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Determination of Voriconazole in Human Plasma by Liquid Chromatography Tandem Mass Spectrometry: Application in Therapeutic Drug Monitoring

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Abstract

A sensitive, accurate and robust Liquid Chromatography Tandem Mass Spectrometry method has been developed and validated to measure voriconazole trough levels in human plasma. The plasma samples were mixed with fluconazole as an Internal Standard and directed to protein precipitation and drug extraction. An aliquot of 1 µl was injected into the chromatographic system and separated by the Acquity BEH C18 column at a flow rate of 0.30 ml/min in a gradient mobile phase consisting of acetonitrile, Ultrapure water (UPW), methanol and formic acid. Voriconazole was detected by a Triple Quadrupole Detector (TQD) operating on Multiple Reaction Monitoring (MRM) and a positive ion mode Electrospray ionization (ESI) Q1 mass: 350.1 m/z, Q3 mass: 281.1 m/z. Method linearity of the calibration curve (0.10 - 8.00 µg/ml) indicated a correlation coefficient $r \geq 0.99$. The intra and inter-assay accuracy was within 85% - 115% and the intra and inter-assay precision was ≤5.76%. Voriconazole recovery percentage was between 97.69 - 119.62%. The method was successively applied in routine voriconazole TDM.

Keywords

Voriconazole, Human Plasma, Liquid Chromatography, Tandem Mass Spectrometry, Therapeutic Drug Monitoring

1. Introduction

Recently, the number of patients with a compromised immune system have increased due to various factors, including chemotherapy, immunosuppressive agents, grafts, prosthetic devices and broad-spectrum antibiotics. This caused the spread of invasive fungal infections in this group of patients [1] [2] [3]. Annually there are over 150 million severe fungal infection cases resulting in 1.7 million deaths globally [4], which increased the demand for antifungal agents. However, antifungal agents can cause toxic side effects due to the structural and metabolic similarities between mammalian and fungal cells [5].

Voriconazole is a broad-spectrum antifungal agent of the drug class triazole antifungals. Clinically, voriconazole is used for the treatment of invasive fungal infections caused by Aspergillus and Candida species, which are usually seen in immunocompromised patients [6] [7]. The mechanism of action of voriconazole is by targeting ergosterol biosynthesis, an important part of the fungal cell membrane, through the inhibition of the fungal cytochrome P450-dependent enzyme called lanosterol 14-α demethylase, causing an accumulation of lanosterol which causes a defect on the fungal cell membrane resulting in cell death [8] [9]. Voriconazole metabolism occurs through the hepatic cytochrome P450 isoenzymes, mainly CYP2C19 [10]. The CYP2C19 gene is highly polymorphic which can result in a fast or slow drug metabolism. The primary metabolite of voriconazole is voriconazole N-oxide which has a poor antifungal effect furthermore, the exact effect of voriconazole metabolites wasn't directly correlated to toxicity [11] [6]. The bioavailability of voriconazole is approximately 90% and it exhibits nonlinear pharmacokinetics (PK) due to the elimination capacity and PK variability between individuals. It depends on the administrated dose and the patient's metabolism [12] [13]. In addition, voriconazole has a narrow therapeutic index [14]. Plasma trough level of voriconazole range from 1.00 - 5.00 μg/ml is considerably recommended for better outcome and to avoid toxicity [15]. A high concentration of voriconazole in plasma is associated with neurotoxicity, liver dysfunction and hepatotoxicity [16] [17]. However, a low concentration of voriconazole in plasma is associated with treatment failure in patients with an invasive infection, which may lead to devastating consequences [18]. Therapeutic Drug Monitoring (TDM) of voriconazole is recommended for patients treated with voriconazole through measuring the plasma trough level to improve the clinical outcomes, avoid adverse events and improve drug efficacy [19] [20]. Several assays were developed to determine voriconazole levels in biological fluids However, Liquid Chromatography Tandem Mass Spectrometry technology is considered to be more sensitive and selective for clinical applications [21] [22].

Our main objective was to establish a sensitive, accurate and robust analytical method for measuring the plasma trough level of voriconazole taking advantage of the sophisticated technology of Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) to assist the clinicians in monitoring the treatment of patients with invasive fungal infections or as prophylaxis to avoid fungal infections

by giving the appropriate dose of voriconazole to prevent toxicity and achieve the most effective and safest dose for each patient.

2. Materials and Methods

2.1. Chemicals and Solutions

Voriconazole pure reference standard was purchased from the United States Pharmacopeia (USP, China) and fluconazole pure reference standard was used as the Internal Standard (IS) supplied by Jazeera Pharmaceutical Industries (Riyadh, KSA). Methanol LC-MS grade and Acetonitrile LC-MS grade were purchased from CARLO ERBA Reagents (Val de Reuil, France). Ethanol HPLC grade and formic acid analytical grade were purchased from Sigma-Aldrich (Saint Louis, USA). Ultrapure water (UPW) (18.2 MΩ cm) was obtained in-house using the Milli-Q® Integral 5 Water Purification System (Millipore, France).

The stock solution of voriconazole analytical standard was prepared by weighing 5 mg of voriconazole pure standard powder, which was dissolved in 50 ml ethanol to obtain a final concentration (100 µg/ml). The stock solution of fluconazole analytical standard was prepared by weighing 25 mg of fluconazole pure standard and dissolved in 250 ml ethanol to obtain a final concentration of 100 µg/ml. The stock solutions were stable at −40˚C for at least 2 months.

2.2. Instrumentation and Chromatographic Conditions

The LC-MS/MS system used was Xevo TQ mass detector with an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters, USA). The Xevo TQ Detector operated at Multiple Reaction Monitoring (MRM) for voriconazole mass of parent ion Q1 mass: 350.1 m/z and daughter ion Q3 mass: 281.1 m/z using Cone: 24 V and Collision energy: 18 eV, for Fluconazole mass of parent ion Q1 mass: 307.1 m/z and daughter ion Q3 mass: 238.0 m/z using Cone V: 26 V and Collision energy: 16 eV, positive ion mode, Electrospray Ionization (ESI) source, Probe: 4.00 KV, Extractor: 3.00 V, Source Temperature: 150˚C, Desolvation Temperature: 500˚C, Desolvation Gas Flow: 700 L/Hr and Collision Gas Flow: 0.15 ml/min. The column used for separation was the Acquity BEH C18 (1.7 µm) (100 × 2.1 mm) UPLC column at 30˚C. The sample manager was set at 4˚C and the injection volume: 1.00 µl. The software used was MassLynx version 4.1 (Micromass, Waters, USA). Waters Acquity Binary Solvent Manager was set at a flow rate of 0.30 ml/min. The mobile phase consisted of mobile Phase A: composed of acetonitrile-UPW-formic acid (50:50:0.1, v/v/v) and mobile Phase B: composed of methanol-UPW-formic acid (25:75:0.1, v/v/v) on a gradient mode for 7.50 min for each sample as shown in **Table 1**.

2.3. Calibration Curve and Quality Control (QC) Preparation

The stock solutions of voriconazole and fluconazole were prepared by accurately weighing an equivalent amount of each pure standard powder which was

Table 1. The gradient mode used for determination of voriconazole and fluconazole.

Time (min)	%Mobile Phase A	%Mobile Phase B
0.00	5	95
1.00	5	95
1.50	95	5
3.50	95	5
4.00	5	95
7.50	5	95

dissolved in ethanol to reach a final concentration of 100 µg/ml. Voriconazole serial solutions were prepared by diluting voriconazole stock solution with (50% acetonitrile: 50% UPW) in prelabeled volumetric flasks accordingly for calibration curve samples (Blank, Standard zero, 1.00, 2.00, 5.00, 10.00, 20.00, 40.00, 60.00 and 80.00 µg/ml) and (1.00, 3.00, 30.00 70.00 µg/ml) for QC samples. Voriconazole calibration curve in the plasma was prepared in ten prelabeled Eppendorf (1.5 ml) tubes as Blank, Standard zero, 0.10, 0.20, 0.50, 1.00, 2.00, 4.00, 6.00 and 8.00 µg/ml and voriconazole QC samples were prepared in four prelabeled Eppendorf tubes as 0.10, 0.30, 3.00, and 7.00 µg/ml. Briefly, 100 µl from each prepared serial solution were added to their corresponding prelabeled Eppendorf tube of calibration curve samples (Calibrators) and QC samples, and 900 µl of blank plasma was added to all samples then vortexed for 2 min and 100 µl of each Calibrator and QC sample were transferred into prelabeled Eppendorf tubes, all standard Calibrators and QCs were stored at −40˚C. The working solution of the fluconazole was prepared by diluting fluconazole stock solution (100 µg/ml) with acetonitrile to reach a final concentration of 10 µg/ml which was used as an IS.

2.4. Sample Preparation

Briefly, the patient samples were collected in heparinized tubes (Green Top Tubes) and ethical approval. However, 100 ul of each plasma sample (patient plasma samples, calibrators, and QCs) were spiked in prelabeled Eppendorf tubes and 100 µl of IS (fluconazole working solution 10 µg/ml) was added to all samples except blank then vortexed for 30 seconds after that 800 µl of acetonitrile was added for direct protein precipitation and drug extraction. All the samples were vortexed for 1 min, and subsequently centrifuged for 5 min at 15,000 r.p.m at 4˚C. In total, 200 µl of each supernatant was transferred to 800 µl UPW in 2ml autosampler glass vials and vortexed for 30 seconds, followed by injecting 1µl of each sample into the chromatographic system.

2.5. Method Validation

The validation of this method was applied according to the guidelines of the US FDA [23]. The method validation was done for selectivity, sensitivity, linearity,

accuracy, precision, recovery, stability and carryover effect. Six samples of blank human plasma were used for the assessment of the method selectivity. The Lower Limit of Quantification (LLOQ) was determined by using six spiked LLOQ samples to detect the method sensitivity. The linearity of the method was determined by plotting calibration curve ranges from 0.10 - 8.00 μg/ml. The assessment of intra and inter assay accuracy and precision was done by using six replicates of four QC levels 0.10, 0.30, 3.00 and 7.00 μg/ml. The voriconazole peak areas in the spiked plasma samples (six of each LLOQ, LQC, MQC and HQC) were compared with the voriconazole samples that have been prepared by spiking of the extracted drug-free plasma, which had the same concentration of voriconazole for recovery determination. IS recovery determination was done by following the previous protocol through comparing the extracted QC samples' mean peak areas with the IS samples prepared by spiking the extracted drug-free plasma with the same concentration of IS. The carryover effect was determined by comparing the peak areas after injection of two blank (Mobile Phase) samples with the Upper Limit of Quantification (ULOQ) in the calibration curve. Run size estimation was done by running 12 samples of LLOQ, LQC, MQC and HQC. The stability testing of voriconazole in the plasma samples were tested under different conditions. Several stability tests were performed, including the stability of the benchtop samples for 6 hours, stability of the analyzed samples, including the stability of the autosampler samples for 24 hours, stability of the wet extract samples for 24 hours, stability of freeze and thaw (three cycles) and stability of the long-term samples (30 days). All the stability testing was done by comparing accuracy and precision between the six replications of each stability sample with freshly prepared samples of LLOQ, LQC, MQC and HQC.

3. Clinical Application

The application of this method was the routine determination of voriconazole trough level (pre-dose) in human plasma samples for patients receiving voriconazole treatment and ethical committee approval was obtained. The blood samples were collected at a steady state in heparinized tubes (green top tubes) prior to receiving a voriconazole dose (15 - 60 min before dose). For each patient, one heparinized blood sample was collected, centrifuged for 5 min at 3000 r.p.m at 4˚C for plasma separation. The plasma was transferred to a new prelabeled (test) tube and stored at −20˚C until the day of analysis. This method has been developed due to the need of a routine voriconazole TDM, and informed consent were exempted in this study.

4. Results

4.1. Selectivity

Determination of the method selectivity was done by running six blank human plasma samples. The chromatograms were checked for any interference by plasma components which may interfere with the voriconazole and IS results.

The resulted chromatograms showed no significant interference detected at voriconazole 3.40 min and IS 2.44 min retention times Figure 1. There was no observed interference with the IS chromatogram at 2.44 min retention time Figure 2.

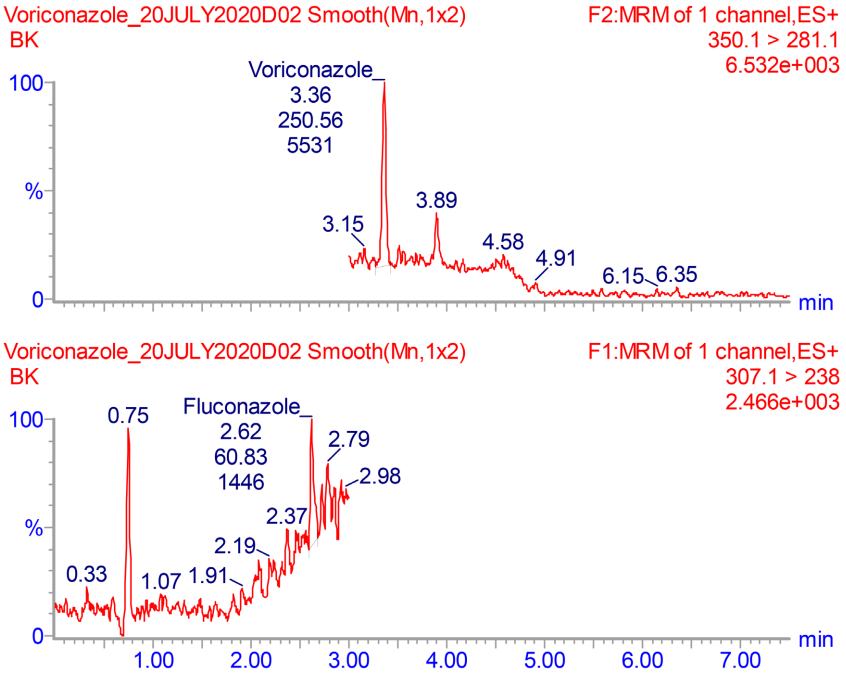

Figure 1. Blank human plasma sample for voriconazole at mass transition 350.1/281.1 (upper chromatogram) and for fluconazole (IS) at mass transition 307.1/238 (lower chromatogram).

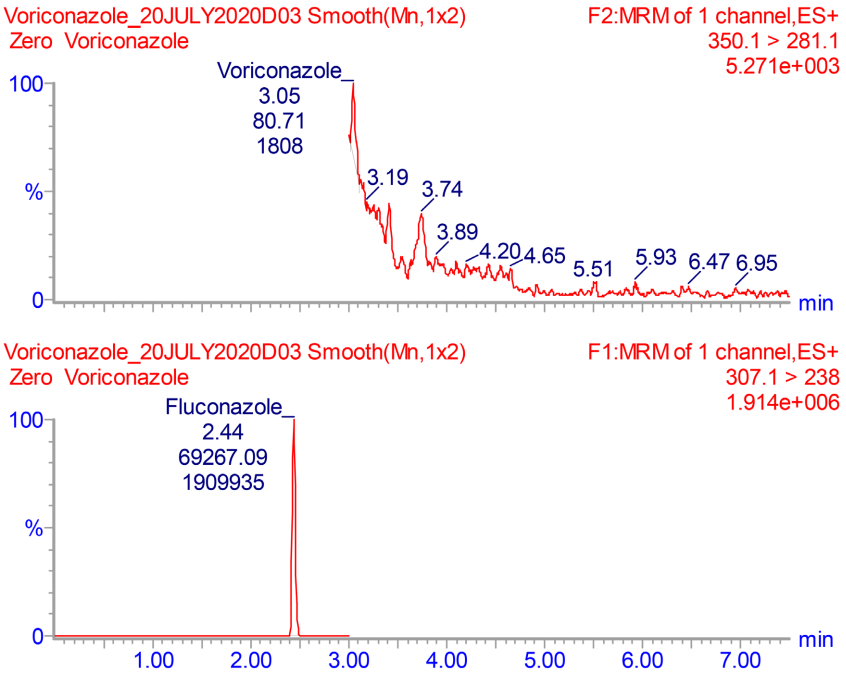

Figure 2. Blank human plasma sample with the addition of IS for voriconazole at mass transition 350.1/281.1 (upper chromatogram) and for fluconazole (IS) at mass transition 307.1/238 (lower chromatogram).

4.2. Sensitivity

The method sensitivity was assessed by running of six spiked LLOQ samples (0.10 µg/ml), which resulted in 95.00% accuracy and 5.76% CV. The chromatogram of LLOQ QC sample Figure 3.

4.3. Linearity

The method linearity determination was done by plotting the calibration curve ranges (0.10 - 8.00 µg/ml). The assessment of the mean correlation coefficient r Figure 4 indicated a good linearity of the calibration curve ≥ 0.99.

4.4. Accuracy and Precision

The intra and inter assay accuracy and precision were determined by running six replications of the four QC levels 0.10, 0.30, 3.00 and 7.00 µg/ml. The accuracy and precision results are displayed in Table 2. Overall, the intra-assay precision was ≤5.76%, and the inter-assay precision was ≤4.45%. The accuracy acceptance criteria ranged between 85% - 115%. However, the accuracy results indicated acceptable accuracy.

4.5. Recovery

The voriconazole recovery was 109.17 ± 9.92% (range 97.69% - 119.62%). The results are shown in Table 3.

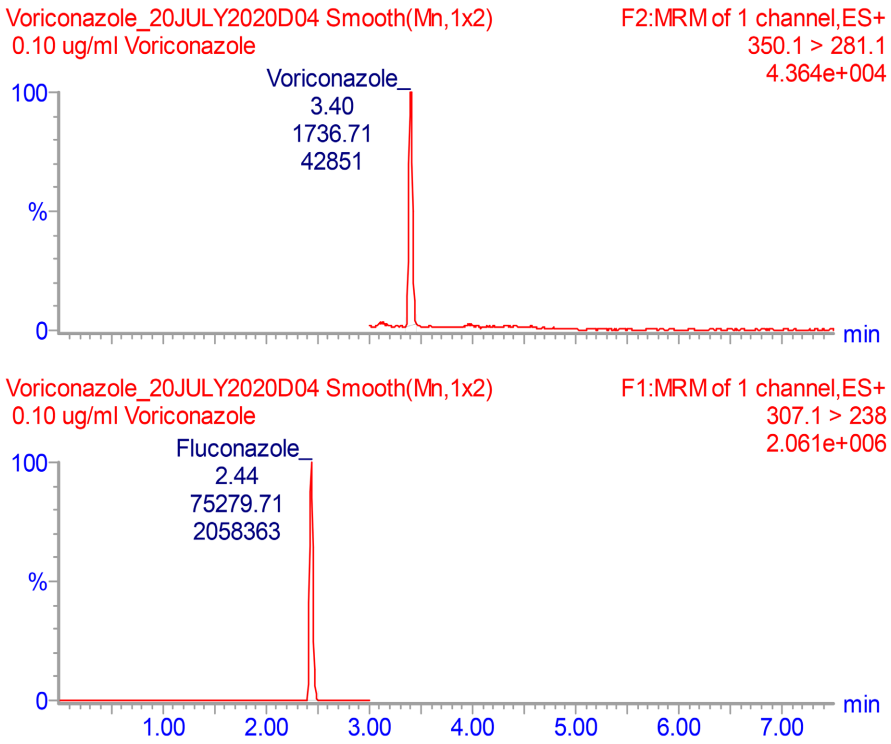

Figure 3. LLOQ QC sample chromatograms for voriconazole at mass transition 350.1/281.1 (Upper chromatogram) and for fluconazole (IS) at mass transition 307.1/238 (lower chromatogram).

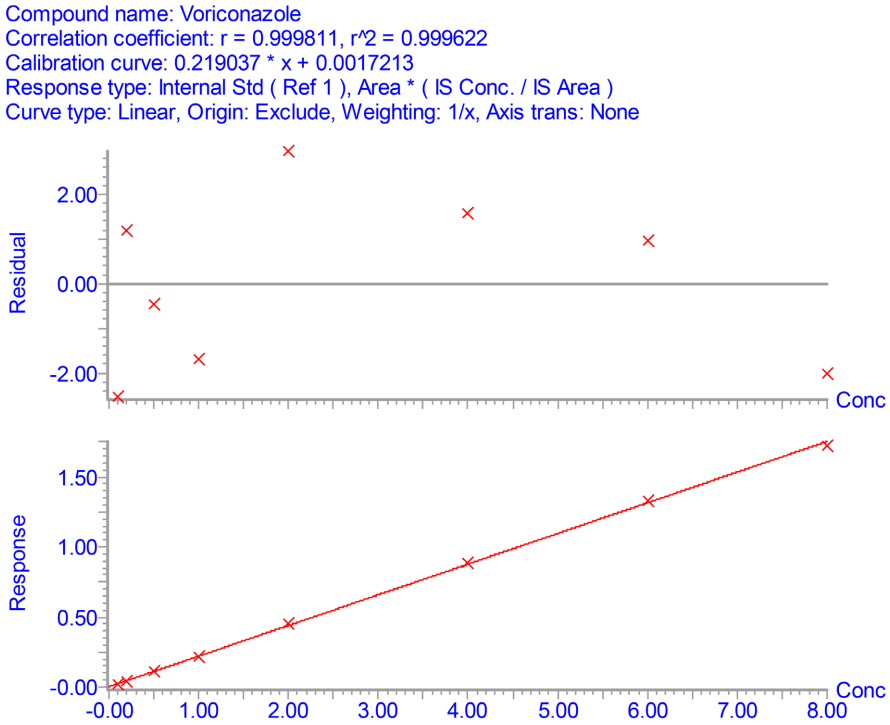

Figure 4. Voriconazole calibration curve ranges (0.10 - 8.00 µg/ml).

Table 2. The intra and inter assay accuracy and precision of the method.

Analyte	QC concentrations (µg/ml)	Intra-assay accuracy and precision (n = 6)			Inter-assay accuracy and precision (n = 6)		
		Measured concentrations (µg/ml) (Mean ± SD)	Accuracy (%)	Precision (%)	Measured concentration (µg/ml) (Mean ± SD)	Accuracy (%)	Precision (%)
Voriconazole	0.10	0.09 ± 0.01	95.00	5.76	0.09 ± 0.00	91.66	4.45
	0.30	0.29 ± 0.01	98.88	2.75	0.29 ± 0.01	99.44	3.29
	3.00	3.12 ± 0.07	104.16	2.26	3.06 ± 0.06	102.27	1.87
	7.00	7.15 ± 0.22	102.26	3.02	7.09 ± 0.13	101.33	1.77

Table 3. The mean recovery of voriconazole and fluconazole (IS) (n = 6).

Analyte	QC concentrations (µg/ml)	Unextracted response (Mean ± SD)	Extracted response (Mean ± SD)	Recovery (%)	Mean recovery (Mean ± SD)	CV %
Voriconazole	0.10	1660 ± 88	1699 ± 106	97.69	109.17 ± 9.92	9.08
	0.30	4646 ± 261	4446 ± 354	104.49		
	3.00	50506 ± 4287	42220 ± 2349	119.62		
	7.00	111172 ± 7630	96779 ± 11743	114.87		
Fluconazole	10	73196 ± 4803	62089 ± 2699	117.88	118.15 ± 10.29	8.71

4.6. Stability

The voriconazole was stable in all the stability tests, included benchtop, auto-

sampler, wet extract, freeze & thaw and long-term sample stability. All the stability results were within the acceptable range (90% - 110%).

4.7. Clinical Samples

Voriconazole trough level of the clinical plasma samples (n = 50) were within the calibration range (0.30 - 7.00 µg/ml). A chromatogram of a clinical sample is in Figure 5.

5. Discussion

The LC-MS/MS method was developed and validated to measure voriconazole concentration in human plasma to improve treatment and management outcome. The method was sensitive, precise and robust which has been successfully applied in routine TDM. The calibration curve ranged from 0.10 - 8.00 µg/ml approximately, which was sufficient for the quantification of the voriconazole plasma trough levels in patients' samples. Comparing our method with a recently published method for voriconazole determination using standard HPLC, the running time of our method was 7.50 min compared to 20.00 min in the HPLC method [24]. The flow rate in our method was 0.30 ml/min, which relatively considered lower than most of the published methods, this has an advantage of reducing solvents consumption [25] [26] [27] [28]. In comparison of the method injection volume with other published methods, the injection volume of our method was very low (1.00 µl), reflecting the high sensitivity and robustness of the method besides, the low injection volume reduces samples contamination and carryover problems [25] [26] [28]. The mobile phase contains simple composition of different ratios of UPW, methanol, acetonitrile and formic acid

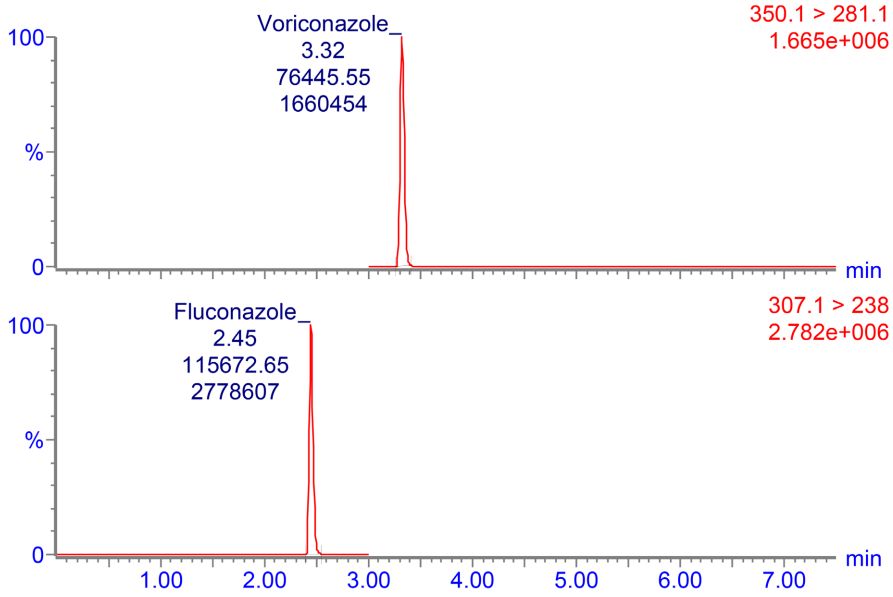

Figure 5. chromatograms of voriconazole at mass transition 350.1/281.1 (Upper chromatogram) and fluconazole (IS) at mass transition 307.1/238 (lower chromatogram) in a clinical sample.

which can be easily prepared. The limitation of this method that it requires LC-MS/MS, which is costly instrument and requires trained staff for LC/MS/MS operation and troubleshooting. The use of fluconazole as an IS rather than the deuterated voriconazole was preferred due to the cost and difficult availability of the deuterated voriconazole. In summary, this fully validated method is considered as an accurate, precise and simply applicable method for the quantification of voriconazole in patient's plasma and it is considered as a good choice for voriconazole TDM. This method can be used in clinical research studies, for example drug-drug interactions and pharmacokinetic-pharmacodynamic studies.

6. Conclusion

The developed LC-MS/MS method was successfully validated and applied in measuring of voriconazole plasma trough levels for the routine voriconazole TDM in clinical settings besides, it's considered as a suitable method for clinical studies and personalized medicine.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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