sample.

3.2.3 Lower limit of quantification and linear calibration range

Calibration curves performed in both MOPS and RPMI-1640 matrices at a pH value of 7, were compared. The linear regressions were y = 0.0277 x - 0.2068 (R^2 = 0.9986) and y = 0.0241 x – 0.3460 (R^2 = 0.9992) for MOPS and RPMI, respectively. A significant difference in the slope value was found proving that there is an effect of the matrix. For this reason, the validation was totally performed with CSF samples in RPMI-1640 matrix.

Data obtained from the chromatographic peak area of CSF were treated using a weighted linear regression ($1/x^2$). In **Table 2** the linear regression equations of the different calibration curves built are given (Response = slope · concentration + intercept).

Table 2: Weighted ($1/x^2$) calibration curves and the coefficient of determination (r^2) obtained in the different validation days

Day	**Curve equation**	**r^2**
1	$y = 0.0217x - 1.1225 \cdot 10^{-5}$	0.9973
2	$y = 0.0232x - 6.6396 \cdot 10^{-5}$	0.9998
3	$y = 0.0241x - 5.4115 \cdot 10^{-5}$	0.9992

The lowest nonzero calibrator was established as the LLOQ with less than 20% of variability (8.1%) in the nominal value. **Figure *2*** shows chromatograms obtained with the injection of the RPMI medium and the injection of this concentration level of CSF prepared in RPMI. At the retention time of CSF, no signal was observed for the blank analysis.

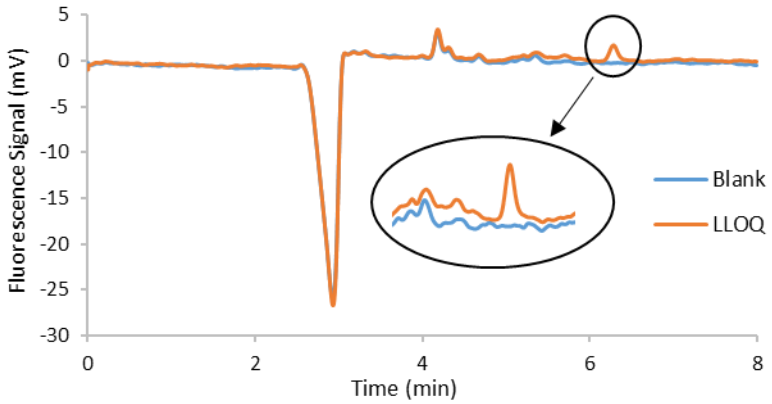

Figure 2: Chromatograms of a blank of RPMI-1940 and CSF at LLOQ concentration (10 µg/L CSF)

3.2.4 Accuracy and precision

The results obtained for accuracy and precision are shown in Table 3. The mean value of the % RE of the nominal concentration was lower than 15 % at all concentration levels in both assays (intra-day and inter-

day). The high variability in the accuracy within days has to be highlighted. In Table 3, apart from the mean value of the three days, the minimum (min) and maximum (max) values obtained are shown.

Table 3: Intra- and inter-day accuracy and precision results (n= 3 days x 5 replicates) in terms of % RE and % RSD, respectively, at four concentration levels (10, 30, 500 and 750 µg/L CSF).

Concentration level (µg/L)	**Intra-day**		**Inter-day**	
	Accuracy (% RE) Mean (min-max)	**Precision** (% RSD) Mean (min-max)	**Accuracy** (% RE)	**Precision** (% RSD)
10 (LLOQ)	13.6 (12.5-15.3)	8.5 (7.5-10.4)	13.6	8.1
30 (LQC)	10.8 (2.2-16.4)	5.6 (4.5-6.1)	10.7	8.8
500 (MQC)	9.6 (0.35-16.1)	7.8 (7.5-8.4)	9.4	9.8
750 (HQC)	7.6 (1.0-12.7)	6.7 (2.6-8.7)	7.6	8.4

3.2.5 Stability

The stock solution of 800 mg/L CSF in $H_2O$was stable at -80 ^oC for 1 month. LQC and HQC samples were stable for 2 hours at room temperature in RPMI. From that time on, a significant loss of CSF was observed. Based on these results, samples must be analysed in a short period of time after the sampling.

The results obtained for selectivity, carryover, linear concentration range, accuracy and precision met the acceptance criteria of the guideline followed, hence the method was successfully validated.

3.3 Analysis of CSF in cell culture medium

To prove that the column-switching-HPLC-FLD method developed here was efficient in cell culture conditions, a kinetic study of cell proliferation in absence and in presence of CSF was performed. The objectives were to measure, at the same time, the amount of free CSF in the cell culture medium and the concentration of cells. Yeast cells were cultured without or with caspofungin (125µg/L) for 24 h at 35 ^oC under agitation. Cell counting was per-formed at different times (see sampling times in Section 2.7) to obtain growth curves (**Figure 4**). In parallel, free caspofungin contained in the cell culture medium was quantified. As expected, treatment with CSF reduced cell proliferation. This result was consistent with the choice of a sub-inhibitory concentration of CSF (MIC/4).Meanwhile, the amount of free CSF was quantified in the culture medium in absence and in presence of cells, the results were similar in both conditions. Indeed, free CSF concentration decreased immediately then it plateaued between 3 h and 8 h before decreasing slowly until 35 g/mL after 24 h. There was a decrease of 70% of CSF amount available in the cell culture medium in 24 h. Fortunately, despite the decrease of free CSF concentration, the remaining amount was enough to reduce cell proliferation during all the experiments. These important variations in free CSF concentration could be due to the poor stability of caspofungin or to the adsorption phenomenon described before (23). However, maybe due to the use of a column switching, degradation products were not observed. However, these results helped to explain the variability in MIC values for CSF determined in different laboratory and highlighted the difficulty to have interpretive criteria for this drug.

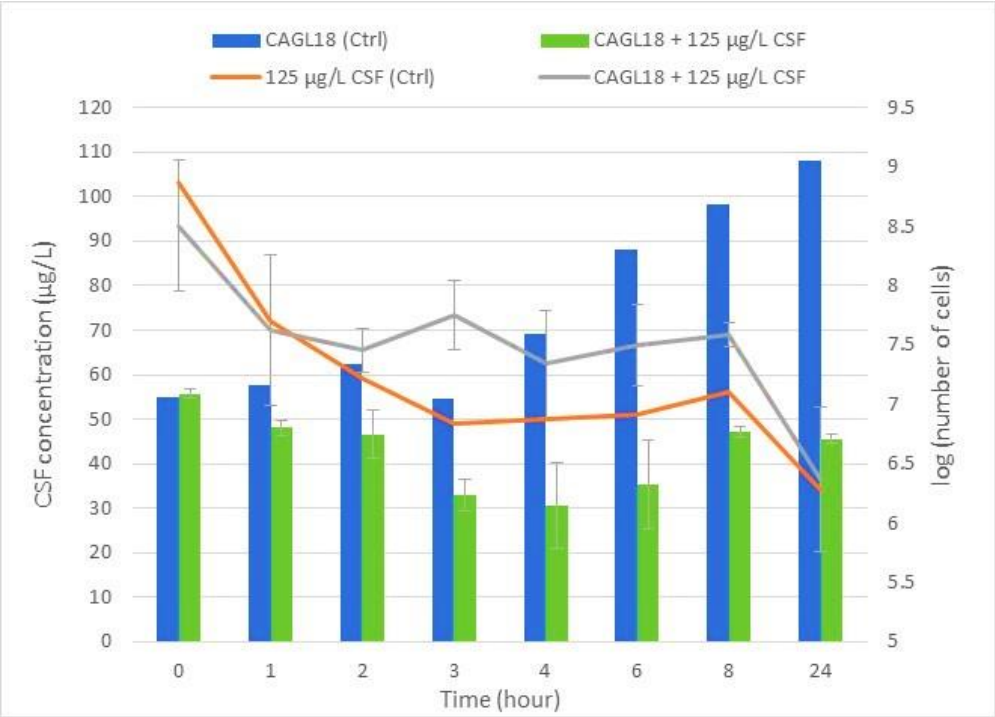

Figure 4: Variation of CSF concentration in absence of fungal cells (orange curve) and in the presence of fungal cells (grey curve, CSF concentration mean of two experiments with error bars representing min and max values), and cell proliferation without CSF (blue histogram) and with a dose of 125 µg/L of CSF (green histogram, log number of cells mean value of two experiments with error bars representing min and max values).

4 Conclusions

The column-switching HPLC-FLD method developed allows the simple and rapid quantification of caspofungin antifungal drug in RPMI-1640 cell culture media. The effect of the matrix in the chromatographic response obliges to use RMPI to build the calibration curve. Nevertheless, no significant differences were observed among the calibration curves built on different days nor among the different RPMI matrices employed. The method showed a high recovery and was successfully validated according to the criteria established in the guideline on bioanalytical method validation of the FDA (29) in terms of selectivity, carryover, linear concentration range, accuracy and precision. The loss of CSF signal over time in the medium was found as a critical variable that could have a negative impact in the accuracy of the method. Due to this reason, the analysis should be performed immediately after the sampling.

Comparing the method developed with the work of Egle *et al.* (24) for the determination of CSF in serum using a column-switching system, this work presents a faster analysis with a total run of 8 min instead of 30 min. Furthermore the extraction stationary phase is C8 instead of diol, which allows the use of a minimum of 5% ACN in the eluent to limit CSF adsorption. Above all, the significant LLOQ decrease has to be mentioned, from 200 µg/L to 10 µg/L.

Thanks to this column-switching-HPLC-FLD method, for the first time, the amount of free caspofungin in cell culture medium, RPMI-1640, was followed during a 24 h kinetic study of cell proliferation. Variations of free CSF concentration occurred during the kinetic study. Due to significant interlaboratory variability in CSF MIC val-ues, EUCAST and CLSI gave up to propose interpretive criteria forthis drug. This analytical approach constitutes an efficient and rapid monitoring to follow CSF concentration in culture media. It willbe of great interest to understand CSF behaviour in various cell culture conditions and also to investigate methodology to propose interpretive criteria for this drug.

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Conflict of interest

Authors declare that they have no conflict of interest.

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