Development and validation of a new method to simultaneously quantify triazoles in plasma spotted on dry sample spot devices and analysed by HPLC-MS

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Received 12 April 2012; returned 13 May 2012; revised 14 June 2012; accepted 27 June 2012

Objectives: Therapeutic drug monitoring (TDM) of triazoles is widely used in clinical practice to optimize therapy. TDM is limited by technical problems and cost considerations, such as sample storage and dry-ice shipping. We aimed to develop and validate a new method to analyse itraconazole, posaconazole and voriconazole in plasma spotted on dry sample spot devices (DSSDs) and to quantify them by an HPLC system.

Methods: Extraction from DSSDs was done using n-hexane/ethyl acetate and ammonia solution. Samples were analysed using HPLC with mass spectrometry (HPLC-MS). Accuracy and precision were assayed by inter- and intra-day validation. The stability of triazoles in plasma spotted on DSSDs was investigated at room temperature for 1 month. The method was compared with a validated standard HPLC method for quantification of triazoles in human plasma.

Results: Mean inter- and intra-day accuracy and precision were <15% for all compounds. Triazoles were stable for 2 weeks at room temperature. The method was linear (r² > 0.999) in the range 0.031–8 mg/L for itraconazole and posaconazole, and 0.058–15 mg/L for voriconazole. High sensitivity was observed; limits of detection were 0.008, 0.004 and 0.007 mg/L for itraconazole, posaconazole and voriconazole, respectively. A high degree of correlation (r² > 0.94) was obtained between the DSSD method and the standard method of analysis.

Conclusions: The method that we developed and validated to quantify triazoles in human plasma spotted on DSSDs is accurate and precise. It overcomes problems related to plasma sample storage and shipment, allowing TDM to be performed in a cheaper and safer manner.

Keywords: voriconazole quantification, posaconazole quantification, itraconazole quantification, HPLC, dry spot, therapeutic drug monitoring

Introduction

The incidence of invasive fungal infections has greatly increased over the past two decades, becoming a serious clinical problem as morbidity and mortality are rising. Many infections are due to the well-known Candida albicans and Aspergillus fumigatus, but new fungal pathogens are emerging, such as non-fumigatus species of Aspergillus, opportunistic yeast-like fungi and more than 17 different species of Candida. Although triazoles have revolutionized the prevention and treatment of invasive fungal infections, their pharmacological properties are still unclear. Itraconazole, posaconazole and voriconazole show high inter- and intra-subject pharmacokinetic variability and, in some cases, show non-linear pharmacokinetics. Triazoles interact with many different co-administered agents. They are substrates for, and inhibitors of, cytochrome P450 enzymes and membrane transporters such as P-glycoprotein. This may cause alteration in pharmacokinetic profiles leading to underdosing or overdosing of both drugs involved. Therefore, therapeutic drug monitoring (TDM) is important to optimize therapy in terms of improving efficacy and reducing toxicity. TDM should be routinely used, especially in high-risk patients, such as individuals undergoing solid-organ, blood and marrow transplantation, individuals undergoing major surgery and immunosuppressive therapy, and those being treated for AIDS, neoplastic diseases and conditions associated with advanced age and premature birth.
important to underline that TDM is limited by technical problems and cost considerations, such as sample storage and dry-ice shipping. An attractive method for collecting samples for TDM and pharmacokinetic studies is using dry blood spots or dried plasma spots. Recently, a dried blood spots technique to quantify posaconazole in human whole blood samples was published.\(^5\) Drug quantification using plasma instead of blood, on dried sample spot devices (DSSDs), may represent a viable option in clinical practice for a cost-effective strategy in sample handling. Drug quantification on DSSDs has been described previously.\(^6\,7\) We aimed to develop and validate a new chromatographic method for simultaneous plasma quantification of itraconazole, posaconazole and voriconazole using DSSDs.

**Methods**

**Chemicals and reagents**

Posaconazole was purchased from Sequoia Research (Pangbourne, UK), while itraconazole, voriconazole and dimethyl diquinoxaline (QX, the internal standard (IS)) were purchased from Sigma–Aldrich (St Louis, MO, USA). Acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from J.T. Baker (Deventer, Holland). Formic acid was purchased from Riedel-de Haen (Seelze, Germany) and HPLC-grade water was purchased in glass tubes and dried in a vacuum centrifuge at 50°C. DSSDs were purchased from Biomicron (Turin, Italy). Blank plasma from healthy donors was kindly supplied by the blood bank of Maria Vittoria Hospital (Turin, Italy).

**Stock solution, standard (STD) and quality controls (QCs)**

Itraconazole, posaconazole and voriconazole stock solutions were made in methanol at 1 mg/mL. A stock solution of QX was prepared in methanol/HPLC-grade water (50:50, v/v). All stock solutions were stored at −20°C and used within 3 months, according to our previous work.\(^8\) For each analysis session we prepared a working solution of IS: 0.833 mg/L QX in methanol/HPLC-grade water (50:50, v/v). The highest concentration calibration standard (STD 9, used for the highest point on the calibration curve) was prepared by spiking stock solution into blank plasma. We used final concentrations of 8 mg/L for itraconazole and posaconazole and 15 mg/L for voriconazole. A calibration curve was obtained by serial dilution from STD 9 to STD 1 with a zero sample (matrix with IS). The calibration ranged from 8 to 0.031 mg/L for itraconazole and posaconazole and from 15 to 0.058 mg/L for voriconazole. QCs were prepared by adding a predetermined volume of stock solutions to blank plasma. The concentrations were: 5 mg/L (QC H), 3 mg/L (QC M), 1.5 mg/L (QC L) and 0.1 mg/L (QC LL) for itraconazole and posaconazole; and 6 mg/L (QC H), 4 mg/L (QC M), 1.5 mg/L (QC L) and 0.2 mg/L (QC LL) for voriconazole. STDs and QCs were stored at −20°C until use, and for no longer than 3 months.

**Sample preparation**

STDs, QCs and patient samples were thawed at room temperature, and 50 μL of plasma was gently and carefully spotted on each DSSD. Devices were dried at room temperature for 30 min. Subsequently, DSSDs were rolled up and put in 2 mL Eppendorf tubes. Then, 50 μL of IS, 1850 μL of extraction solution [1750 μL of n-hexane/ethanol acetate (75:25, v/v) and 100 μL of 15% ammonia solution] were added to each tube. Tubes were turned for 15 min at 40 rpm at room temperature and then kept at −80°C for 15 min. The organic phase was transferred in glass tubes and dried in a vacuum centrifuge at 50°C. Samples were then suspended in 100 μL of HPLC-grade water/acetonitrile (50:50, v/v), vortexed for 15 s and transferred to microvials. Aliquots of 50 μL of the samples were injected in the HPLC-MS system.

The DSSD method was compared with a standard method of extraction (plasma not spotted on DSSDs) to confirm the robustness of the developed assay. Patient samples were quantified using both methods, and correlation between the two assays was evaluated by linear regression analysis. The extraction procedure from plasma samples was done according to our previous work.\(^8\) Briefly, 50 μL of IS was added to 200 μL of each plasma sample. Proteins were precipitated with 200 μL of acetonitrile. After vortexing and centrifugation, the supernatant was diluted 1:1 with water, and 50 μL was injected in the HPLC-MS system.

**Chromatography**

Chromatographic separation was performed according to our previous published work.\(^8\) Briefly, a C-18 Atlantis T-3 5 μm column (150 mm×4.6 mm i.d.; Waters, Milford, CT, USA) protected by a Security Guard C-18 pre-column (4.0 mm×3.0 mm i.d.; Phenomenex, Torrence, CA, USA) at 35°C was used. The mobile phases were water with formic acid (0.05%) and acetonitrile with formic acid (0.05%). Detector settings were: electrospray ionization, positive polarity ionization; capillary voltage, 3.5 kV; source temperature, 110°C; desolvation temperature, 350°C; nitrogen desolvation flow, 400 L/h; and nitrogen cone flow, 50 L/h. The ions monitored for mz were the ion protonated ‘sions’ from each transition: 705.6→353.2 for itraconazole, 700.8→351.0 for posaconazole, 349.3→350.3 for voriconazole and 312.4→313.4 for QC. Cone voltage was 25 V for itraconazole, posaconazole and voriconazole, and 50 V for QC.

**Validation of the assay**

**Accuracy, precision, recovery and matrix effect**

Intra- and inter-day accuracy and precision were determined by five replicates of QC samples (high, medium and low). Accuracy was evaluated as percentage deviation of the mean from the true value. Precision was expressed as relative standard deviation (RSD) at each QC concentration. Recovery was calculated by comparing the peak areas of extracted and non-extracted QCs. Non-extracted QCs were prepared by spiking stock solutions of itraconazole, posaconazole, voriconazole and QX in the HPLC-grade water/acetonitrile solution. The matrix effect was assayed according to Matuszewski et al.\(^2\) the analytes were spiked after extraction into different blank plasma extracts, and the peak areas were compared with those of extracted QCs.

**Stability**

Long-term stability of spotted plasma samples was assayed by comparing the peak area of QC samples stored at room temperature for 1 month with QCs spotted immediately before the analysis. Post-preparative
stability was assayed by analysing extracts kept in an autosampler for 24 h at room temperature.

Specificity
The specificity of the method was assayed with blank plasma spiked with some potentially co-administered antibiotics and antifungals (ciprofloxacin, levofloxacin, moxifloxacin, ceftiraxone, ertapenem, imipenem, meropenem, amikacin, gentamicin, teicoplanin, vancomycin, linezolid, tigecycline, rifampicin, daptomycin, caspofungin, anidulafungin, amphoterin C B, sulfamethoxazole, tazobactam, trimethoprim and ceftazidime).

Lower limit of quantification (LLOQ) and limit of detection (LOD)
The LLOQ was considered the lowest point of the calibration curve (STD 1) that could be determined with a <20% deviation from the nominal concentration. This gave a signal to noise ratio >5. The LOD was calculated by serial dilutions of STD 1. LOD is the plasma concentration that had a signal to noise ratio >3.

The assay was also validated by comparing concentrations of patient plasma spotted on DSSDs with those obtained after ‘standard’ extraction, using 13, 15 and 23 patient samples (by both methods) for itraconazole, posaconazole and voriconazole, respectively. A linear regression analysis was used to correlate the results. ANOVA was used to assess the statistical significance of the linear regression.

All validation procedures were performed according to FDA guidelines.

Results
Retention times were 4.54 ± 0.20, 4.95 ± 0.20, 6.99 ± 0.20 and 10.14 ± 0.20 min for voriconazole, QX (IS), posaconazole and itraconazole, respectively. Calibration curves were created by plotting the height ratio of each drug relative to the IS against the various drug concentrations in the spiked plasma standards. A quadratic forced-through-zero calibration curve was used for all drugs. Regression coefficients were >0.999 for the three drugs quantified.

We obtained mean intra- and inter-day accuracies of −6.33% and −2.41%, 2.77% and 3.06%, and −9.44% and 0.48%, for itraconazole, posaconazole and voriconazole, respectively. The corresponding mean intra- and inter-day precisions were 9.50 and 10.59, 8.54 and 12.76, and 9.92 and 9.84. Recovery (RSD) values were 49.7 (13.6%), 50.5 (10.6%), 81.44 (13.4%) and 18.4 (14.1%) for itraconazole, posaconazole, voriconazole and QX, respectively. In the matrix effect assay, the deviations (%) of the peak areas at the three concentrations for all analytes were similar (never exceeding −15.0%), showing the absence of a matrix effect.

Drug and IS stability in stock solutions was reported in our previous work. Long-term stability testing of plasma samples spotted on DSSDs showed that plasma samples spotted on the DSSDs and stored at room temperature for 1 month were stable, with degradations of <19% for posaconazole and voriconazole and <21% for itraconazole (Figure 1). In particular, in the first week we observed a mean degradation <5% for the three triazoles; at 2 weeks, mean degradation was <17%. Autosampler stability testing showed absence of degradation during 24 h in an autosampler at room temperature. Selectivity tests showed absence of interference with drugs co-administered to the patients.

The LLOQ for itraconazole and posaconazole was 0.031 mg/L, and the LLOQ for voriconazole was 0.058 mg/L. The LODs were 0.008 mg/L for itraconazole, 0.004 mg/L for posaconazole and 0.007 mg/L for voriconazole.

Finally, we compared plasma concentrations obtained by quantification on DSSDs with those obtained by ‘standard’ quantification in plasma samples. The variation between the two methods was <20% for the three drugs. The high correlations (r = 0.944 for itraconazole, r = 0.992 for posaconazole and r = 0.959 for voriconazole) confirmed the robustness of the method (Figure 2). ANOVA analysis showed statistical significance (P < 0.0001) for all drugs tested.

Discussion
TDM of triazoles is essential in clinical practice to optimize therapy. As laboratories able to perform this analysis of drugs are not available at all hospitals, transportation is commonly required to perform analysis. Therefore, a simple and cost-effective procedure of shipping samples with DSSDs could help clinicians to routinely perform TDM. The method we developed and validated on DSSDs for triazoles has the following advantages: (i) the procedure for storage and shipment of plasma samples is cheap and simple; (ii) the method shows a good

![Figure 1](https://academic.oup.com/jac/article-lookup/doi/10.1093/jac/dkab230) Long-term stability of QC plasma samples spotted on DSSDs and stored at room temperature for 30 days. ITC, itraconazole; POS, posaconazole; VRC, voriconazole.
LLOQ, which allows low plasma levels to be identified with a small amount of sample, especially important for special patient populations such as paediatric patients; (iii) the calibration curves cover a wide range of plasma concentrations; and (iv) there is good correlation between the DSSD method and the ‘standard’ method. The standard method is highly reliable; it was also used to measure the three drugs in plasma samples for the International Interlaboratory Quality Control Program for Therapeutic Drug Monitoring of Antifungal Drugs (Lab. no. 26). Accuracy obtained by the Quality Control Program was <15% for the three triazoles assayed.

The results of the long-term stability tests confirm the suitability of the use of DSSDs for clinical practice, allowing plasma samples to be stored at room temperature. Considering the higher degradation over 30 days compared with 2 weeks, we suggest samples should be analysed within 15 days of preparation. In a TDM clinical setting the turnaround time should be <5 days.

Quantification of drugs on dried blood spots could be influenced by haematocrit values. In the study by Reddy et al., it was reported that this analytical method gave acceptable results for haematocrit values in the range 25%-41%. Thus, their technique might not be suitable for all patients. The method we validated can be applied to all patients regardless of haematocrit. Moreover, plasma concentration (rather than blood concentration) better reflects efficacy and toxicity cut-off values considered in clinical practice.

Conclusions

We have developed and validated a new method to simultaneously quantify itraconazole, posaconazole and voriconazole in plasma samples spotted on DSSDs and analysed by HPLC-MS. The method correlates well with a ‘standard’ and validated analytical method. Therefore, it could be used for routine TDM, and allows shipping of samples in a safe and cheap manner for hospitals without TDM laboratories.

Funding

This study was supported by internal funding.

Transparency declarations

None to declare.

References

Quantification of triazoles on dry sample spot devices

7 Kolocouri F, Dotsikas Y, Loukas YL. Dried plasma spots as an alternative sample collection technique for the quantitative LC-MS/MS determination of gabapentin. Anal Bioanal Chem 2010; 398: 1339–47.


