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Research paper

A rapid ultra-performance LC-MS/MS assay for determination of serum unbound fraction of voriconazole in cancer patients

Jieli Li[a], Jing Ma[b], Elizabeth A. Wagar[a], Dong Liang[b], Qing H. Meng[a,*]

[a] Department of Laboratory Medicine, The University of Texas MD Anderson Cancer Center, Houston, TX, United States
[b] Department of Pharmaceutical and Environmental Health Sciences, Texas Southern University, Houston, TX, United States

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ABSTRACT

Background: Voriconazole (VOR), an antifungal agent, is clinically monitored to guide therapeutic dosing and avoid toxicity. It is believed that measurement of serum unbound VOR provides more accurate information, especially in hypoalbuminemia patients. We developed and validated an accurate, simple and fast test with ultrafiltration and ultra-performance liquid chromatography (UPLC)-tandem mass spectrometry (MS/MS) to measure unbound VOR in human serum.

Methods: The Agilent UPLC system coupled with a SCIEX QTRAP4000 MS with a positive ionization mode was developed and validated for VOR analysis.

Results: A good linearity was demonstrated from 0.02 to 2.5 μg/ml for unbound VOR ($r^2 = 0.9969$). The within-run and between-run accuracy and precision was < 5% and < 6%. The levels of total VOR were well correlated with reference laboratory results. Serum unbound VOR levels were correlated with the total VOR levels ($r = 0.78$, $p < 0.0001$). There was a reverse correlation between unbound VOR fractions and plasma albumin levels ($p < 0.05$). In hypoalbuminemia patients, the unbound VOR levels were increased to a higher degree than total VOR.

Conclusion: This assay is suitable for monitoring both unbound and bound VOR in cancer patients especially in those with hypoalbuminemia in clinical laboratories. Measurement of unbound VOR offers a better approach in prediction of VOR toxicity.

1. Introduction

Aspergillosis is the top one invasive fungal infections. It is also the major cause of infection-related death in hematopoietic stem cell transplant recipients and patients who receive prolonged immunosuppression [1, 2]. Voriconazole (VOR) is currently indicated as the first-line treatment of invasive aspergillosis in non-neutropenic patients [3]. The correlation between the plasma concentration of VOR and clinical efficacy have not been established in any of the large prospective randomized studies, due to the non-linear pharmacokinetics profile of VOR [4]. Thus, the use of total VOR concentrations in certain patient groups may misguide therapy.

Most of the drugs bind to plasma proteins such as albumin. The unbound (free) fraction of drugs is the only fraction available for distribution to the target site, having pharmacological effect, and clearance from the plasma. Plasma protein binding affects the unbound drug concentration available for drug metabolism and efficacy. Since the total plasma concentrations are not a reliable indication of drug effect, direct measurement of unbound drug concentrations offers a more meaningful approach.

Serum albumin concentrations usually decrease in patients with chronic infection, chronic liver disease, or malnutrition. Hypoalbuminemia occurs in approximately 60% of critically ill cancer or patients receiving immunosuppressants [5, 6]. The presence of hypoalbuminemia is known to reduce the protein binding of VOR, leading to higher unbound drug concentrations in plasma [7]. Both the ratio of unbound/total VOR concentration and the total VOR concentration may be altered accordingly. Therefore, it is difficult to accurately estimate the unbound VOR concentration from the total concentration. VOR is a narrow therapeutic window drug. It is possible that the total VOR concentration is within the therapeutic range while the unbound VOR reaches toxicity level. Measurement of the concentration of the unbound form of VOR might provide better and additional critical information for physicians to predict therapeutic efficacy and side effects than total concentration.

* Corresponding author.
E-mail address: QHMeng@mdanderson.org (Q.H. Meng).

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2. Materials and methods

2.1. Chemicals and reagents

VOR, formic acid, and HPLC grade acetonitrile and water were purchased from Sigma-Aldrich. VOR-d3 (internal standard, IS) was purchased from Toronto Research Chemicals.

2.2. Standards and quality controls

Stock solutions of VOR (1 mg/ml) and internal standard (IS) (1 mg/ml) were prepared individually by dissolving each substance in acetonitrile (VOR) or methanol (IS), and stored at 4 °C until used. Fresh standards were prepared on a monthly basis. A series of standards of VOR were prepared by diluting the stock solution with acetonitrile and water (1:1 ratio) and then spiked into blank serum (patient samples pool without VOR administration) to obtain the following testing concentrations: 0.05, 0.1, 0.5, 1.0, 2.5, 5, 7.5 and 10 μg/ml for total VOR. Various concentrations of free VOR (at 0.05, 0.1, 0.5, 1.0 and 2.5 μg/ml) were prepared by spiking the same series of standards into filtered blank serum with Centrifree-10 K tubes (Millipore, Burlington, MA). The low, medium and high levels of quality control (QC) samples were prepared in the same way as the standards at 0.4, 2 and 8 μg/ml for total VOR, 0.4 and 2 μg/ml for free VOR, respectively. IS working solution was prepared by diluting the stock solution with acetonitrile and water (1:1 ratio) to 0.13 μg/ml for total VOR and 0.27 μg/ml for free VOR.

2.3. Sample preparation

To measure total VOR, briefly, 50 μl of standard, QC, or blank serum sample was extracted and deproteinized by adding 100 μl of mixture of acetonitrile and IS. After vortex mixing for 30 s, the mixture was centrifuged for 30 min at 20,800 ×*g* at room temperature. An aliquot of 100 μl of the supernatant was taken for UPLC-MS/MS analysis.

To measure unbound VOR, serum samples were placed in a rolling incubator at room temperature for 1 h prior to ultrafiltration. Ultrafiltration tubes were pretreated by addition of 500 ml of distilled H_2O in the sample reservoir and centrifuged for 30 min at 20,800 ×*g* at room temperature to remove glycine as recommended by the supplier. The sample reservoir was weighed before and after centrifugation to obtain the precise volume of the filtrate, followed by same speed and time of centrifuge at room temperature. After the addition of a 100 μl of solution of IS in acetonitrile to the 50 μl of ultrafiltrate (2:1 ratio) to remove small molecular weight of proteins, the mixtures were vortex mixed for 30 s and then centrifuged again. An aliquot of 100 μL of the supernatant was taken for LC-MS/MS analysis.

2.4. Chromatographic conditions

The chromatographic separation was achieved by a Shimadzu Nexera ×2 series UHPLC Super C18 column (50 × 2.1 mm, 2 μm, ACE Excel, Canadian Life Science) with a flow rate of 0.2 ml/min. The mobile phase was 100% HPLC water (A) and 100% acetonitrile (B), with the gradient as 98% acetonitrile 2.5 mins, and 25% acetonitrile 0.5 mins. Each sample injection volume was 2 μl. The MS analysis was performed by an API 4000 QTRAP triple quadrupole mass spectrometer with a Turbo Ion Spray ion source (Applied Biosystems/MDS SCIEX). The quantification was performed by selected reaction monitoring (SRM) at positive mode to detect the specific precursor to product ion transitions *m/z* 350 → 281 for VOR, and *m/z* 353 → 284 for IS. The source parameters were set as follows: ionspray voltage, 4500 V; ion source temperature, 500 °C; nebulizer gas, 55 psi; heater gas, 40 psi; curtain gas, 25 psi; and the collision gas, high. The LC-MS/MS system was controlled and data was acquired by Analyst software version 1.5.

2.5. Method validation

2.5.1. Linearity and sensitivity

Calibration curves in blank serum were created by plotting the peak area ratio of VOR and IS against the known concentrations of the VOR from 0.1–10 μg/ml (total VOR) and 0.02–2.5 μg/ml (free VOR). Total analytical run time was 3 min. The least-squares linear regression method with no weighting (total VOR) or 1/x weighting (unbound VOR) was applied to generate the slope, intercept, and correlation coefficient of each linear regression equation. As defined in CLSI EP17-A [8], lower limit of detection (LOD) is determined by utilizing both the measured lower limit of blank (LOB) and replicates of a sample known to contain a low concentration of total/free VOR (0.005 μg/ml), flowing the Eq.

$$\mathrm{LOD} = \mathrm{LOB} + 1.645^{*}\mathrm{SD}_{(\text{low concentration sample})} \quad (1)$$

Lower limit of quantitation (LOQ) was determined as Eq.

$$\mathrm{LOQ} = \mathrm{LOD} + 2^{*}\mathrm{SD}_{(\text{low concentration sample})} \quad (2)$$

2.5.2. Matrix effect

To examine the matrix effect, the QC at two or three concentration levels were evaluated. The matrix effect were calculated according to Eq. (3).

$$\text{Matrix effects, \%} = \text{peak area of (post-extracted samples /unextracted samples)} \times 100 \quad (3)$$

Experiments were conducted in triplicates.

2.5.3. Precision and accuracy

Following CLSI EP5-A3 [9], the within- or between-run precision of the UPLC-MS/MS method was determined by analyzing the QC samples via calibration curves created on the same day or three different days. Experiments were conducted at least three times. Accuracy was performed as comparing our results with the reference lab.

2.5.4. Stability

All stability studies were conducted at low, medium and high QC levels using three replicates at each concentration level. All samples were compared with freshly prepared samples at the same concentrations. Short-term stability samples were freshly prepared and left on the bench-top at room temperature and 4 °C for 3 and 7 days, respectively. Long-term storage stability samples were freshly prepared and stored at −80 °C for 14 days.

2.5.5. Free VOR concentration

The volume of filtrates were precisely correlated with the weight difference of ultrafiltration before and after filtration (data not shown). Based on the volume of filtrate, free fraction of VOR calculation followed the Eq. (4).

$$\text{Free VOR concentration} = (\text{measured concentration} \times \text{volume of ultrafiltrate})/\text{total volume of pre} - \text{ultrafiltration} \quad (4)$$

2.6. Statistics

Statistical analysis was performed using EP Evaluator 11 (Data Innovations) and Microsoft Excel by One-way ANOVA. All the statistical tests considered as significant for a $p < 0.05$. Data were described with means ± standard deviations (SD). Univariate correlations were investigated using scatterplots combined with Spearman's rank correlation coefficients.