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Development and validation of a liquid chromatography-tandem mass spectrometry assay for the simultaneous quantitation of 5 azole antifungals and 1 active metabolite

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ABSTRACT

Background: Azole antifungal medications are often administered to prevent or treat invasive fungal infections. These infections are deadly in the immunocompromised population. Therapeutic drug monitoring of the azole antifungal medications may potentially decrease morbidity and mortality in patients undergoing azole treatment.

Methods: To assist with azole therapeutic drug monitoring, a liquid chromatography tandem mass spectrometry (LC-MS/MS) method was developed and validated for 6 azole analytes: fluconazole, voriconazole, posaconazole, isavuconazole, itraconazole, and its active metabolite hydroxyitraconazole.

Results: The validated method solely required a protein precipitation step before subsequent dilution and injection onto the LC-MS/MS system. Furthermore, the analysis time was < 2 min per sample.

Conclusions: This method enables measurement of all 6 of these analytes into a single LC-MS/MS assay.

1. Introduction

Invasive fungal infections (IFI) are deadly and prevalent in certain high-risk patient populations: patients with hematological malignancies, the immunosuppressed, and the critically ill [1–3]. Antifungal medications are routinely administered to these susceptible patients to either treat or prevent IFI. One common class is the azole drugs, so named because they contain a 1,2,4-triazole ring [4]. These medications inhibit lanosterol 14α-demethylase, disrupting ergosterol biosynthesis and accumulating14-α-methyl-3,6-diol, a toxic sterol. This toxic sterol causes severe membrane stress on the fungal cells [5].

Therapeutic drug monitoring (TDM) may be utilized to assist with azole administration. Generically, TDM is favored when the medication demonstrates a large pharmacokinetic (PK) variability, a relationship between measured levels and efficacy, and a narrow therapeutic range, amongst other considerations. For the azole medications, PK can be affected by a variety of factors: polymorphisms of cytochrome P450 enzymes, drug-drug interactions, renal insufficiency, hepatic insufficiency, formulation, fasting status, gastric acidity, and dosing intervals [6–10]. At our hospital, clinicians requested the offering of an azole assay to assist with dosing strategies.

We developed and validated a rapid and accurate assay for the quantification of selected azoles that are routinely administered in our hospital system. There are 3 major analytical methodologies for azole quantification: bioassays, liquid chromatography (LC), and LC coupled to mass spectrometry (MS) [11]. Due to its superior speed, analyte specificity and sensitivity, analytical measuring range (AMR), and multiplexing ability, LC-MS has emerged as the current platform of choice [12–20]. Several reports in the literature take advantage of the methodology's inherent multiplexing ability, and quantify several azoles in one method [13–15,18–20]. All the recent reports utilizing MS only require minimal amounts of patient specimen (< 100 μl) with analytical times < 5 min.

With these advantageous characteristics in mind, an LC-MS/MS method was developed and validated for 6 analytes: fluconazole, voriconazole, posaconazole, isavuconazole, itraconazole, and its active metabolite hydroxyitraconazole. To our knowledge, this represents one of the first clinical-use methods to include all 6 of these analytes into a single LC-MS/MS assay [18].

2. Materials and methods

2.1. Materials

Burdick and Jackson LC-MS grade acetonitrile and methanol was

Abbreviations: IFI, invasive fungal infection; TDM, therapeutic drug monitoring; PK, pharmacokinetics; AMR, analytical measuring range; MRM, multiple reaction monitoring; HESI, heated electrospray ionization

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purchased through Fisher Scientific. Ultrapure water was prepared with a Milli-Q water purification system (Millipore Synergy). Fluka formic acid was purchased through Sigma-Aldrich and certified ACS plus hydrochloric acid from Fisher. The charcoal-stripped serum was from SeraCare. Fluconazole, fluconazole-$^{13}C_3$, voriconazole, voriconazole-D_3, posaconazole, posaconazole-D_4, itraconazole, itraconazole-D_4, and hydroxyitraconazole-D_4 were from Cerilliant. Isavuconazole and hydroxyitraconazole were purchased from Toronto Research Chemicals. Isavuconazole-D_4 was purchased from Medical Isotopes. Hydroxyitraconazole, hydroxyitraconazole-D_4, and isavuconazole were obtained as exact weight powders. Prior to use they were dissolved in 1% (v/v) 1 mol/l HCl in methanol to a concentration of 1 mg/ml. Calibrators were prepared in charcoal-stripped serum, via serial dilution, at the following concentrations: 0.2, 0.3, 0.6, 1.3, 2.5, 5.0, and 10.0 μg/ml for voriconazole, posaconazole, isavuconazole, itraconazole, and hydroxyitraconazole. The fluconazole calibrators were prepared at 0.5, 0.9, 1.9, 3.8, 7.5, 15.0, and 30.0 μg/ml, via serial dilution, in charcoal-stripped serum. The protein precipitation solution was acidified acetonitrile [0.1% (v/v) 1 mol/l HCl] containing the internal standards for all 6 analytes (1 μg/ml).

2.2. Sample preparation

Fifty microliters of sample (serum or lithium heparin plasma) was precipitated with 250 μl of the precipitation solution. After 60 s of vortex mixing, the sample was centrifuged at 18,900 × *g* for 10 min. A 50 μl aliquot was then diluted with 200 μl 0.1% (v/v) formic acid in ultrapure water, which was then ready for LC-MS/MS analysis.

2.3. LC-MS/MS method

Utilizing a Transcend LC system (Thermo Scientific), 20 μl of the diluted supernatant was injected onto a reversed-phase column (Accucore RP-MS, 50 × 2.1 mm, 2.6 μm). Solvent A was comprised of 0.1% (v/v) formic acid in ultrapure water and solvent B was 0.1% (v/v) formic acid in acetonitrile. At a flow rate of 0.8 ml/min, the gradient was ramped from 40% B to 60% B over 20 s, held at 60% B for 30 s, and finally equilibrated back at 40% B for 60 s. A Thermo Scientific TSQ Vantage triple quadrupole mass spectrometer, operating in multiple reaction monitoring (MRM) mode, was utilized as the detector. The source was positive heated electrospray ionization (HESI) operating with a spray voltage of 4000 V, a vaporizing temperature of 350 °C, a sheath gas of 50, an auxiliary gas of 10, and a capillary temperature of 200 °C. Two mass-to-charge (*m/z*) transitions were monitored for each analyte (quantifier and qualifier) and 1 transition for each internal standard (Table 1).

Table 1
MRM transitions monitored for the azole analytes and their internal standards.

Analyte	Quantifier transition	Qualifier transition
Fluconazole	307.2 ➔ 238.1	307.2 ➔ 220.1
Fluconazole-$^{13}C_3$	310.2 ➔ 223.1	–
Voriconazole	350.2 ➔ 127.0	350.2 ➔ 281.1
Voriconazole-D_3	353.1 ➔ 127.0	–
Posaconazole	701.5 ➔ 683.4	701.5 ➔ 614.3
Posaconazole-D_4	705.6 ➔ 687.5	–
Isavuconazole	438.3 ➔ 224.0	438.3 ➔ 127.0
Isavuconazole-D_4	442.2 ➔ 224.0	–
Itraconazole	705.4 ➔ 392.2	705.4 ➔ 432.3
Itraconazole-D_4	709.5 ➔ 396.2	–
Hydroxyitraconazole	721.4 ➔ 408.2	721.4 ➔ 430.2
Hydroxyitraconazole-D_4	725.5 ➔ 412.2	–

2.4. Method validation

To validate the method, the following experiments were performed: ion suppression, mixing study, interference, AMR, carryover, stability, precision, and method comparisons. From the stability experiment onwards, quality control samples at 2 levels were analyzed with every batch. The levels for all analytes, except fluconazole, were 0.7 and 5.0 μg/ml for the low and high control, respectively. The fluconazole controls were 1.9 and 15.0 μg/ml, for the low and high, respectively. The use of leftover patient samples was approved by the Cleveland Clinic Institutional Review Board.

2.4.1. Ion suppression and mixing study

The absolute ion suppression was evaluated by infusing a 2 μg/ml solution of each analyte and the respective internal standard through a connection tee post-column. While the solution was being infused at 25 μl/min, a solvent blank and 10 blank patient serum samples (5 males and 5 females) were consecutively injected onto the LC column. The patient blank samples were extracted according to procedure in the sample preparation section, however, without internal standards. The signal intensity from each analyte and internal standard were monitored throughout the chromatogram. The chromatogram was observed for a reduction or enhancement in signal intensity with the patient samples versus the solvent blank for each analyte and internal standard.

The acceptability of a candidate matrix for calibrator preparation was determined via a mixing study. The candidate matrix (charcoal-stripped serum) spiked with 10.0 μg/ml of each analyte was mixed 1:1 with patient blank serum samples (3 males and 3 females). The candidate matrix, the patient blank serum samples, and the mixture were then extracted. The response ratio (analyte over internal standard) of each mixture was then monitored. The candidate matrix was accepted if the response ratio of each mixture was within 20% of the theoretical response ratio.

2.4.2. Interferences

Interferences from both endogenous and exogenous sources were investigated. Four endogenous conditions were evaluated for each analyte: lipemic (L index: 689), hemolyzed (H index 303), icteric (I index: 26), and uremic (blood urea nitrogen: 32 mg/dl) samples. This was investigated at 2 analyte concentrations (1.0 μg/ml and 10.0 μg/ml) in spiked charcoal stripped serum, by mixing the spiked serum 1:1 with the analyte-free interferent samples. Significant interference was determined if the response ratio of any mixture was > 20% different than the theoretical response ratio.

To evaluate the effects of potential exogenous interferences, commercial controls [Lyphochek Quantitative Urine Quality Control, Liquid Assayed Multiqual, Liquichek Immunoassay Control, and Liquichek Urine Toxicology Quality Control (Bio-Rad)] were extracted. The controls contained > 100 therapeutic drugs and common endogenous substances. Interference was determined by observing the chromatograms for any peaks with similar retention times as the analytes.

2.4.3. AMR

The AMR of each analyte was evaluated at 8 levels in pooled patient blank serum: blank, 0.2, 0.3, 0.6, 1.3, 2.5, 5.0, and 10.0 μg/ml for voriconazole, posaconazole, isavuconazole, itraconazole, and hydroxyitraconazole. For fluconazole, the 8 levels were a blank, 0.5, 0.9, 1.9, 3.8, 7.5, 15.0, and 30.0 μg/ml. Each level was extracted in triplicate and analyzed. The AMR was deemed acceptable if each level demonstrated a recovery within 100 ± 20%, CV ≤ 20%, and a signal-to-noise ratio > 10.

2.4.4. Carryover

Sample carryover was evaluated for each analyte by performing the following sequence: low concentration sample ➔ a high concentration sample ➔ 2nd injection of the low concentration sample in serum. The high concentration sample was approximately double the upper limit of quantitation (LOQ). The low concentration samples were approximately 0.3 μg/ml for all analytes except fluconazole, which was 1.0 μg/ml. The samples were extracted and analyzed in triplicate. Sample