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Journal of Chromatography B, 865 (2008) 74–80
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of voriconazole in human plasma and saliva using high-performance liquid chromatography with fluorescence detection

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Received 27 August 2007; accepted 17 February 2008
Available online 10 March 2008

Abstract

Voriconazole is a widely used triazole antifungal agent with a broad spectrum including *Aspergillus* species. A simple, sensitive and selective high-performance liquid chromatography method for the determination of voriconazole in human plasma and saliva was developed. Drug and internal standard (UK-115 794) were extracted from alkaline plasma and saliva with *n*-hexane–ethyl acetate (3:1, v/v) and analyzed on a Luna C 18 column with fluorimetric detection set at excitation and emission wavelengths of 254 and 372 nm, respectively. The calibration curve was linear through the range of 0.1–10 μg/ml using a 0.3 ml sample volume. The intra- and inter-day precisions were all below 6.1% for plasma and below 9.1% for saliva. Accuracies ranged from 94 to 109% for both matrices. Mean recovery was 86 ± 4% for voriconazole. The method showed acceptable values for precision, recovery and sensitivity and is well suited for routine analysis work and for pharmacokinetic studies.

Keywords: Voriconazole; HPLC; Fluorescence detector; Plasma; Saliva

1. Introduction

Voriconazole (VRC) ((2*R*,3*S*)-2-(2,4-difluorophenyl)-3-(5-fluoro-4-pyrimidinyl)-1-(1*H*-1,2,4-triazol-1-yl)-2-butan-2-ol)) (Fig. 1) is a broad spectrum antifungal agent with potent activity against *Aspergillus* and *Candida* species [1–5] and emerging fungal pathogens, such as *Scedosporium* and *Fusarium* species [6–8]. Voriconazole has been in clinical use for several years for a wide range of invasive and opportunistic fungal infections. Voriconazole displays non-linear pharmacokinetics in adults [9–11] but has linear pharmacokinetics in children [12] and is extensively metabolized in the liver, mainly by CYP2C9, CYP2C19 and CYP3A4 isoenzymes [13]. Inter-individual variability is generally high, both in children and in adults, and diverse manifestations of toxicity are possibly attributed to high voriconazole concentrations [14,15]. Elevated voriconazole levels may be caused by hepatic dysfunction, hepatic polymorphism of the isoenzyme CYP 2C19 or drugs that inhibit this enzyme [15,16]. Although routine monitoring of voriconazole levels is not recommended, both under- and overexposure to voriconazole may have serious consequences. Clinical failure may be related to subtherapeutic drug exposures [17–19] and high drug exposures may result in significant toxicities such as hepatotoxicity [20,21] and a therapeutic drug monitoring may be helpful to individually optimize therapy with voriconazole.

Several methods are reported to detect voriconazole in biological fluids by using HPLC with UV detection [9,22–30], HPLC–MS [31–33], capillary zone electrophoresis [34] and by a simple agar well diffusion bioassay [24] but to our knowledge no method for determination of voriconazole in biological fluids using fluorescence detection has been described so far. The bioassay is easier than the HPLC methods and needs no special equipment but suffers from a greater degree of variability and lacks the required sensitivity for voriconazole and is therefore not sufficient for pharmacokinetic studies. Stopher and Gage [22,23] developed two different HPLC methods with UV detection. The first method is complex and technically difficult, because it involves column-switching of three columns [22]. The second method is easier, using protein precipitation with acetonitrile as the only sample preparation and an internal standard is not required [23]. Perea et al. [24] and Pascual et al. [25] also use acetonitrile precipitation of 500 μl plasma followed by reversed HPLC on a C18 column and UV detection. Deproteinization by precipitation is a simple and rapid procedure. The

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doi:10.1016/j.jchromb.2008.02.009

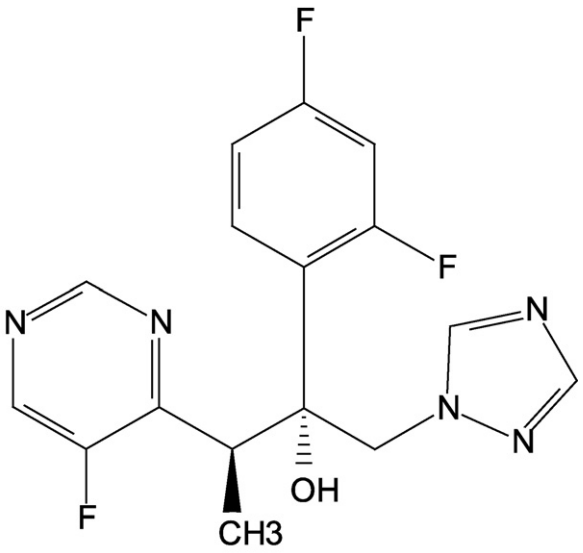

Fig. 1. Chemical structure of voriconazole.

disadvantage is the eventual loss of analytes by adsorption at the precipitate and the lack of a concentration step. Péhourcq et al. [26] used a direct plasma injection HPLC micro method and detection by UV (255 nm). Linear response was observed over the concentration range 0.5–10 μg/ml and the lower limit of quantitation (LOQ) was 0.2 μg/ml. Pennick et al. [27] reported a HPLC method using UV detection for analysis of voriconazole with solid-phase extraction using two different solutions for the washing step and methanol–glacial acetic acid (99:1, v/v) for elution. The limit of quantitation was 0.2 μg/ml and the method requires a sample volume of 500 μl plasma and an internal standard. The method given by Wenk et al. [28] works with a Chromolith RP 18e monolithic silica rod column for determination of voriconazole in human plasma with a flow rate of 3.5 ml/min and a total run time of 4 min. Khoschsorur et al. [29] developed a simple HPLC-UV method for simultaneous determination of voriconazole, itraconazole and its metabolite hydroxyitraconazole in human serum with a linearity from 0.1 to 8.0 μg/ml. Chhun et al. [30] reported simultaneous quantification of voriconazole and posaconazole using a 100 μl plasma sample. Both methods using liquid–liquid extraction and UV detection at 255 nm. To our knowledge three different methods are described using HPLC–MS techniques for the determination of voriconazole in aqueous humor [31] and serum [32,33]. These methods require only small sample volumes and minimal sample preparation, but are expensive and generally not available. One assay using liquid extraction and separation by HPLC and UV detection was described in [11] for determination of voriconazole in saliva samples. The lower limit of quantification was 0.01 μg/ml and the upper limit of the calibration curve was 2.0 μg/ml. The pharmacokinetic profiles for saliva followed a pattern similar to those observed for plasma and there was a highly significant correlation between plasma and saliva voriconazole concentrations.

In the current paper, we describe a simple HPLC method for clinical routine use and for pharmacokinetic studies using fluorescence detection, which shows sufficient specificity, sensitivity and simplicity for the measurement of voriconazole in human plasma and saliva samples. Detection based on fluorescence is generally more sensitive than UV absorption and provides high reliability in the identification and determination of compounds of interest when patients are on combined therapy. It is of particular importance that the proposed method requires as little as 300 μl of plasma or saliva, because the procedure presented here has been approved to a pharmacokinetic study to investigate the pharmacokinetic and pharmacodynamic behavior of the parenteral formulation of voriconazole in immunocompromised children (2 to <12 years of age). Based on the findings that voriconazole levels in saliva may have some potential use in predicting plasma levels [11] we also want to investigate, if measurement of voriconazole in saliva is a useful and suitable alternative to plasma. Especially in therapeutic drug monitoring an easier sample collection being non-invasive and painless is more acceptable to patients, particularly children.

2. Experimental

2.1. Chemicals and standards

VRC (UK-109 496) and I.S. (UK-115 794) were kindly supplied by Pfizer Central Research (Sandwich, United Kingdom). HPLC grade methanol, *n*-hexane, acetonitrile and water were obtained from J.T. Baker (Deventer, The Netherlands). Phosphoric acid (85%) and TEMED (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine) were purchased from Sigma–Aldrich (Steinheim, Germany). Potassium dihydrogen phosphate was obtained from Merck (Darmstadt, Germany). A pH 9.0 borate buffer (0.1 M) was prepared by mixing 3.9 g of boric acid (Merck, Darmstadt, Germany) and 7.3 g of disodium tetraborate (J.T. Baker, Deventer, The Netherlands) and adding water until 1000 ml. The standard stock solution of VRC was prepared at a concentration of 1000 μg/ml in methanol and diluted to 100 and 10 μg/ml in methanol for the working solutions. The I.S. was prepared at 1000 μg/ml in methanol and diluted to 50 μg/ml in methanol for the working solution. Stock solutions and working solutions were stored at −20 °C. Two sets of calibration standards, one for plasma at 0.1, 0.2, 0.4, 1.0, 2.0, 4.0 and 10.0 μg/ml, and one for saliva at 0.1, 0.4, 1.0, 4.0 and 10 μg/ml were prepared by spiking blank plasma and saliva samples with the appropriate working solutions. In the same manner, plasma and saliva quality controls (QC) were prepared to perform fluorimetric experiments and storage stability tests. The saliva samples were collected by chewing on the cotton wool swabs of a saliva-collecting device (Salivette®) (Sarstadt; Nümbrecht, Germany) impregnated with 20 mg citric acid. Standards were stored at −20 °C until analysis.

2.2. Instruments and chromatographic conditions

Chromatographic conditions were identical for plasma and saliva samples. Analyses were carried out on a Gynkotek apparatus (Gynkotek, now Dionex, Germering, Germany) consisting

of a model M480 pump, a model GINA 50 autosampler fitted with a Rheodyne 7010 injector (100 μl loop) and a model FP-2020Plus Intelligent Fluorescence Detector (Jasco Corporation, Hachioji, Tokyo, Japan). Fluorimetric experiments were conducted by analyzing replicates of plasma QC samples with varying emission and excitation wavelengths to select a optimum for the fluorimetric detection of VRC and I.S. Fluorescence was measured with emission and excitation wavelengths set at 372 and 254 nm, respectively. Detector gain was set at 100. The data was acquired and processed by means of Gynkosoft Data System 5.30 (Gynkotec). Chromatographic separation was carried out by a reversed phase HPLC column (LUNA 5u C18 100A, 250 mm × 3.0 mm × 5 mm, Phenomenex Aschaffenburg, Germany) protected by a 4 mm × 2.0 mm C18 guard column (Phenomenex Aschaffenburg, Germany). The column temperature was maintained at 30 °C with a Model 2/1-LL column heater (Sunchrom, Friedrichsdorf, Germany). The mobile phase, which is similar to Pennick et al. [27], consisted of 0.01 M potassium dihydrogen phosphate buffer (containing 0.01 M TEMED as modifier; pH was adjusted with phosphoric acid to pH 6.8) and acetonitrile (55:45, v/v). The mobile phase was degassed by helium and a flow rate of 0.5 ml/min was used.

2.3. Sample preparation

Of each plasma or saliva sample a 300 μl aliquot was pipetted into a 2 ml polypropylene tube (Eppendorf, Hamburg, Germany). After the addition of 500 μl of a 0.1 M borate buffer (pH 9.0) and 10 μl internal standard working solution the tube was vortexed. The analytes were extracted with 1 ml of *n*-hexane–ethyl acetate (3:1, v/v) by mixing for 2 min, followed by centrifugation for 4 min at 6000 × *g*. The upper organic layer was separated in a glass tube and the aqueous residue was extracted a second time with 1 ml of *n*-hexane–ethyl acetate (3:1, v/v). After mixing and centrifugation, the organic layer was added into the glass tube and evaporated under nitrogen at 40 °C. The residue was reconstituted in 150 μl of mobile phase and 20 μl aliquots of this solution were injected onto the HPLC system.

2.4. Accuracy, precision and limit of quantitation

Validation of the HPLC method was performed by determining the intra-day and inter-day accuracy and precision under the extraction and analytical condition as described in Sections 2.2 and 2.3. The method was validated for human plasma samples using heparin as the anticoagulant and partially validated for saliva samples. Six replicate plasma calibration samples were assayed intra-day at five (0.1, 0.4, 1, 4 and 10 μg/ml) and inter-day at seven different concentrations (0.1, 0.2, 0.4, 1, 2, 4 and 10 μg/ml) of VCR. The calibration curve was obtained using seven calibration points, ranging from 0.1 to 10.6 μg/ml. Five replicate saliva calibration samples were assayed intra- and inter-day at five different concentrations (0.1, 0.4, 1, 4 and 10 μg/ml) of VCR. The calibration curve was obtained using five calibration points, ranging from 0.1 to 9.3 μg/ml. Furthermore five QC saliva samples (0.1, 0.4, 1.0, 4.0 and 10.0 μg/ml) were analyzed with a routine standard curve in plasma.

Accuracy was calculated as a percentage error, whereas precision was expressed as the relative standard deviation (R.S.D.) of each calculated concentration. Precision was expected to be less than 15% at all concentrations, except for the LOQ, for which 20% was acceptable. For all calibration curves, a linear regression with a $1/x$ weighting factor was used, considering analyte-to-I.S. peak height ratio. A correlation of more than 0.99 was desirable for each calibration curve.

2.5. Recovery

Absolute recovery of VCR and I.S. was determined by comparing peak heights of VCR and I.S. obtained after injection of the processed plasma calibration samples with those achieved by direct injection of the same amount of drugs in the mobile phase at 0.1, 0.4, 1.0, 4.0 and 10 μg/ml (six samples for each concentration level).

2.6. Stability

Since other authors previously showed that VCR in pooled plasma was stable through two freeze–thaw cycles at −25 °C up to 14 months, no stability study in plasma was conducted [22]. All plasma samples were analyzed within 14 months since their freezing. The short-term stability in saliva samples was examined by analyzing replicates of the low and high saliva QC samples at room temperature for 24 h. The effect of repeated freezing and thawing in saliva samples was also studied for up to three cycles. Each freeze–thaw cycle consisted of a minimum of 24 h frozen at −20 °C followed by a complete thaw at room temperature. Samples were analyzed prior to being frozen and after the third freeze–thaw cycle. Stability of VCR in human saliva was tested after storage at −20 °C for 30 days. Post-preparative stability was determined for a set of processed and reconstituted low and high saliva QC samples left at −20 °C for 48 h (extract stability) and at room temperature for 24 h (autosampler stability). For each concentration and each storage condition, five replicates were analyzed in one analytical batch. The concentration of VCR after each storage period was related to the initial concentration as determined by samples that were freshly prepared and immediately processed. Samples were considered stable if the deviation of the freshly prepared standard was less than 10%.

3. Results

3.1. Chromatography

Retention times of voriconazole and internal standard were 7.9 and 8.9 min, respectively. Representative chromatograms of the blank human plasma extract, plasma sample at the lowest and highest spiked VRC concentration (0.1 and 10 μg/ml) and a plasma sample obtained from patient under polymedication (ciprofloxacin, metronidazole, valacyclovir, furosemide, granisetron, esomeprazole, zopiclone, folic acid) are shown in Fig. 2. The calibration curve was linear in the range from 0.1 to 10.6 μg/ml with mean values ($n = 6$) for slope of

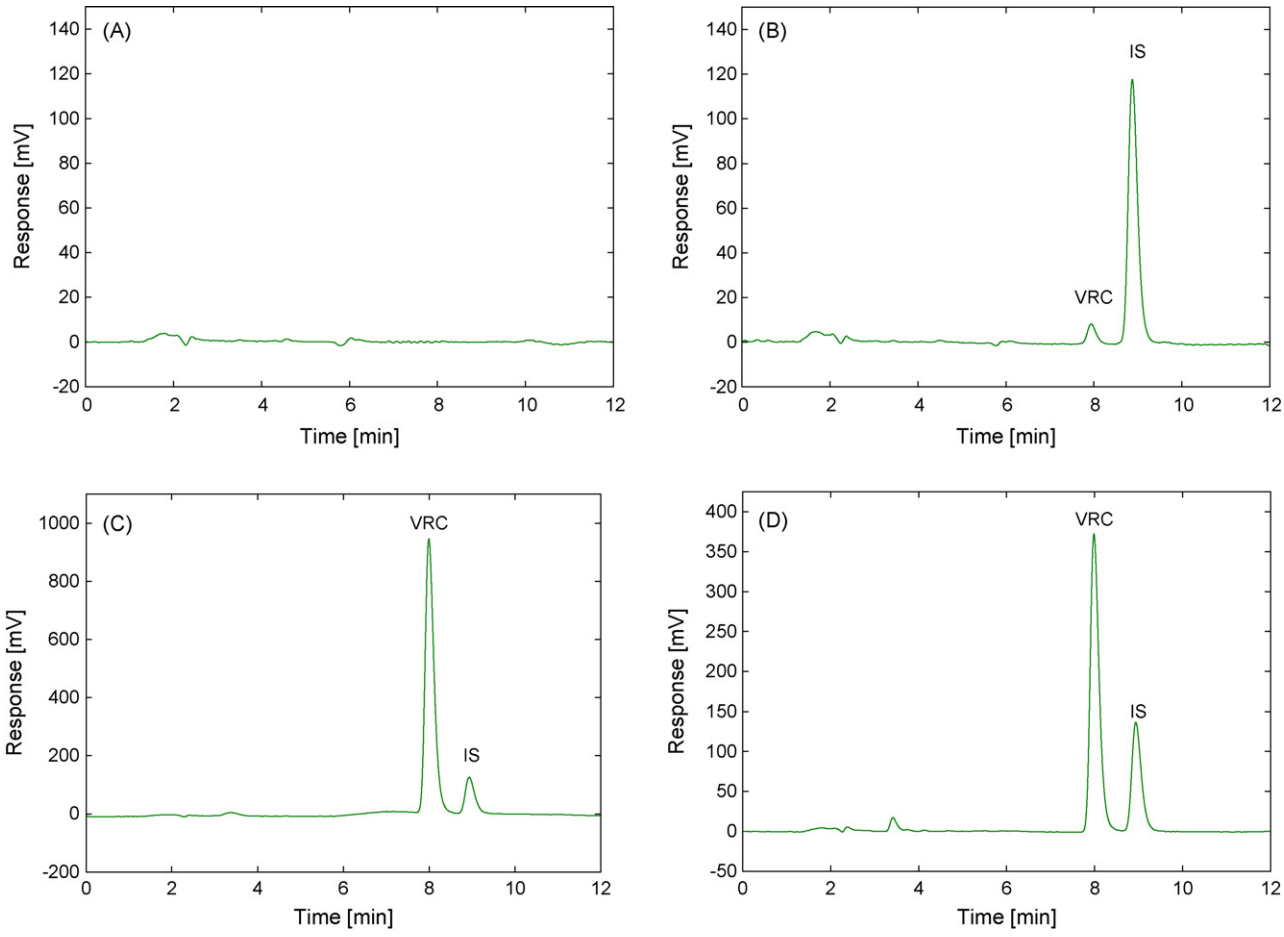

Fig. 2. Typical chromatograms of (A) blank human plasma, (B) blank plasma spiked with 0.1 μg/ml of VRC (LOQ) and 3.3 μg/ml of IS, (C) spiked plasma with 10.6 μg/ml VRC and 3.3 μg/ml IS and (D) plasma sample obtained from patient under polymedication with VRC concentration of 4.1 μg/ml.

0.72135 ± 0.0263, intercept of 0.00325 ± 0.00376 and correlation coefficient of 0.9999 ± 0.00009.

Typical chromatogram of a saliva sample from patient under polymedication (moxifloxacin, metronidazole, acyclovir, granisetron, ondansetron, doxepin, furosemide, esomeprazole, folic acid) with VRC concentration of 2.9 μg/ml is shown in Fig. 3. The calibration curve was linear in the range from 0.1 to 9.3 μg/ml with mean values ($n = 5$) for slope of 0.60536 ± 0.0323, intercept of 0.00354 ± 0.00323 and correlation coefficient of 0.9998 ± 0.00008.

3.2. Specifity and selectivity

The specifity of the method was evaluated by analyzing plasma and saliva samples from six volunteers in which no interference by endogenous components was noted (see Fig. 2A). Plasma and saliva samples from patients under polymedication receiving voriconazole were measured and examined for potential drug interferences (see Figs. 2D and 3). The samples were collected from ICU patients with broad comedication such as antibiotics (ciprofloxacin, moxifloxacin, metronidazole, gentamycin, amikacin, vancomycin, teicoplanin, co-trimoxazole, piperacillin, imipenem, meropenem), antivirals (valacyclovir, acyclovir), cyclosporin A, methotrexate, melphalan, vincristine, cyclophosphamide, dexamethasone, prednisolone, thiamine, pyridoxine, cyanocobalamin, ascorbic acid, folic acid, loperamide, magaldrate, esomeprazole, allopurinol, furosemide, granisetron, ondansetron, enoxaparin, methyldigoxin, zopiclone, doxepin, midazolam, nitrazepam,

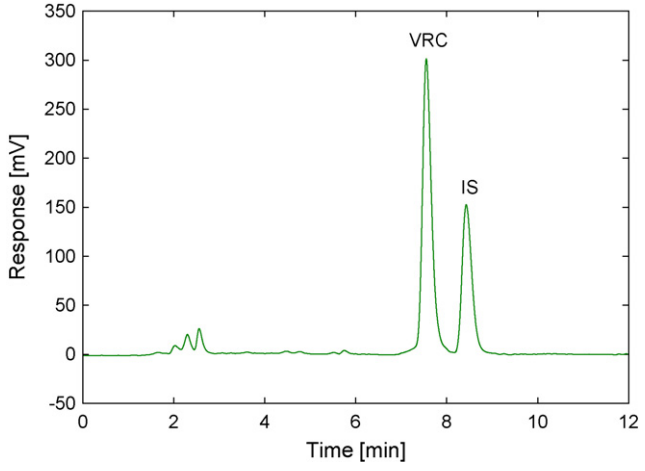

Fig. 3. Typical chromatogram of saliva sample obtained from patient under polymedication with VRC concentration of 2.9 μg/ml.

Table 1
Intra-day precision, accuracy and average recovery (measured each within one day) and inter-day precision and accuracy (measured in 15 consecutive days) of voriconazole in human plasma calibration samples (six individually prepared samples at each calibration level)

Added (μg/ml)	Found (mean μg/ml ± S.D.)	Precision (%R.S.D.)	Accuracy (%)	Recovery VRC (mean% ± S.D.)
Intra-day				
0.099	0.108 ± 0.003	2.60	108.6	83.3 ± 1.3
0.392	0.385 ± 0.008	2.16	98.3	91.4 ± 5.1
0.992	0.940 ± 0.057	6.02	94.8	87.4 ± 10.9
3.881	3.831 ± 0.076	1.98	98.7	82.2 ± 1.7
10.573	10.675 ± 0.349	3.27	101.0	85.6 ± 3.2
All		3.2 ± 1.6	100.3 ± 5.2	86.0 ± 3.7
Inter-day				
0.099	0.095 ± 0.002	2.28	96.2	
0.199	0.200 ± 0.006	2.89	100.5	
0.392	0.401 ± 0.01	2.55	102.3	
0.992	0.986 ± 0.036	3.65	99.4	
1.937	2.024 ± 0.084	4.13	104.5	
3.881	4.023 ± 0.066	1.64	103.7	
10.573	10.338 ± 0.525	5.08	97.8	
All		3.2 ± 1.2	100.6 ± 3.1	

Mean: arithmetic mean. Accuracy (%) = measured concentration/spiked concentration × 100%.

dimenhydrinate, paracetamol, metamizole and morphine. Due to the high specificity of fluorescence detection no interference by drugs, most frequently co-described, was observed at the retention time of voriconazole and internal standard under the conditions employed in this method. Possible interferences of other antimycotics were investigated by analyzing one-by-one pure solutions of amphotericin B, fluconazole, ketoconazole, itraconazole, hydroxyitraconazole and nystatin. None of these compounds caused interference with voriconazole or the internal standard.

3.3. Accuracy, precision, and limit of quantitation

The method showed good precision and accuracy in plasma and saliva samples. Table 1 summarizes the intra- and inter-day precision and accuracy for voriconazole in plasma samples. Intra-day (R.S.D.%) and inter-day precisions (R.S.D.%) of the HPLC determinations gave mean standard deviations as 3.2 ± 1.6 and 3.2 ± 1.2%, respectively. Intra-day and inter-day accuracies were 100.3 ± 5.2 and 100.6 ± 3.1%, respectively. The sensitivity of the method is satisfactory since concentrations

Table 2
Partial validation of voriconazole determination in human saliva calibration samples containing five various concentration levels with five individually prepared samples at each calibration level: (A) Intra-day precision and accuracy (each measured within one day); (B) inter-day precision and accuracy (measured in 5 consecutive days) and (C) calibration of QC saliva samples with a routine standard curve in plasma

Added (μg/ml)	Found (mean μg/ml ± S.D.)	Precision (%R.S.D.)	Accuracy (%)
(A) Intra-day			
0.092	0.094 ± 0.007	7.08	102.1
0.377	0.371 ± 0.008	2.17	98.4
0.916	0.870 ± 0.079	9.08	94.9
3.824	3.657 ± 0.137	3.74	95.6
8.768	8.980 ± 0.227	2.53	102.4
All		4.9 ± 3.0	98.7 ± 3.5
(B) Inter-day			
0.096	0.096 ± 0.004	4.41	99.2
0.406	0.401 ± 0.010	2.48	98.7
0.979	0.926 ± 0.039	4.26	94.6
3.637	3.906 ± 0.068	1.75	107.4
9.349	9.133 ± 0.202	2.21	97.7
All		3.0 ± 1.2	99.5 ± 4.7
(C) Calibration of QC saliva samples with routine standard curve in plasma			
0.092	0.089 ± 0.007	7.93	97.5
0.377	0.386 ± 0.009	2.23	102.4
0.916	0.920 ± 0.084	9.18	100.4
3.824	3.900 ± 0.146	3.75	102.0
8.768	9.594 ± 0.290	2.53	109.4
All		5.1 ± 3.2	102.3 ± 4.4

Mean: arithmetic mean. Accuracy (%) = measured concentration/spiked concentration × 100%.

Table 3
Stability of voriconazole in saliva

Sample concentration (μg/ml)	Concentration found (mean μg/ml ± S.D.)	Precision (%R.S.D.)	Accuracy (%)
Short-term stability for 24 h at RT ($n = 5$)			
0.097	0.103 ± 0.004	3.55	105.8
4.012	4.087 ± 0.058	1.42	101.9
Three freeze and thaw cycles ($n = 5$)			
0.097	0.093 ± 0.002	1.84	96.2
4.012	3.897 ± 0.099	2.54	97.1
Autosampler stability for 24 h ($n = 5$)			
0.097	0.100 ± 0.004	4.27	103.1
4.012	3.958 ± 0.386	9.75	98.7
Extract stability after 48 h at −20 °C ($n = 5$)			
0.097	0.095 ± 0.002	1.61	98.5
4.012	4.076 ± 0.077	1.89	101.6
30 days stability at −20 °C ($n = 5$)			
0.097	0.094 ± 0.002	2.15	97.4
4.012	3.938 ± 0.019	0.47	98.2

of voriconazole in plasma usually range from 0.2 to 8 μg/ml. The limit of quantitation (LOQ) was set at the lowest calibration standard value (0.1 μg/ml; R.S.D. ≤2.6%). The limit of detection (LOD) was 0.044 μg/ml at a signal-to-noise level of 3:1.

Results of validation in saliva samples are shown in Table 2. Intra-day (R.S.D.%) and inter-day precisions (R.S.D.%) of the HPLC determinations gave mean standard deviations as 4.9 ± 3.0 and 3.0% ± 1.2%, respectively. Intra-day and inter-day accuracies were 98.7 ± 3.5 and 99.5 ± 4.7%, respectively. The results of validation experiments in plasma and saliva do not significantly differ from each other and are therefore comparable in each matrix. The routine standard curve in plasma was used for determination of voriconazole in saliva samples. There was no significant difference in precision and accuracy between the plasma and saliva data. The precision ranged from 2.2 to 9.2 with an accuracy of 97.5–109.4% well within the acceptable range of the assay.

3.4. Recovery

Table 1 shows the results of the recovery tests for the five levels tested (0.1, 0.4, 1, 4 and 10 μg/ml). The extraction recoveries in plasma samples ranged from 82 to 91% for voriconazole at all concentration levels. The mean recovery of the internal standard was determined to be 102.8 ± 8.4% at the spiked concentration of 3.33 μg/ml.

3.5. Stability

Saliva QC samples of voriconazole at two concentrations (0.1 and 4.0 μg/ml) were used for stability experiments. Stability experiments were conducted to evaluate the influence of all possible storage conditions between sample collection and analysis. Table 3 presents the results for the short-term (24 h at room temperature) and 30 days stability at −20 °C, freeze/thaw stability (three cycles), stability of the processed samples in the autosampler (24 h at room temperature) and the extract stability (48 h at −20 °C).

Three freeze–thaw cycles and 24 h room temperature storage for low and high quality controls samples indicated that voriconazole was stable in human saliva under these conditions. Saliva QC samples were stable for at least 30 days if stored frozen at approximately −20 °C. Testing of post-preparative stability of QC samples indicated that voriconazole is stable when kept at room temperature for up to 24 h and at −20 °C for up to 48 h. The deviation of the mean test responses to the freshly prepared standard was less than 10% at any of the investigated conditions.

4. Discussion and conclusion

This paper describes the validation of a HPLC method for the quantification of voriconazole in plasma and saliva samples. According to the literature research, voriconazole has been determined using a fluorescent detector for the first time. Fluorimetric detection allows sensitive and reliable determination of voriconazole when patients are on combined therapy. This validated method covers the wide range of linearity for voriconazole (0.1–10 μg/ml) and is suitable for the estimation of voriconazole at different therapeutic dose levels for pharmacokinetic studies as well as for therapeutic drug monitoring. Our liquid–liquid extraction procedure is easy and economical. The proposed method requires a small volume of 300 μl plasma or saliva, which can be advantageous in clinical pharmacokinetic studies, especially if children participate. The proposed method was successfully applied to a pharmacokinetic study (approved by the Ethics Committee of the University of Leipzig; study is ongoing) to investigate the pharmacokinetic and pharmacodynamic behavior of the parenteral formulation of voriconazole in immunocompromised children (2 to <12 years of age) with a higher voriconazole dosage per kg body weight compared to adults (20–60 years of age). The procedure has been employed for over 1 year during which time we measured over 400 patient samples. This method is simple and efficient with good accuracy, precision and reproducibility.

Acknowledgement

Financial support for this study was provided by Pfizer Pharma GmbH (Karlsruhe, Germany) and is gratefully acknowledged.

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