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RESEARCH ARTICLE

Method for Therapeutic Drug Monitoring of Voriconazole and its Primary Metabolite Voriconazole-N-oxide in Human Serum using LC-MS/MS

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OBJECTIVES: his study aims to present a strategy to optimize a liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) method for voriconazole and voriconazole-N-oxide with an stable isotopically labelled internal standard.

METHODS: Protein precipitation was used as extraction method and detection was carried out with LC-MS/MS using $^{13}C_2$-$^{2}H_3$-voriconazole as internal standard.

RESULTS: Voriconazole and voriconazole-N-oxide concentrations can be detected with good accuracy and reproducibility within the therapeutic range (ref. 1-6 mg/L). Accuracy ranged from -3.0%-4.2% for voriconazole and -1.0%-3.8% for voriconazole-N-oxide and overall coefficients of variation (CV) ranged from 2.9%-7.5% for voriconazole and 4.0%-10.8% for voriconazole-N-oxide. However, voriconazole-N-oxide is relatively unstable at room temperature (Low QC sample: -81.1% after 120 hours), therefore samples should be cooled (2-8°C) after sampling to detect reliable voriconazole-N-oxide concentrations.

CONCLUSIONS: An accurate and simple assay for the analysis of voriconazole and voriconazole-N-oxide to enable therapeutic drug monitoring was developed and validated for all critical parameters.

KEYWORDS: voriconazole, voriconazole-N-oxide, LC-MS/MS, therapeutic drug monitoring

INTRODUCTION

Voriconazole is a commonly used anti-fungal drug for treatment and prophylaxis of invasive fungal disease [1]. Metabolism of voriconazole is primarily by cytochrome P450 (CYP) 2C19 iso-enzymes, with a smaller contribution of CYP3A4 and CYP2C9 iso-enzymes [2]. The main metabolite of voriconazole is voriconazole-N-oxide, which is the result of N-oxidation of the fluoropyrimidine ring [2]. Trough concentra-

tions of voriconazole are correlated with its efficacy and toxicity.
Target through concentrations range from 1 to 6 mg/L in serum. Concentrations lower than 1 mg/L are associated with decreased efficacy, while concentrations higher than 6 mg/L are associated with adverse effects, such as visual disturbances and hepatic toxicity [3]. Voriconazole shows a large inter- and intra-individual pharmacokinetic variability [1]. To improve efficacy and safety, routinely use of therapeutic drug monitoring (TDM) for voriconazole has become standard care [4].
Voriconazole-N-oxide shows negligible antifungal activity compared to voriconazole [5]. However, TDM of this metabolite can be useful, to determine the extent of voriconazole metabolism, which gives a better understanding of the variability in voriconazole serum concentrations [6]. Metabolism of voriconazole can be increased in ultra-rapid metabolizers [7], or in case of drug-drug interactions with CYP inducers, such as rifampicin and phenytoin [8]. Inflammation [9], liver impairment [10] and drug-drug interactions with CYP inhibitors, such as omeprazole and clarithromycin [8] can lead to decreased voriconazole metabolism. Furthermore, metabolism of voriconazole can be decreased in poor and intermediate metabolizers [7].
A low voriconazole concentration with a corresponding low voriconazole-N-oxide concentration may indicate non-compliance or malabsorption, while a high voriconazole concentration with a corresponding high voriconazole-N-oxide concentration may indicate an overdose.
To date only a few published high performance liquid chromatography (HPLC) methods included the metabolite in their assay [5,11,12]. To optimize voriconazole treatment, it would be useful to optimize the assays that are only measuring the parent drug [13,14]. In addition, using stable isotopically labeled (SIL) internal standards instead of structure analogues would help to improve accuracy and precision as shown by international proficiency testing for antifungal drugs [15].
Therefore, this study aimed to present a strategy to optimize a LC-MS/MS method to be more informative for clinicians by including also the voriconazole-N-oxide metabolite and to increase its analytical performance by using a SIL internal standard.

MATERIALS AND METHODS

Chemicals, reagents and materials

Voriconazole, voriconazole-N-oxide and $^{13}C_2$-$^{2}H_3$-voriconazole (the internal standard) were purchased from Alsachim (Illkirch Graffenstaden, France). For the chemical structures, see **Figure 1**. Trifluoroacetic acid and acetonitrile were supplied by Biosolve BV (Valkenswaard, The Netherlands). Ammonium acetate, methanol Lichrosolv (LC-MS grade)® and acetic acid were provided by Merck (Darmstadt, Germany) and ammonium formate from Acros (Geel, Belgium). The aqueous precipitation reagent consisted of 25 µg/L $^{13}C_2$-$^{2}H_3$-voriconazole in a mixture of 160 mL/L methanol and 840 mL/L acetonitrile. Bovine serum was purchased from Life technologies Europe BV (Bleiswijk, the Netherlands). Human blank serum was purchased from MerckMillipore (Amsterdam, the Netherlands).

Instrumentation and settings

The analyses were carried out on a triple quadrupole TSQ® Quantum Access MAX LC-MS/MS system with a Surveyor® MS pump and a Surveyor plus® autosampler. A 50 mm × 2.1 mm C18, 5-µm HyPURITY Aquastar analytical column was used for the chromatographic separation, with a column temperature of 20°C. The autosampler temperature was set to 10°C. All instrumentation was from Thermo Fisher Scientific (Waltham, MA, USA). The mobile phase (pH 3.5) contained an aqueous buffer (A) (acetic acid 35 mL/L, ammonium acetate 5 g/L, and trifluoroacetic acid 2 mL/L), water (B) and acetonitrile (C). The flow rate was 0.5 ml/min. The elution gradient is shown in **Table 1**. The injection volume was 5 µL.
The TSQ® Quantum Access MAX mass selective detector operated in electrospray posi-

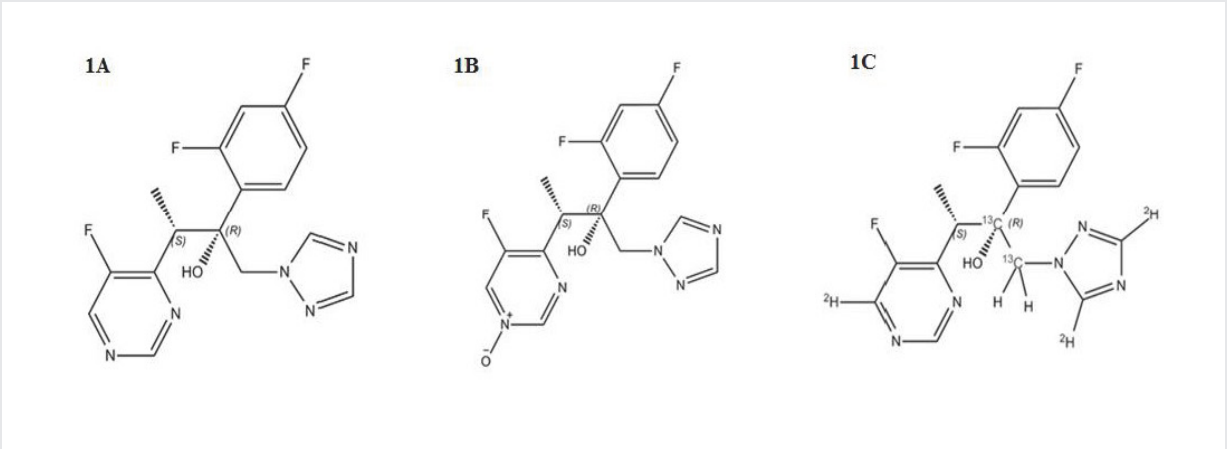

Figure 1. Chemical structures of voriconazole (1A), voriconazole-N-Oxide (1B) and $^{13}C_2$-$^{2}H_3$-voriconazole (1C)

tive ion mode with selected reaction monitoring (SRM). The following settings were used: Ion source spray voltage: 3500 V, sheath gas pressure: 35 Arbitrary units (Arb.), auxiliary gas pressure: 10 Arb., collision gas pressure1.5 mTorr and the capillary temperature 350°C. The subsequent mass parameters were used at a unit resolution of 0.5 m/z: voriconazole m/z 350.1 → m/z 280.9 (collision energy 17 eV), voriconazole-N-oxide m/z 366.1 → m/z 224.1 (collision energy 16 eV) and $^{13}C_2$-$^{2}H_3$-voriconazole m/z 355.1 → m/z 284.0 (collision energy 18 eV). $^{13}C_2$-$^{2}H_3$-voriconazole was used as SIL internal standard for both voriconazole and voriconazole-N-oxide. We used Xcalibur® software version 2.0.7 SP1 (Waltham, MA, USA) for peak height integration.

Standard stocks and serum samples

Preparation of the calibration samples was carried out by spiking stock solution A (voriconazole 201.6 mg/L, voriconazole-N-oxide 202.0 mg/L in methanol) to bovine serum and quality control (QC) samples were prepared by spiking stock solution B (voriconazole 199.8 mg/L, voriconazole-N-oxide 199.2 mg/L in methanol) to bovine serum. Calibration standards (voriconazole: 0.10-0.2-0.50-1.01-2.02-4.03-6.05-8.06-10.08 mg/L, voriconazole-N-oxide: 0.10-0.51-1.01-2.02-4.04-6.06-8.08-10.10 mg/L) and QC samples (see **Table 2**) were stored at -20°C.

Table 1. Gradient elution

Time (min)	% elution		
	A	B	C
0.00	5	95	0
1.50	5	0	95
2.20	5	0	95
2.21	5	95	0
2.60	5	95	0

A: aqueous buffer, B: water, C: acetonitrile

Procedure of sample preparation

10 µL bovine serum with 750 µL precipitation reagent was homogenized for 1 min in a 2.0 mL autosampler vial and subsequently centrifuged (5 minutes at 11,000 x g). 5 µL of the supernatant was used for injection into the LC-MS/MS.

Validation

Validation parameters included selectivity, linearity, accuracy, precision, recovery and stability, and were based on FDA and EMA guidelines [16,17]. Because we updated our previous method, the stability tests for voriconazole and the selectivity/interference tests were not performed extensively [13].

A calibration curve was obtained on each analytical day. For the determination of accuracy and precision, five different QC samples were used. They included lower limit of quantification (LLOQ), low, medium, high and 10 times over the curve dilution (OC) samples. Samples were analyzed in quintuplicate at three different days. One-way analysis of variance (ANOVA) was used as statistical test.

Peak height of QC samples in bovine serum (low, medium, high) were compared with corresponding control samples to calculate extraction recovery, matrix effects, and process efficiency (n = 5) [18]. QC samples were prepared by prespiking voriconazole and voriconazole-N-oxide with $^{13}C_2$-$^{2}H_3$-voriconazole in a mixture of methanol/acetonitrile (4:21, v/v), control samples were prepared by postspiking voriconazole and voriconazole-N-oxide with $^{13}C_2$-$^{2}H_3$-voriconazole in a mixture of methanol/acetonitrile (4:21, v/v).

The extraction recovery was calculated by comparing peak heights of voriconazole and voriconazole-N-oxide in prespiked samples with peak heights of voriconazole and voriconazole-N-oxide in post-spiked samples. The matrix effects were determined by comparing peak heights of voriconazole and voriconazole-N-oxide in post-spiked samples with peak heights of voriconazole and voriconazole-N-oxide in the extraction fluid. Total process efficiency was determined by comparing peak heights of voriconazole and voriconazole-N-oxide in prespiked samples with peak heights of voriconazole and voriconazole-N-oxide in the extraction fluid.

As this method was developed for the analysis of voriconazole in human serum, a matrix comparison between bovine serum and human serum was performed. The calibration curve was prepared in bovine serum on seven levels, control samples were prepared in human serum also on seven levels. The analysis was performed in triplicate and the bias between both samples matrixes was calculated to determine whether there was a matrix effect.

To evaluate the selectivity of the method, six lots of blank human serum samples were analyzed in order to investigate potential interferences at the retention times of voriconazole and voriconazole-N-oxide. Furthermore, six lots of blank human serum were spiked with

Table 2. Accuracy and precision

	QC levels of VRZ					QC levels of VNO				
	LLOQ	LOW	MED	HIGH	OC	LLOQ	LOW	MED	HIGH	OC
Mean concentration (mg/L)	0.100	0.50	4.00	7.99	20.0	0.100	0.498	3.98	7.97	19.9
Bias (%)	4.2	2.0	-0.7	2.6	-3.0	1.6	-0.2	0.3	3.8	-1.0
Within-run CV (%)	4.9	2.8	1.9	3.6	2.0	10.8	5.6	3.6	4.3	4.0
Between run CV (%)	5.7	6.0	3.2	0.0	2.1	0.0	7.7	5.5	0.4	0.0
Overall CV (%)	7.5	6.7	3.7	3.6	2.9	10.8	9.5	6.6	4.4	4.0

QC: Quality control, VRZ: Voriconazole, VNO: Voriconazole-N-Oxide, LLOQ: Lower limit of quantification, MED: medium, OC: 10 times over the curve dilution.

LLOQ standards of voriconazole and voriconazole-N-oxide and analyzed.
The stability of voriconazole in serum was investigated earlier [13] as well as autosampler stability, which remained valid as sample preparation was similar as before. The same method of stability testing was used for voriconazole-N-oxide. Voriconazole-N-oxide concentrations were measured in QC samples (low, high) after 4 cycles of freeze–thaw, after 0, 24, 48, 72, 96, 120 hours at room temperature and after 0, 24, 48, 72, 96, 120 hours in the autosampler (10°C). The concentrations of voriconazole-N-oxide were reported as percentage of the concentration of the freshly made QC samples .

RESULTS AND DISCUSSION

Method validation

For the chromatograms of blank human serum and $^{13}C_2$-$^{2}H_3$-voriconazole and the LLOQ of voriconazole, and voriconazole-N-oxide, see **Figure 2**. All calibration curves were linear by using a weight factor of 1/x over a range 0.05–10 mg/L for voriconazole and voriconazole-N-oxide.
The validated LLOQs were 0.1 mg/L for both voriconazole and voriconazole-N-oxide. The results of accuracy and precision are summarized in **Table 2**. Calculated biases were -3.0 to +4.2% for voriconazole and -1.0 to 3.8% for voriconazole-N-oxide. The overall CV (%) varied between 2.9 and 7.5% for voriconazole and between 4.0 and 10.8% for voriconazole-N-oxide. Interassay variability of the calibration curves are shown in **Table 3**.
The recovery, matrix effects and total process efficiency for low, medium and high QC samples, calculated on substance/internal standard ratios are shown in **Table 4**. Matrix comparison showed no difference between the analysis of voriconazole and voriconazole-N-oxide in bovine serum and in human serum (bias <15%). No interference of blank human serum was observed at retention times of voriconazole and voriconazole-N-oxide (detected peaks <20% of LLOQ). The CV (%) of six human serum lots spiked with LLOQ standards was < 20%.
Measured concentrations of voriconazole-N-oxide were 6.9% (QC low) and 14.2% (QC high) lower than the nominal concentration after 24 hours at room temperature, 3.9% (QC low) and 2.2% (QC high) lower than the nominal concentration after 4 freeze-thaw cycles and 6.9% (QC low) and 7.5% (QC high) lower than the nominal concentration after 72 hours in the autosampler (10°C). The results of the stability study are displayed in **Figure 3**. Remarkably, after 120 hours at room temperature, the voriconazole-N-oxide concentration was 81.1% (QC low) and 84.1% (QC high) lower than the nominal concentration.

Clinical practice

Voriconazole and voriconazole-N-oxide concentrations are measured trice weekly at the University Medical Center Groningen. TDM of voriconazole is routinely performed in our hospital, especially for patients with invasive fungal infections who are treated with voriconazole. This method has also been used for research purposes in our hospital [9]. Between April 2016 and December 2017, 717 voriconazole and 590 voriconazole-N-oxide concentrations were determined. The median voriconazole concentration was 2.5 mg/L (interquartile range 1.3-4.2 mg/L) and the median voriconazole-N-oxide concentration was 2.6 mg/L (interquartile range 1.6-3.7 mg/L). See **Figure 4** for a graphical

Table 3. Interassay variability of the calibration curves.

Component	Slope + (SD)	Intercept + (SD)	Correlation Coefficient
Voriconazole	0.676 (0.00571)	-0.0361 (0.00742)	0.99911
Voriconazole N-oxide	0.108 (0.000921)	-0.000599 (0.00139)	0.99920

SD: Standard deviation.

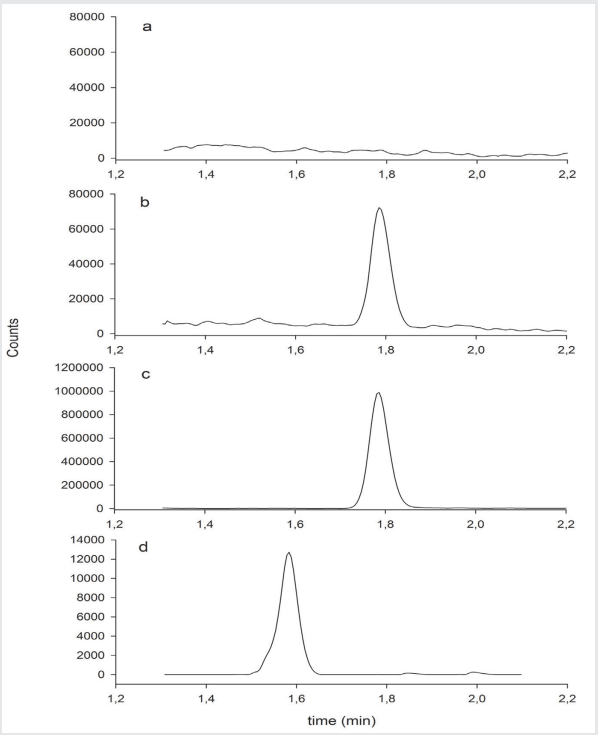

Figure 2. Chromatograms of blank human serum (a), lower limit of quantification (LLOQ) of voriconazole (b), internal standard $^{13}C_2$-$^{2}H_3$-voriconazole (25 µg/L) (c) and LLOQ of voriconazole-N-oxide (d).

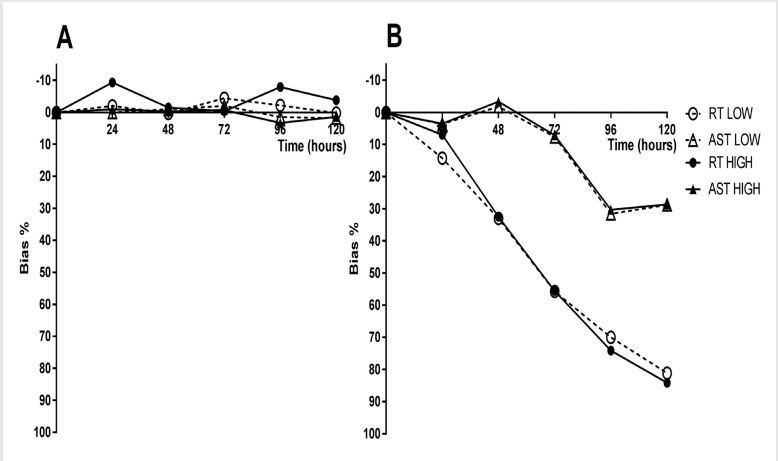

Figure 3. Bias (mean) at room temperature (circles) and autosampler (10°C; triangles) for voriconazole (3A) and voriconazole-N-oxide (3B). Solid symbols represent high concentrations and open symbols represent low concentrations. All measurements were performed in quintuplicate; RT: Room temperature; AST: Auto sampler temperature

Table 4. Recovery (%), Matrix effects (%), and total process efficiency.

	Voriconazole			Voriconazole-N-oxide		
	LOW	MED	HIGH	LOW	MED	HIGH
Recovery(%)	101.1	107.2	98.6	104.7	105.0	95.0
Matrix effects (%)	102.4	99.7	103.7	100.2	98.5	103.3
Total process efficiency (%)	103.5	106.9	102.3	104.9	103.4	98.2

MED: Medium

representation of these data. The addition of voriconazole-N-oxide to the method gives us more insight into the metabolism of voriconazole and the metabolic capacity of the liver. For example, this method was used in a study to investigate the effect of inflammation on voriconazole metabolism. We observed that metabolism of voriconazole is decreased during inflammation, as reflected by a reduction in the formation of voriconazole-N-oxide. Therefore, this study confirmed that inflammation contributes to the large inter- and intra-individual variability in voriconazole metabolism, and emphasizes the need for measurement of voriconazole-N-oxide concentrations [9].

DISCUSSION

We developed an accurate and simple assay for the analysis of voriconazole and voriconazole-N-oxide that was validated for all critical parameters.

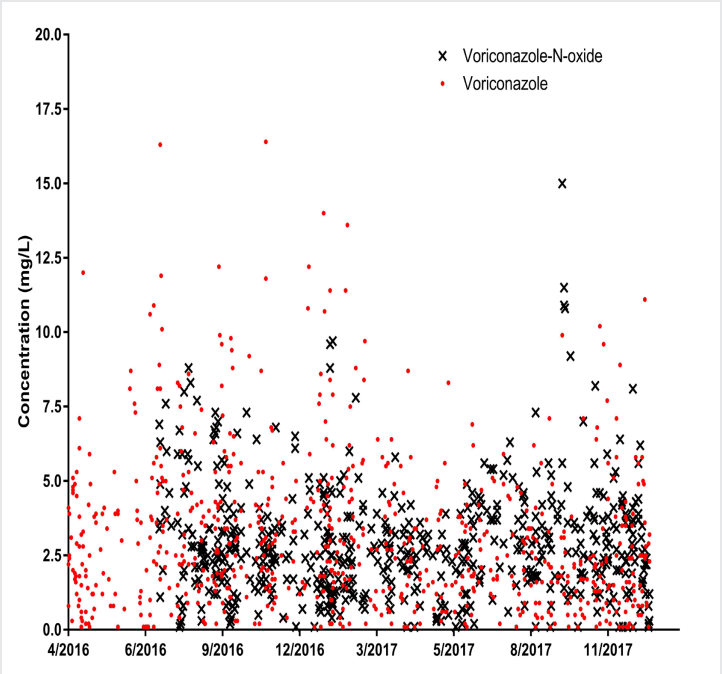

Figure 4. Voriconazole and voriconazole-N-oxide concentrations measured between April 2016 and December 2017.

Voriconazole-N-oxide is relatively unstable at room temperature, with a bias of 14.2% and 81.1% for low QC samples and 6,2% and 84.1% for high QC samples after 24 hours and 120 hours at room temperature respectively. Therefore, blood samples for this analysis have to be centrifuged as soon as possible. The collected serum should be stored at -20°C as soon as possible, but within 24 hours, in order to detect reliable voriconazole-N-oxide concentrations. These results are in concordance with other publications [5,11].

Compared to our previously published method [13], this method has the advantage that voriconazole-N-oxide levels can be determined as well. As stated in the introduction, the analysis of voriconazole-N-oxide can be useful in situations in which the voriconazole concentration cannot be explained properly. Several LC-MS/MS methods that determine voriconazole concentrations have been published before [19]. However, to our knowledge there are only three published HPLC methods that determine both voriconazole and voriconazole-N-oxide [5,11,12]. Both Yamada et al. and Eiden et al. used an isocratic HPLC method with UV detection. These methods have the disadvantage of being less sensitive and selective compared to our LC-MS/MS method. In addition, sample preparation for these methods is more time consuming, and runtimes are longer.

Similar to our method, Decosterd et al. developed a LC-MS/MS method, which can determine both voriconazole and voriconazole-N-oxide with a SIL compound as internal standard [11]. Their method not only determined voriconazole and its main metabolite, but also five other anti-fungal agents and one metabolite. There are a few disadvantages to their method. First, sample preparation requires 100 µL sample while our method only requires 10 µL, which is ideal when microsampling is required or desirable, like in pediatric patients, patients suffering from venous damage and in small animals. Furthermore, Decosterd et al. [14] also used protein precipitation as sample preparation, however their method is more extensive compared with our method of precipitation and injection in one vial. The relatively long runtime of 7 minutes described by Decosterd et al. [14] interferes with fast turnaround times and urgent patient sample analysis. For example, one urgent patient requires the analysis of at least 13 injections (1 blank, 8 standards, 3 QC samples and 1 patient), which takes 91 min, while our method with 2.6 minutes, runtime takes less than 34 min. A shorter run time is of critical importance in a clinical setting. It could mean the difference between reporting a result the same day or the next day, especially if you take into account that the equipment is not solely used for voriconazole but also for other drugs and patient sample analysis.

Compared to our previously published method, the advantages of the updated method are the faster turnaround time (2.6 minutes instead of 3.6 minutes) and the use of a SIL parent compound as internal standard for voriconazole [13]. Although the validation results comply with the criteria using the SIL parent compound as internal standard, the use of a SIL for the metabolite would be preferable for obtaining the best results and most robust assay. Unfortunately, the SIL for the metabolite was not yet available at the time of validation.

A limitation of this study is that we did not validate our method in hemolyzed or lipemic samples, however with the use of a SIL internal standard, correction for such samples is possible.

Furthermore, the EMA reported that voriconazole was light sensitive [20]. As voriconazole-N-oxide has a comparable molecular structure, it is possible that voriconazole-N-Oxide is light sensitive as well. Further research is necessary to investigate the influence of light on the stability of voriconazole and voriconazole-N-oxide in serum.

This example shows the importance to update analysis methods, in order to adapt to the needs of the clinic and to continue to perform state of the art analyses. Quality control

programs are a valuable tool for the quality improvement of analyzing methods, as these programs can compare results between different types of assays, in order to validate whether the results match to the expectations of the quality necessary for patient care.

CONCLUSION

In conclusion, an accurate and simple assay for the analysis of voriconazole and voriconazole-N-oxide to enable TDM was developed, using a SIL internal standard and validated for all critical parameters.

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