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Simultaneous determination of voriconazole, posaconazole, itraconazole and hydroxy-itraconazole in human plasma using LCMS/MS

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ABSTRACT

Introduction: Invasive fungal infections are an increasing cause of mortality and morbidity in high risk patient populations such as those on immunosuppressive therapy. Triazole antifungals are recommended for the prevention and treatment of such infections.

The aim of this study was to develop and validate a simple, sensitive and robust LCMS/MS method for the simultaneous analysis in human plasma of three frequently used antifungal drugs: voriconazole, posaconazole, and itraconazole.

Methods: Precipitation reagent, containing deuterated internal standards, is added to 50 µL of plasma. The vials are vortexed before centrifugation. The organic supernatant is transferred to a polypropylene vial and 1 µL is injected into the Waters Acquity® Ultra Performance Liquid Chromatography system coupled with a Waters Acquity® TQ Detector system. Chromatographic separation is achieved on a BEH C_{18} column using gradient elution with mobile phases consisting of 2 mM ammonium acetate with 0.1% formic acid in water and methanol. Run time is < 5 min between injections.

Results: The evaluation of the LCMS/MS triazole method showed good precision (intra-assay CVs < 6.7%, inter-assay CVs < 8.3%).

The lower limit of quantitation for all antifungal triazoles tested was 0.10 mg/L.

Passing Bablok comparisons of voriconazole (n = 50) and posaconazole (n = 50) showed good correlation with the current HPLC method (Voriconazole LCMS = 0.94(HPLC) + 0.03, r^2 = 0.99; Posaconazole LCMS = 1.18(HPLC) − 0.04, r^2 = 0.95).

Passing Bablok comparisons of itraconazole and hydroxy-itraconazole (n = 18) showed good agreement with an external referral laboratory's antifungal LCMS/MS method (Itraconazole LCMS = 1.00(referral lab) + 0.01, r^2 = 0.99; Hydroxy-Itraconazole LCMS = 1.05(referral lab) + 0.04, r^2 = 0.99).

External quality assurance samples for posaconazole and voriconazole (n = 12, UK NEQAS Antifungal Pilot Panel) were assayed 'blind' and results were in good agreement with consensus mean values (both r^2 = 0.99).

Conclusion: The rapid pre-analytical sample preparation procedure, short chromatographic time, limit of quantitation and linear range make this LCMS/MS method suitable for determination of plasma voriconazole, posaconazole, itraconazole and hydroxy-itraconazole levels in a high throughput laboratory.

1. Introduction

Invasive fungal infections are an increasing cause of mortality and morbidity especially in high risk patient populations such as those who are on immunosuppressive therapy. Triazole antifungals are recommended for the prevention and treatment of such infections.

The triazole antifungals work by inhibiting the cytochrome P450-dependent enzyme lanosterol 14-alpha-demethylase [1]. This enzyme is necessary for the conversion of lanosterol to ergosterol, a vital component of the cellular membrane of fungi. Disruption in the biosynthesis of ergosterol causes significant damage to the cell membrane by increasing its permeability, resulting in cell lysis and death.

The pharmacokinetics of triazole antifungals show large inter- and intra-individual variability which can partly be explained by non-linear pharmacokinetics, differences in bioavailability, drug-drug interactions and cytochrome P450 polymorphisms [2]. As observational studies have shown a correlation between triazole plasma concentrations and their efficacy, it has now become routine practice to monitor plasma

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triazole concentrations to ensure efficacy in the treatment of serious fungal infections and, in the case of voriconazole, to avoid toxicity.

A number of methods for the quantitation of triazole antifungals in biological fluids have been described. Liquid chromatography-tandem mass spectrometry (LCMS/MS) and high performance liquid chromatography (HPLC) with UV detection are the most widely used.

In order to maximise the clinical benefit of therapeutic drug monitoring (TDM), accurate quantitative results are required with minimal turn-around times. With HPLC methods pre-treatment of the samples by liquid-liquid extraction can be time consuming and the use of hazardous organic solvents is undesirable.

Ultra performance liquid chromatography coupled with tandem mass spectrometry allows the selective and sensitive quantification of several drugs in a single analytical run, resulting in substantial reductions in analytical time, turnaround time, and costs.

The aim of this study was to develop and validate a simple, sensitive and robust LCMS/MS method for the simultaneous analysis in human plasma of three recommended and frequently used antifungal drugs and one metabolite; voriconazole, posaconazole, itraconazole and its active metabolite hydroxy-itraconazole.

2. Materials and methods

2.1. Instrumentation

An Acquity® Ultra Performance Liquid Chromatography system with a binary solvent manager, sample manager and column holder (Waters, Milford, USA) was coupled with an Acquity® TQ Detector system (Waters, Milford, USA). Chromatographic separation was achieved using an Acquity® UPLC BEH C18 1.7 μm × 2.1 × 50 mm column together with an Acquity® UPLC BEH C18 1.7 μm Vanguard Pre-Column (Waters, Milford, USA).

All data manipulation was carried out using the Masslynx V4.1 software (Waters, Milford, USA).

2.2. Chemicals and reagents

Methanol and acetonitrile (Merck, Darmstadt, Germany) were LCMS/MS grade LiChrosolv®. LCMS/MS grade water was generated using a Milli-Q water purification system (Millipore, Molsheim, France). Ammonium acetate and formic acid (FA) were obtained from Sigma-Aldrich (St. Louis, USA). Voriconazole, posaconazole, itraconazole and hydroxy-itraconazole were purchased from Toronto Research Chemicals Inc. (Toronto, Canada). The deuterated internal standards; d3-voriconazole, d4-posaconazole, d5-itraconazole, and d5-hydroxyitraconazole were also purchased from Toronto Research Chemicals Inc. (Toronto, Canada).

2.3. Calibrators and QCs

The calibration curve was established using the Recipe ClinCal Calibrator Set lyophilised for Antimycotics (Recipe, Munich, Germany).

Three levels of controls (Lyophilised Plasma Itraconazole, Posaconazole, and Voriconazole Tri-Level Control, Chromsystems, Germany) were reconstituted as per the company's recommendation.

The internal standard was prepared in acetonitrile with a final concentration of 0.5 mg/L for each of the four deuterated triazoles.

2.4. Sample preparation

Blood was collected and processed following our routine clinical laboratory procedure. The EDTA plasma was aliquoted and stored at − 30 °C until analysis.

Precipitation reagent containing the internal standards (100 μL) was added to 50 μL of calibrator, quality control or patient sample. The vials were then vortexed before centrifugation at 13,000 rpm. The organic supernatant was transferred to a polypropylene vial for injection into the LCMS/MS.

Table 1
Multiple reaction monitoring (MRM) acquisition parameters.

Compound	Parent (*m/z*)	Daughter (*m/z*)	Collision (V)	Comments
Voriconazole	350	281	20	Quantifier
Voriconazole	350	224	20	Qualifier
Posaconazole	701.4	683.3	35	Quantifier
Posaconazole	701.4	127	35	Qualifier
Itraconazole	705.4	392.2	35	Quantifier
Itraconazole	705.4	432.4	35	Qualifier
Hydroxy-Itraconazole	721.4	408.3	35	Quantifier
Hydroxy-Itraconazole	721.4	392.3	35	Qualifier

2.5. Chromatographic and mass spectrometric conditions

The mobile phase was composed of 10 mM ammonium acetate in ultra-pure water plus 0.1% FA (solvent A) and methanol plus 0.1% FA (solvent B).

The flow rate was 0.4 mL/min and the solvent gradients were progressively modified over a run time of 4.0 min. Sample injection volume was 1 μL and column temperature was 45 °C. The tandem mass spectrometer was operated in positive mode electron spray ionisation (ESI) with multiple reaction monitoring acquisition parameters shown in Table 1. The source and desolvation temperature were set at 120 °C and 350 °C respectively. Nitrogen was used as a desolvation gas and flow was set at 800 L/h.

2.6. Method validation

2.6.1. Linearity

Linearity of the assay was assessed using patient EDTA plasma samples that were assayed neat, then diluted with fresh frozen plasma and assayed over four evenly spaced dilutions. Measured concentrations were compared to expected concentrations. Linearity of the assay was confirmed by weighted linear regression, aiming for a correlation coefficient r^2 value > 0.99.

2.6.2. Lower limit of detection (LOD) and lower limit of quantitation (LLOQ)

The lower limit of detection (LOD) was determined as the smallest detectable peak in extracted plasma above baseline noise. This was established by using the formula LOD = 3.3(SD of the blank/slope of the calibration curve) (n = 20). The lower limit of quantitation (LLOQ) was determined using a plasma sample with low levels of triazoles. The concentrations were measured (n = 6) over 2 days and the %CV and deviation from the theoretical target value determined. The LLOQ was assigned to the lowest concentration with both a CV and mean value from the theoretical target of < 20%.

2.6.3. Imprecision

Assay imprecision was assessed using commercial quality control material (n = 10) as well as by spiking fresh frozen drug-free EDTA plasma with pure triazole stock standards (n = 10). These samples were analysed repeatedly (same vial, n = 10) within a single analytical run to determine intra-assay imprecision. Aliquots of these samples were analysed in duplicate over 5 days to determine inter-assay imprecision. Precision was assessed as a function of variation (%CV).

2.6.4. Accuracy

To calculate accuracy, clinical patient samples (n = 4) with low to mid-range triazole concentrations were spiked with a range of