

# A New HPLC Validated Method for Therapeutic Monitoring Of Triazoles in Human Plasma: First Results in Leukaemic Patients

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## Abstract

Therapeutic drug monitoring of triazoles is widely used in clinical practice to optimize therapy, especially in those patients who need antifungal treatment as co-medications. Here it is described development and validation of a new chromatographic method in order to quantify voriconazole and posaconazole in human plasma by ultraviolet detection.

After liquid extraction of analytes from plasma using acetonitrile, samples are evaporated to dryness and then reconstituted in mobile phase for chromatographic separation. Analysis is achieved on C18 reverse phase column and eluate is monitored at 250 nm. Mobile phase consisted of 35% water, 15% methanol, 50% acetonitrile. Flavone was used as internal standard; retention times (minutes) were, respectively, for voriconazole 3.9, posaconazole 7.9, flavone 7.1.

Accuracy and variability were assayed by inter- and intra-day validation, conducted on three separate days. Methodology was used for the analysis of plasma samples of acute myeloid leukaemia patients in therapy with voriconazole or posaconazole for treatment of fungal infections, in order to perform therapeutic monitoring of antifungal drugs levels.

Mean inter- and intra-day accuracy and variability were acceptable for both compounds; thus, method developed resulted linear in the range 0.125–8 µg/mL. Limits of quantification and limits of detection for voriconazole and posaconazole are, respectively, 0.100 and 0.050 µg/mL, and 0.030 and 0.020 µg/mL.

It has been observed that voriconazole and posaconazole circulating levels achieved in patients are on average below the therapeutic range defined in literature.

In conclusion, method developed and validated in order to quantify voriconazole and posaconazole in human plasma is accurate and precise; it is easily applicable and reproducible, and, therefore, it could be an useful tool in clinical routine to better manage patients in case of multi-therapy approach.

**Keywords:** HPLC; Voriconazole; Posaconazole; Quantification; Leukemia

## Introduction

Due to polypharmacy and complexity of comorbidities in haematology patient population, antifungal therapy may be often associated with significant toxicity or drug interactions, leading to sub-therapeutic antifungal drug concentrations and poorer clinical outcomes [1]. Antifungal agents of the azole class are metabolized by the cytochrome P450 (CYP450) system, such as voriconazole (VRC, Figure 1), a widely used broad-spectrum triazole substrate of

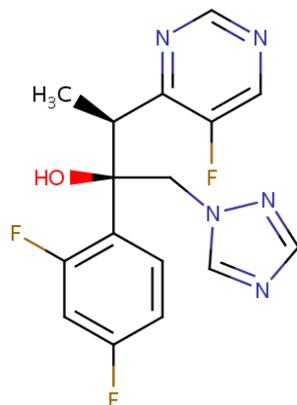


Figure 1: Structure of Voriconazole

CYP2C19, CYP3A4 and CYP2C9 isoforms [2]. Posaconazole (PSC, Figure 2), instead, likewise broad-spectrum triazole, is also substrate of CYP3A4 but primarily undergoes glucuronidation [3,4], leading to a potential for drug-drug interactions minor than that for VRC [1]. Then, co-administration of drugs that induce CYP450 metabolism can result in undetectable circulating levels of VRC and PSC, while co-administration of CYP450 inhibitors may cause an increase of these levels. On the other hand VRC inhibits CYP3A4 isoform, and even more stronger inhibitor is PSC, leading to an increase of circulating levels of drugs metabolized by CYP3A4. Although antifungal therapy does not typically fulfill all criteria needed for Therapeutic Drug Monitoring (TDM) indication [5], assessment of circulating levels achieved in patients may be helpful for antifungal agents where non-compliance,

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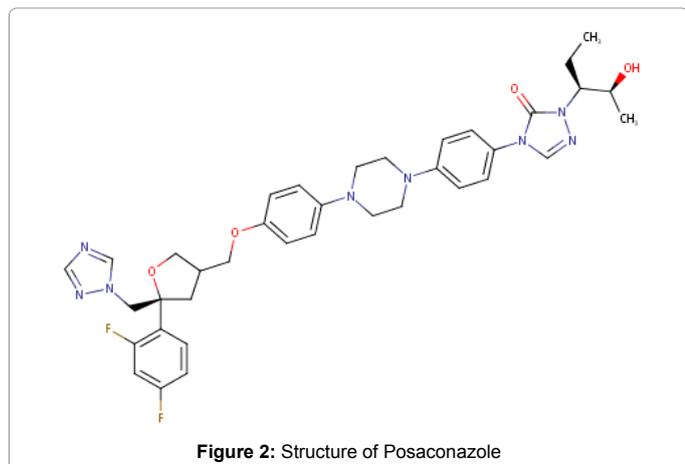


Figure 2: Structure of Posaconazole

non-linear pharmacokinetics, a narrow therapeutic window, drugs interactions or unexpected toxicity are encountered, all factors that may even worsen for haematology population.

VRC exhibits ca.100-fold variability in drug levels in individuals receiving the same dose [6], showing non-linear saturable pharmacokinetics [7]. To our knowledge available recommendations suggest that VRC levels should be maintained > 1 g/mL and that levels > 5.5 g/mL have been reported to be possibly associated with toxicity [8]. Furthermore, polymorphisms in the CYP2C19 isoform are associated with slow VRC metabolism and may lead to two- to four folds higher VRC exposure [9]. Corroborating the potential of drug-drug interactions, it's known that in patients with haematologic malignancy, the correlation between VRC concentration and daily dose is poor [10].

Less data are available in literature for PSC. It is known that PSC shows high inter-patient variability [11]. Furthermore, exposure-response analyses revealed a clear relationship between an higher incidence of clinical failure and lower PSC circulating concentrations, suggesting that it is necessary to ensure adequately high plasma exposure to PSC, in terms of prophylaxis and treatment of fungal infections both [7,11,12].

TDM has become recently an essential tool for the management of patients with different pathologies and may be useful, then, also for haematologic patients treated with azoles for fungal infections, especially if drugs administered for haematologic disorder may tamper with antifungal agents.

Daunorubicin and cytarabine are current therapy for acute myeloid leukaemia (AML) treatment. These drugs are all metabolized by CYP3A4; furthermore, daunorubicin, inhibits CYP450 isoforms, especially CYP3A4. Then, drug interactions involving CYP450 in AML patients treated with azoles for fungal infections may play a central role.

Therefore, quantification of circulating azoles levels is an important issue in clinical practice to improve efficacy, minimizing toxicity, especially in selected populations, such as AML patients.

In recent years, many methods to quantify azoles in human plasma individually and simultaneously have been published. Several reported the use of high performance liquid chromatography coupled with ultraviolet determination (HPLC-UV)[4,13-24]. More recently, liquid chromatographic methods based on mass spectrometry (LC-MS/MS) detection have been developed to this purpose [25-32], although MS facilities are not always available in standard hospital laboratories, because they are expensive compared with a simple HPLC system.

The aim of the current work was to develop and validate a reliable HPLC-UV method for the simultaneous determination of VRC and PSC in human plasma, easily suitable in routine TDM for selected population such as AML patients.

## Materials and Methods

### Chemicals and reagents

VRC was kindly provided by Pfizer (Sandwich, United Kingdom) and PSC was kindly provided by MSD/Schering-Plough (Wicklow, Ireland); flavone, used as internal standard (IS), because structurally analogue of voriconazole and PSC, was purchased from Sigma-Aldrich (Milan, Italy). Acetonitrile and methanol HPLC grade were purchased from VWR International (Milan, Italy); HPLC grade water was produced with Milli DI system coupled with a Synergy 185 system by Millipore (Milan, Italy). Blank plasma from healthy donors was kindly supplied by the Blood Bank of San Luigi Hospital (Orbassano, Italy).

### Stock solutions, calibration standards and quality controls

Stock solutions of VRC, PSC and flavone were prepared by dissolving an accurately weighed amount of drug in methanol to obtain a final concentration of 1 mg/mL, then stored at -20°C for no longer than 3 months, based on stability data shown in previously published works [33,34]. A working solution of IS was prepared daily at a concentration of 25 µg/mL in methanol. Stock solutions of VRC and PSC were used to prepare plasma calibration standards samples (STDs) and quality controls (QCs). The highest calibration standard (STD7: 8 µg/mL) and 4 QCs, QC high (5 µg/mL), QC medium (1 µg/mL), QC low (0.25 µg/mL for VRC and 0.2 µg/mL for PSC) and QC as lower limit of quantification (LLOQ; 0.1 µg/mL for VRC and 0.03 µg/mL for PSC), were prepared adding a determined volume of stock solution to blank plasma. Others STDs were prepared daily by serial dilution from STD7 to the lowest calibration standard (STD1: 0.125 µg/mL) with blank plasma, to obtain 7 different spiked concentrations. A blank sample plus IS (STD0) was also included. Calibration range, from STD7 to STD1, and QCs concentrations are listed in table 1. STDs and QCs were stored at -20°C for no longer than 3 months, based on stability data shown in previously published works [33,34].

### Patients

Plasma samples were obtained in TDM routine activity from AML patients admitted to the Haematology ward of San Luigi Gonzaga Hospital (Orbassano, Italy) undergoing treatment with VRC (n = 23: 13 males, 10 females; 200 mg twice daily) or PSC (n = 14; 11 males, 3 females; 200 mg three times daily) for prophylaxis or therapy purposes. All patients were treated for at least 7 days before blood sampling, so that collection was done at steady state. Blood samples were collected in lithium heparin tube at the C trough (12 ± 3 hours after last drug intake). Plasma samples were obtained by centrifugation at 700 g for 10 min at 4°C, then frozen at -20°C till analysis. Blood samples were taken immediately before drug intake. Written informed consensus was

VORICONAZOLE											
STD <sub>1</sub>	STD <sub>2</sub>	STD <sub>3</sub>	STD <sub>4</sub>	STD <sub>5</sub>	STD <sub>6</sub>	STD <sub>7</sub>	QC <sub>l</sub>	QC <sub>m</sub>	QC <sub>h</sub>	LLOQ	LOD
0.125	0.25	0.5	1	2	4	8	0.25	1	5	0.1	0.05
POSACONAZOLE											
STD <sub>1</sub>	STD <sub>2</sub>	STD <sub>3</sub>	STD <sub>4</sub>	STD <sub>5</sub>	STD <sub>6</sub>	STD <sub>7</sub>	QC <sub>l</sub>	QC <sub>m</sub>	QC <sub>h</sub>	LLOQ	LOD
0.125	0.25	0.5	1	2	4	8	0.2	1	5	0.03	0.02

Table 1: Calibrations Standards from STD<sub>1</sub> to STD<sub>7</sub>, Quality Controls (low, medium, high), Lower Limit of Quantification (LLOQ) and Lower Limit of detection (LOD) concentrations (µg/mL) for voriconazole and posaconazole.

reached from patients and approval for the research was obtained from local ethics committee.

Developed method was also used to measure the two drugs in plasma samples for the International Interlaboratory Quality Control Program for Therapeutic Drug Monitoring of Antifungal Drugs [35].

### Samples preparation

STDs, QCs and patient samples were thawed at room temperature, and 500  $\mu$ L of plasma were pipetted into a 2 mL polytetrafluoroethylene tube; 25  $\mu$ L of IS working solution were added to each tube. Samples were extracted by protein precipitation using 750  $\mu$ L of acetonitrile. Each sample was vortexed for 30 s and then centrifuged at 8,000 g for 10 min. Supernatant was resuspended in 250  $\mu$ L of mobile phase after evaporation to dryness and then transferred to vials for injection in column (50  $\mu$ L). All procedure was carried out at room temperature.

### Chromatographic system and conditions

HPLC was performed with a LaChrom Elite system (VWR International, Milan, Italy) equipped with autosampler, spectrophotometer, and heated column compartment. System management and data acquisition were performed with the EzChrom Elite software. Separation was achieved with a C18 Reverse Phase column (LiChroCART<sup>®</sup> 250-4 LiChrospher<sup>®</sup> 100 RP 18, 5  $\mu$ , VWR International, Milan, Italy) preceded by a Security precolumn (LiChroCART<sup>®</sup> 4-4 LiChrospher<sup>®</sup> 100 RP 18, 5  $\mu$ , VWR International, Milan, Italy). Mobile phase consisted of 35% water, 15% methanol, 50% acetonitrile. Analysis was carried out at the constant flow rate of 1 ml/min at 35°C and eluate was monitored at 250 nm. Each run time was 10 min.

### Method validation

Method was validated following recommended FDA procedures over 3 days [36]. Every day, 2 calibration curves and 8 QCs (specifically, 2 samples for each QC concentration) were extracted using the protocol described above and then analyzed to assess linearity, variability, and accuracy.

**Linearity:** Range within method linearity was tested was from 0.125 to 8  $\mu$ g/mL, according to data available in literature [6,8,11]. Calibration curves, over the concentrations range chosen, were built with the spike height ratios of each STD and IS, and fitted using linear regression. Totally, 6 calibration curves were analyzed, 2 curves for each validation day.

**Variability:** Variability was assessed as intra and inter day parameter. Intra-day was defined as relative standard deviation (RSD) calculated from the values measured for 8 QCs performed each day at concentration of 5, 1, 0.25 and 0.1  $\mu$ g/mL for VRC and 5, 1, 0.2 and 0.03  $\mu$ g/mL for PSC.

Inter-day variability was defined as RSD calculated using the values measured from 24 samples (8 samples/day) at concentration of 5, 1, 0.25 and 0.1  $\mu$ g/mL for VRC and 5, 1, 0.2 and 0.03  $\mu$ g/mL for PSC.

The variability was considered acceptable for each QC if it did not exceed 15%, except for the LLOQ for which was accepted 20% of variation.

**Accuracy:** Accuracy was assessed as intra- and inter-day parameter. Intra-day was defined as the medium percent deviation from the nominal concentration for 8 QCs performed each day at concentration of 5, 1, 0.25 and 0.1  $\mu$ g/mL for VRC and 5, 1, 0.2 and 0.03  $\mu$ g/mL for PSC.

Inter-day was defined as the medium percent deviation from the

nominal concentration for 24 samples (8 samples/day) at concentration of 5, 1, 0.25 and 0.1  $\mu$ g/mL for VRC and 5, 1, 0.2 and 0.03  $\mu$ g/mL for PSC.

The accuracy was considered acceptable for each QC if it did not exceed 15%, except for the LLOQ for which was accepted 20% of variation.

**Recovery, limit of detection (LOD) and LLOQ:** Percent recovery was obtained from the spike height ratio between extracted sample and drug in methanol at equal concentration (0.25  $\mu$ g/mL for VRC and PSC, 0.05  $\mu$ g/ml for IS). Final value was obtained as mean from 15 ratios.

As requested by international guidelines [36], LOD in plasma was defined as the concentration that yields signal to noise ratio of 3/1, while LLOQ, assessed for intra- and inter-day variability and accuracy, was considered the lowest concentration level that could be determined with a RSD < 20%, giving a signal to noise ratio 5/1.

**Selectivity, stability and reproducibility:** Interference from endogenous compounds was investigated by analysis of different blank plasma samples.

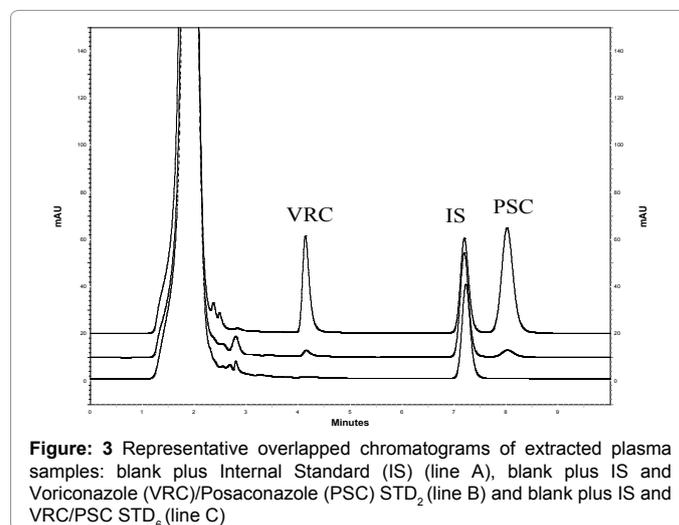
Stability of VRC and PSC stock solutions, STDs and QCs were investigated prior to start validation of the assay within 3 months of storage at -20°C, assessed by variation of heights. If the measured concentration remained within 20% of nominal concentration, analytes were considered stable.

As requested by international guidelines [36], reproducibility was defined as precision of the method under the same operating conditions over a short period of time. It has been calculated as RSD of values measured for 10 repetitions of the same sample extracted at concentration of 1  $\mu$ g/mL for VRC and PSC.

### Results

Different percentages of water, methanol and acetonitrile for constitution of mobile phase were tested (10% water, 10% methanol, 80% acetonitrile; 20% water, 20% methanol, 60% acetonitrile). Different flow rates (0.8; 1.2 ml/min), oven temperatures (32; 37°C) and UV wavelength (230; 250 nm) were also tested.

Time of analytical run was chosen at 10 min, according to the retention times of substances and their good separation. VRC and PSC retention times were 3.9  $\pm$  0.1 and 7.9  $\pm$  0.1, respectively, while it was 7.1  $\pm$  0.1 for IS. Representative chromatogram of a blank plasma (plus IS) and VRC and PSC STD<sub>2</sub> and STD<sub>6</sub> overlying is shown in figure 3.



## Method validation

**Linearity:** Calibration curves were linear over the concentrations range selected for validation (0.125-8 µg/mL), with a mean regression coefficient ( $r^2$ ) of 0.99 for both analytes.

Variability, accuracy, recovery, LOD and LLOQ.

Variability and accuracy, assessed as intra- and inter-day parameters, are listed in table 2. All observed data were below 15.0%, with the only exception for QC medium value of PSC that was 15.78% for intra-day accuracy. Observed recovery was around 100 % for both analytes. LLOQ was set at 0.1 µg/mL for VRC and 0.03 µg/mL for PSC, prior to start validation of the assay, while LOD was set at 0.05 µg/mL for VRC and 0.02 µg/mL for PSC.

**Selectivity, stability and reproducibility:** No signal increase due to endogenous plasma substances was observed at the retention time of analytes of interest. VRC and PSC stock solutions, plasma STDs and QCs were stable within 3 months of storage at -20°C; measured concentration remained within 20% of nominal concentration. RSD observed of method reproducibility was 0.29 % for VRC and 0.88% for PSC.

**Patients:** Validated assay has been applied to azoles quantification of 65 plasma samples of 23 AML patients treated with VRC and 40 plasma samples of 14 AML patients treated with PSC.

Patients data are reported in table 3, divided by sex. VRC C trough ranged from 0 to 7 µg/mL, while PSC C trough ranged from 0.08 to 2.06 µg/mL, according to data present in literature [6,8,11].

Positive results obtained from proficiency testing program<sup>35</sup> showed that the method allows correct quantification of analytes in plasma samples, becoming useful for clinical routine of antifungal therapy TDM.

## Discussion

Antifungal TDM is an important tool in clinical practice, especially

for patients with haematological disorders, in order to avoid drug dose related toxicity and improve clinical outcome. Due to specific metabolic pathways of triazoles, drug interactions in selected patients, such as AML patients, may often cause sub-therapeutic concentrations. In particular, VRC is a substrate and inhibitor of CYP2C19, CYP2C8/9 and CYP3A4 [1,2] and PSC is an inhibitor of CYP3A4, metabolized by glucuronidation<sup>4</sup>. Due to the metabolic bind especially at CYP3A4 site between azoles and current therapy for AML treatment, and to therapeutic window (1-5,5 µg/mL) reported for VRC [8], and thresholds of efficacy reported for PSC (0.7 µg/mL for prophylaxis and 1.25 mg/mL for treatment)<sup>11,12</sup>, antifungal TDM is gaining increasingly importance for selected populations such as AML patients. Considering this interest, we have developed and fully validated a new HPLC-UV methodology to measure VRC and PSC in human plasma. During development and optimization of our methodology, best results were achieved at 35 % water, 15 % methanol, 50 % acetonitrile of mobile phase, constant flow rate of 1 ml/min, 35°C and 250 nm. In our opinion, more complete assay reported in literature using HPLC-UV is that from Gordien et al. [37]. With description of methodology able to simultaneously quantify fluconazole, PSC, VRC, ketoconazole, itraconazole and its metabolite. Although this method allows quantification of the five azoles plus metabolite in a single assay, plasma samples were purified by solid-phase extraction procedure, which is more expensive than our developed procedure for extraction. They also used linezolid as IS, an antibiotic which may be co-administered in clinical practice, while the use of flavone, as we set in our methodology, makes our method more applicable to the analysis of immunocompromized patients. More recently LC-MS/MS methods have been developed to the purpose of antifungal TDM [25-32], but MS facilities are not always available in standard hospital laboratories. Our developed method, based on HPLC-UV system, reveals a good performance to this aim. Even if VRC and PSC are not co-administered, our chromatographic method allows the quantification of both analytes using a single calibration curve, extraction procedure, and HPLC system. Therefore, plasma samples of patients taking VRC or PSC could be analyzed simultaneously,

VORICONAZOLE															
QC <sub>l</sub> (0.25 µg/mL)				QC <sub>m</sub> (1 µg/mL)				QC <sub>h</sub> (5 µg/mL)				LLOQ (0.1 µg/mL)			
Accuracy		Variability		Accuracy		Variability		Accuracy		Variability		Accuracy		Variability	
intra-day	inter-day	intra-day	inter-day	intra-day	inter-day	intra-day	inter-day	intra-day	inter-day	intra-day	inter-day	intra-day	inter-day	intra-day	inter-day
10.28	10.26	3.50	5.71	7.07	7.40	3.85	7.28	3.64	3.41	3.30	3.26	13.39	13.02	8.42	8.97
POSACONAZOLE															
QC <sub>l</sub> (0.2 µg/mL)				QC <sub>m</sub> (1 µg/mL)				QC <sub>h</sub> (5 µg/mL)				LLOQ (0.03 µg/mL)			
Accuracy		Variability		Accuracy		Variability		Accuracy		Variability		Accuracy		Variability	
intra-day	inter-day	intra-day	inter-day	intra-day	inter-day	intra-day	inter-day	intra-day	inter-day	intra-day	inter-day	intra-day	inter-day	intra-day	inter-day
10.43	10.87	7.70	10.78	15.78	14.71	4.28	4.76	5.38	5.16	3.18	3.25	13.78	13.18	7.88	7.88

**Table 2:** Validation data for Quality Controls (low, medium, high) and Lower Limit of Quantification (LLOQ) concentrations for voriconazole and posaconazole. Values are expressed as percent Relative Standard Deviation.

VORICONAZOLE			
SEX	MEAN C <sub>TROUGH</sub>	UPPER C <sub>TROUGH</sub>	LOWER C <sub>TROUGH</sub>
MALE (N=13)	1.46 ± 1.40	6.81	0
FEMALE (N=10)	1.29 ± 1.55	7.00	0
POSACONAZOLE			
SEX	MEAN C <sub>TROUGH</sub>	UPPER C <sub>TROUGH</sub>	LOWER C <sub>TROUGH</sub>
MALE (N=11)	0.49 ± 0.42	2.06	0.08
FEMALE (N=3)	0.57 ± 0.46	1.19	0.08

\*: number of patients.

**Table 3:** Pharmacokinetic data of patients treated with voriconazole and posaconazole. Concentrations are expressed as µg/mL. Mean C<sub>Trough</sub> values are expressed with standard deviation.

reducing costs and time required for analysis. Furthermore our calibration curves, cover all drug concentrations achieved in patients followed at our institution and reported in published pharmacokinetics studies [21,38-41], making our method useful for routine analysis, especially for TDM application, where receiving a report quickly may help clinicians to eventually modify antifungal drug dose during therapy. Mean regression coefficient of calibration curves obtained during validation indicates an excellent linearity of the methodology developed; accuracy, intra- and inter-day variability data, listed in Table 2, are all acceptable but one. High extraction efficiency shows a good final recovery and absence of interference peaks at the analytes retention times, lets an accurate measurement of azoles plasma levels. The LLOQ (0.1 and 0.03 µg/mL for VRC and PSC, respectively) of the developed assay makes methodology suitable to perform azoles therapeutic monitoring in AML patients treated with these drugs for fungal infections. Moreover, the total run time is shorter than those proposed using UV detection in literature (10 min vs 19 and 15 min) [29,37].

The developed method is currently used in our laboratory service to monitor VRC and PSC concentrations in plasma of patients for antifungal therapy. To the purpose of this paper, the methodology developed was applied to 65 plasma samples obtained from 23 AML patients receiving VRC and 40 plasma samples of 14 AML patients receiving PSC. There were no samples with concentrations above the upper limit of quantification (8 µg/mL for both analytes). Although the concentrations of patients samples varied widely, they were well encompassed by the concentrations range of our standard curves. Of 105 samples analyzed, a high number of sub-therapeutic plasma concentrations were observed. Especially for PSC, where only 12 of 40 samples (30 %) were above the expected threshold of efficacy. For VRC, among the 65 samples analyzed, 29 (44.6%) were in the expected range (1-5.5 µg/mL), 34 (52.3%) were < 1 µg/mL, while 2 (3%) were 5.5 µg/mL. Data observed indicate need to increase antifungal dose in patients treated for AML, but, on the other hand, lead to pay attention to that AML treatment can be affected by azoles therapy via interaction at CYP3A4 site.

Finally no statistically significant difference was observed between azoles concentrations gender related, even if male patients achieved a major mean concentration of VRC levels than females ( $1.46 \pm 1.40$  vs  $1.29 \pm 1.55$ ), while for PSC trend observed was reverse ( $0.49 \pm 0.42$  vs  $0.57 \pm 0.46$ ).

## Conclusion

A simple, robust and reliable HPLC-UV method for the determination of VRC and PSC in human plasma has been developed, validated and described.

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