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 C18 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, r² = 0.99; Posaconazole LCMS = 1.18(HPLC) − 0.04, r² = 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, r² = 0.99; Hydroxy-Itraconazole LCMS = 1.05(referral lab) + 0.04, r² = 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 r² = 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 concentrations of these antifungal agents.
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 hydroxyitraconazole.

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-hydroxy-itraconazole 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, Chromsys, 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.

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(σ of the blank/slope of the calibration curve) ($n = 10$). 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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Parent (m/z)</th>
<th>Daughter (m/z)</th>
<th>Collision (V)</th>
<th>Comments</th>
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</thead>
<tbody>
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<td>Voriconazole</td>
<td>350</td>
<td>281</td>
<td>20</td>
<td>Quantifier</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>350</td>
<td>224</td>
<td>20</td>
<td>Qualifier</td>
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<td>Posaconazole</td>
<td>701.4</td>
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<td>701.4</td>
<td>127</td>
<td>35</td>
<td>Qualifier</td>
</tr>
<tr>
<td>Itraconazole</td>
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<td>392.2</td>
<td>35</td>
<td>Qualifier</td>
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<td>432.4</td>
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<td>408.3</td>
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<tr>
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<td>721.4</td>
<td>392.3</td>
<td>35</td>
<td>Qualifier</td>
</tr>
</tbody>
</table>
concentrations of triazole solutions. Spiked samples were assayed in triplicate and the mean % triazole recovery was calculated from measured versus expected concentrations. The goal was to achieve 90–110% mean analytical recovery across the spiked range.

2.6.5. Ion suppression

Ion suppression was assessed by continuous post-column infusion of the 4 triazoles and their respective internal standards. These were infused directly into the mass spectrometer during the chromatographic analysis of 5 unique blank EDTA plasma extracts.

A standard solution of all analytes at 1 μg/mL was infused at a flow rate of 10 μL/min during the chromatographic analysis of the 5 unique blank plasma extracts.

The chromatographic signals of each selected MS/MS transition were examined to check for any signal perturbation of the MS/MS signal at the analytes' retention times. Deviations of > 10% from baseline signal were considered significant [3,4].

2.6.6. Ion ratios

The ratio of the qualifier ions relative to the quantifier ions (calculated by dividing the lower by the higher response) was used for analyte confirmation. The ion ratios for six standards were averaged and this was used as a reference ion ratio. The ratio tolerance was set at ± 20%

2.6.7. Method comparison

Voriconazole and posaconazole patient EDTA plasma samples (n = 50) were analysed by both the LCMS/MS method and the routine HPLC method currently in use in the laboratory.

Itraconazole and hydroxy-itraconazole patient EDTA plasma and lithium heparin samples (n = 18) were analysed by both the new LCMS/MS method and by an external referral laboratory's LC/MS/MS method.

Concentrations determined by each method were compared by Passing Bablok analysis. Additionally our laboratory participates in the External Quality Assurance program - UK NEQAS Antibiotic Assays (http://www.ukneqasaa.win-uk.net). Plasma samples distributed by this scheme were analysed by the LCMS/MS method, and the values obtained were compared using Passing Bablok analysis to those obtained by other LCMS/MS users in the program.

2.6.8. Statistics

Linearity and method comparison analysis were performed with Analyse-it version 2.21 software.

3. Results

The described LCMS/MS method enables the simultaneous quantification of three antifungal triazoles and one triazole metabolite. Using a 50 μL sample volume (for each calibrator (n = 4), control (n = 3), or patient sample (n = 10)) and a simple protein precipitation step using the internal standard solution prepared in acetonitrile, the total sample preparation time for this LC-MS/MS method was approximately 20 min. The analytical run time is < 5 min per injection.

Retention times observed were 1.52, 2.40, 2.73 and 2.42 min for voriconazole, posaconazole, itraconazole and hydroxy-itraconazole respectively. A typical chromatogram is shown in Fig. 1.

The assay was found to be linear using linear regression for all compounds; Voriconazole between 0.13 and 6.54 mg/L (slope: 1.00, intercept: − 0.18, r² = 0.99); Posaconazole between 0.16 and 5.66 mg/L (slope: 0.99, intercept: − 0.06, r² = 0.99); Itraconazole between 0.18 and 3.64 mg/L (slope: 1.00, intercept: − 0.04, r² = 0.99); OH-Itraconazole between 0.18 and 3.81 mg/L (slope: 1.02, intercept: 0.01, r² = 0.99).

The LOD for voriconazole, posaconazole, itraconazole and hydroxy-itraconazole were 0.09 mg/L, 0.07 mg/L, 0.02 mg/L and 0.04 mg/L respectively.

The LLOQ, defined by a CV and target deviation of < 20% for all antifungal triazoles tested was 0.10 mg/L. The following results were achieved: voriconazole CV 4%, bias 13%; posaconazole CV 9%, bias 11%; itraconazole CV 5%, bias 11%; and hydroxy-itraconazole CV 6%, bias 16%.

Intra and inter-day precision for each compound is shown in Table 2. All intra assay CVs were < 6.8%, and bias < 9.0%. All inter assay CVs were < 8.3% and bias < 8.0%.

This method has been in routine use in our laboratory for over 2 years, and shows good robustness as seen in long term internal quality control accuracy and precision (CV < 10%).

The assay shows good accuracy with mean (SD) recoveries from aqueous standard solutions of 95% (2%), 101% (15%), 96% (3%) and 93% (6%) for voriconazole, posaconazole, itraconazole and hydroxy-itraconazole respectively (n = 4 for each analyte).

The matrix effect was investigated by assaying five unique blank plasma samples.

Quantification of the matrix effect shows ion enhancement for itraconazole and ion suppression for voriconazole, posaconazole and hydroxy-itraconazole. The obtained percentages of ion enhancement and suppression were as follows; voriconazole 1%, posaconazole 10%, itraconazole 8% and hydroxy-itraconazole 2%. As the deviation of the peak area never exceeded 10% the effect is considered negligible within the dynamic range of the assay.

Voriconazole (n = 50) and posaconazole (n = 50) samples were analysed and the Passing Bablok comparisons showed a good correlation with our current HPLC method (Voriconazole LCMS = 0.94(HPLC) + 0.03, r² = 0.99, Posaconazole LCMS = 1.18(HPLC) − 0.04, r² = 0.95) (Figs. 2 and 3).

Itraconazole and hydroxy-itraconazole (n = 18) samples were also analysed and the Passing Bablok comparisons showed good agreement with an external referral laboratory's antifungal LCMS/MS method (Itraconazole LCMS = 1.00(external referral lab) + 0.01, r² = 0.99, Hydroxy-Itraconazole LCMS = 1.05(external referral lab) + 0.04, r² = 0.99).

External quality assurance samples for posaconazole and voriconazole (n = 12, UK NEQAS Antifungal Pilot Panel) were assayed 'blind' and the results were in good agreement with consensus mean values (Voriconazole LCMS = 1.01(UK NEQAS LCMS Mean) − 0.02, r² = 0.99, Posaconazole LCMS = 0.99(UK NEQAS LCMS Mean) − 0.03, r² = 0.99)). No quality assurance samples containing itraconazole or hydroxy-itraconazole were available for analysis.

4. Discussion

We have developed a rapid triazole antifungal LCMS/MS method requiring minimal pre-analytical sample preparation. The chromatographic run time is 5 min and the sample volume required is 50 μL. The small sample volume allows TDM in special populations such as pediatric patients.

All compounds were detected in plasma with good accuracy and reproducibility at the limit of quantification making this method suitable for both TDM and for pharmacokinetic-pharmacodynamic studies.

Over recent years, TDM of triazole agents has become routine daily practice. These drugs demonstrate large inter- and intra-individual pharmacokinetic variability and can also show non-linear pharmacokinetics. Based on current evidence, levels of recommendation for TDM with antifungal agents have been published [5,6].

Serum voriconazole levels ranging from 0.5 mg/L to 5.5 mg/L have been associated with improved clinical response, decreased breakthrough infections and reduced mortality [6–10]. Voriconazole exhibits non-linear pharmacokinetics. Increasing the dose of voriconazole by 50% can result in a 150% increase in serum concentration and a
Table 2
Intra and inter-day accuracy and precision.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Nominal Concentration (mg/L)</th>
<th>Calculated Concentration (SD) (mg/L)</th>
<th>Intra-day Precision</th>
<th>Inter-day Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Accuracy (%)   CV (%)</td>
<td>Calculated Concentration (SD) (mg/L)</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>0.96</td>
<td>0.93 (0.04)</td>
<td>97               4.1</td>
<td>0.92 (0.04)</td>
</tr>
<tr>
<td></td>
<td>2.19</td>
<td>2.29 (0.10)</td>
<td>96               4.4</td>
<td>2.21 (0.16)</td>
</tr>
<tr>
<td></td>
<td>4.28</td>
<td>4.62 (0.20)</td>
<td>93               6.7</td>
<td>4.50 (0.26)</td>
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<tr>
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<td>93               6.7</td>
<td>0.55 (0.03)</td>
</tr>
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<td></td>
<td>1.14</td>
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<td>91               3.0</td>
<td>1.19 (0.08)</td>
</tr>
<tr>
<td></td>
<td>4.60</td>
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<td>96               3.6</td>
<td>4.77 (0.31)</td>
</tr>
<tr>
<td>Itraconazole</td>
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<td>100              4.4</td>
<td>0.29 (0.02)</td>
</tr>
<tr>
<td></td>
<td>0.60</td>
<td>0.57 (0.01)</td>
<td>95               2.4</td>
<td>0.55 (0.03)</td>
</tr>
<tr>
<td></td>
<td>1.50</td>
<td>1.36 (0.03)</td>
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<td>1.43 (0.12)</td>
</tr>
<tr>
<td>Hydroxy-Itraconazole</td>
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<td>98               6.8</td>
<td>0.43 (0.03)</td>
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<tr>
<td></td>
<td>0.83</td>
<td>0.89 (0.04)</td>
<td>93               4.1</td>
<td>0.87 (0.03)</td>
</tr>
<tr>
<td></td>
<td>2.29</td>
<td>2.45 (0.06)</td>
<td>93               2.6</td>
<td>2.45 (0.18)</td>
</tr>
</tbody>
</table>

Fig. 1. Chromatogram of voriconazole (RT 1.52), posaconazole (RT 2.40), itraconazole (RT 2.73) and hydroxy-itraconazole (RT 2.42).

Fig. 2. Passing-Bablok curve of validated LCMS/MS voriconazole method versus established HPLC voriconazole method (n = 50).

Fig. 3. Passing-Bablok curve of validated LCMS/MS posaconazole method versus established HPLC posaconazole method (n = 50).
significant increase in serum half-life due to saturation of its metabolism and systemic clearance [8]. Certain voriconazole-associated toxicities are associated with higher serum concentrations [6,11].

The pharmacokinetics of posaconazole are characterised by an increased absorption in a fatty environment, saturable absorption that significantly impacts its bioavailability, a long half-life and linear elimination [12].

Although posaconazole displays predictable pharmacokinetic properties, gastric acidity and gastrointestinal integrity can influence its bioavailability. Patients receiving acid-suppressive therapy, those with mucositis, or those with gastrointestinal graft-versus-host disease (GVHD) are likely to have decreased posaconazole levels [6]. Conversely, elderly patients and those with hepatic impairment may show higher posaconazole levels.

Presently, posaconazole TDM appears to be limited to patients with GVHD of the gastrointestinal tract, severe diarrhoea, potential for drug interactions that lower posaconazole levels and those receiving proton pump inhibitors [6].

Itraconazole TDM is useful to optimise dosing due to inter- and intra-patient pharmacokinetic variability, suboptimal absorption of itraconazole particularly in people with impaired gastrointestinal integrity, and drug-drug interactions.

A concentration-effect relationship between itraconazole levels and toxicity has been identified with levels ranging from 0.25 mg/L to 1.0 mg/L associated with pharynxis and invasive fungal infection treatment efficacy [13]. However, there are no studies that support a direct correlation relationship between serum levels and itraconazole toxicity [14,15].

As triazole antifungals are used for the prevention and treatment of systemic infections, often over long periods of time, the high risk of side effects and toxicity justify TDM.

For high throughput analysis of large sample numbers, a rapid and simultaneous method of analysis for triazole antifungals is needed. Having the same method of analysis for four commonly used triazole antifungal drugs enables daily TDM of these compounds, without requiring additional changes in columns or mobile phases. Our method has a rapid extraction procedure and an analysis time of < 5 min. This results in cost effective use of a routine MS instrument, allowing real time processing of patient blood samples. Clinicians are able to respond promptly to sub-therapeutic or toxic concentrations of these triazole agents to optimise treatment of critically ill patients.

To date there are few methods published for the simultaneous quantification of triazole antifungals in human plasma [16–22]. In the literature several plasma triazole methods have been described with conventional methods like HPLC-UV or HPLC-fluorescence using liquid-liquid extraction (LLE) or solid phase extraction (SPE) but these often lack sensitivity and selectivity, the sample preparation is time consuming, and the longer run times result in delayed turnaround times for TDM.

Although SPE and LLE are cleaner than protein precipitation, these methods often result in an increase in method complexity, run time and costs. On line SPE reduces ion suppression effects and can increase sensitivity but is unable to be used for all of the different antifungal agents and is therefore less suitable for routine TDM [17].

Using chromatographic separation together with MS/MS detection ensures that potential interferences from inactive metabolites and other compounds are reduced.

To date, most published methods use only one mass transition to detect the compound and another transition to detect the internal standard. In this method we used a second mass transition for the analyte and its corresponding internal standard. This allows ion ratio monitoring which adds to method selectivity enabling detection of potential interferences [23]. With this method we found that the ion ratios were consistent across a range of concentrations, with relative standard deviations (RSDs) generally < 10%. Ion ratio deviation was usually only seen when samples exhibited low signal/noise ratios such as those with concentrations close to the LLOQ.

Electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) are the most commonly used soft ionisation sources in mass spectrometry [24,25].

In this method we used positive ESI mode, which has been shown to be more susceptible to matrix effects than APCI. This may result in analytical problems such as poor reproducibility, elevated baseline and divergent curves. However, we did not come across any of these difficulties during our method validation process and did not find any significant matrix effect for this method.

The use of an appropriate internal standard compound is essential for a reliable and precise LCMS/MS method. In LCMS/MS methods deviations in ion yield can vary markedly between different compounds. Therefore, it is important that any short term changes in ionisation yield of a target analyte and its respective internal standard due to in source variables such as contamination is similarly affected. This is ideally provided by stable isotope labelled internal standards.

This method was developed using deuterated isotopic compounds of the tested triazoles and metabolite. Despite a suboptimal chromatographic separation of posaconazole and hydroxy-itraconazole within the 5 minute gradient program, the use of deuterated internal standards avoided any reciprocal influence on their respective ionisation efficiencies.

A disadvantage of this method is that it uses commercially available calibrators which limit the linearity of the compounds to the highest calibrator concentration. Some clinical samples may have concentrations above the working range of the calibrators, however dilutions are easily prepared using drug free plasma.

Our method does not measure all antifungal agents, for example we do not measure caspofungin or fluconazole. Using our current gradient program fluconazole elutes at the same retention time as voriconazole and due to the high ionisation signal of voriconazole it is subsequently difficult to measure fluconazole using this method. In our hospital based clinical setting, monitoring of plasma fluconazole levels is rarely requested due to the reliable bioavailability and the benign toxicity of the drug.

We did not perform any stability studies however the long term stability of antifungal agents in plasma has been described previously [18,19,21,26,27]. Triazole antifungals in plasma EDTA have been found to be stable at −30 °C for over 2 months.

5. Conclusion

The rapid pre-analytical sample preparation procedure, short chromatographic time, limit of quantitation and linear range make this LC-MS/MS method suitable for determination of plasma voriconazole, posaconazole, itraconazole and hydroxy-itraconazole levels in a high throughput laboratory, providing a TDM service with clinically timely results.

References


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