Ultrafast Method for Simultaneous Measurement of Triazole Antifungals in Human Serum Using Online SPE/MS/MS

Application Note

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Abstract

Clinical research laboratories traditionally rely on HPLC and LC/MS/MS for quantitative analysis of antifungal drugs. This Application Note describes a rapid and robust online SPE/MS/MS analytical method for high-throughput and accurate measurement of five antifungal drugs (voriconazole, ketoconazole, fluconazole, itraconazole, and posaconazole) in human serum. This method employs protein precipitation followed by dilute and shoot analysis on a SPE/MS/MS system, enabling the analysis of all five antifungals at a rate of 14 seconds per sample. This analytical method produces > 10x savings in analysis time and solvent consumption compared to typical HPLC-based methods.

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Introduction

Traditional analytical measurements of antifungal drugs in serum use microbiological and chromatographic methods that do not overcome the interferences of measuring multiple analytes in this class, or address the desired time to results of < 24 hours[1]. The need for greater throughput and faster turn-around times has increased demands on these traditional methods. The Agilent RapidFire High-throughput Mass Spectrometry System is an ultrafast SPE/MS/MS system capable of analyzing samples with cycle times under 15 seconds per sample. In this study, we developed an ultrafast SPE/MS/MS method for the simultaneous analysis in human serum of five antifungal drugs: (voriconazole, ketoconazole, fluconazole, itraconazole, and posaconazole) with much faster sample cycle times and similar analytical results compared to LC/MS/MS assays[2].

A simple protein precipitation methodology followed by dilute and shoot analysis on a RapidFire SPE/MS/MS system allows for the accurate and precise measurement of these analytes in human serum over a linear range of 0.2–25 µg/mL. Samples were analyzed on the RapidFire system at 14 seconds per sample, providing a much higher throughput method of analysis. This rapid analytical method has the specificity and accuracy necessary for an efficient quantitative workflow.

Experimental

The Agilent RapidFire/MS/MS system consisted of the following modules: Agilent RapidFire 365, Agilent 6460 Triple Quadrupole Mass Spectrometer using Agilent MassHunter Triple Quadrupole Acquisition Software (B.04.01) with Qualitative Analysis (B.05.00), Quantitative Analysis (B.05.00), and Agilent RapidFire Acquisition Software.

Samples were analyzed at a rate of 14 seconds per sample. Quantitative and qualitative ions for all five antifungals and internal standards were monitored simultaneously in all experiments (Table 1). Agilent Mass Hunter Quantitative software automatically calculated qualifier ion ratios.

Table 1. Agilent RapidFire/MS/MS conditions.

RapidFire conditions	
Buffer A (pump 1)	10 mM ammonium acetate in LC/MS grade water + 0.1 % formic acid + 0.01 TFA; 1.5 mL/min flow rate
Buffer B and C (pumps 2 and 3)	100 % LC/MS grade methanol; 1.25 and 0.8 mL/min flow rate, respectively
Aqueous wash	HPLC grade water
Organic wash	HPLC grade methanol
Injection volume	10 µL
SPE cartridge	Agilent RapidFire cartridge C (reversed-phase C18, G9205A)
RF State 1	600 ms
RF State 2	3,000 ms
RF State 3	0 ms
RF State 4	8,000 ms
RF State 5	2,000 ms
Triple quadrupole conditions	
Electrospray, positive ionization	
Gas temperature	300 °C
Gas flow	13 L/min
Nebulizer	45 psi
Sheath gas flow	12 L/min
Sheath gas temperature	375 °C
Nozzle voltage	500 V
Capillary voltage	3,000 V
Peak width	0.07

Chemicals and reagents

All of the antifungal analytes were purchased from Sigma Aldrich (St. Louis, MO) or Santa Cruz Biotech (Santa Cruz, CA). All of the stable-labeled isotopic internal standards were purchased from Santa Cruz Biotech. The two levels of quality controls were obtained from Utak Laboratories (Valencia, CA). All other solvents and reagents were purchased from Fisher Scientific (Hanover Park, IL).

Sample preparation

The samples, calibrators (0.2, 1, 2.5, 5, 10, and 25 µg/mL) and QC levels were prepared using the following procedure. First, 200 µL of sample was added to a 1.5-mL microcentrifuge tube. Next, 50 µL of internal standard mix in methanol at a concentration of 1 mcg/mL was added and the sample was gently mixed. Acetonitrile, 800 µL, was then added, followed by vigorous vortexing for 3 minutes. The samples were centrifuged at 13,000 rpm for 10 minutes. A portion of the supernatant from each tube (100 µL) was added into the well of a deep well plate containing 900 µL of 2 % ammonium hydroxide in LC/MS grade water. The plate was then sealed with an Agilent PlateLoc Thermal Microplate Sealer, mixed and centrifuged prior to RapidFire/MS/MS analysis.

Data analysis

System control and data acquisition were performed by Agilent MassHunter Triple Quadrupole Data Acquisition Software.

Calibration curves were constructed using linear and quadratic least squares regression with 1/X weighting for the multiple reactions monitoring (MRM). The quantitation using MassHunter Quantitative software was performed by spectral peak area ratio to a known concentration of the internal standards.

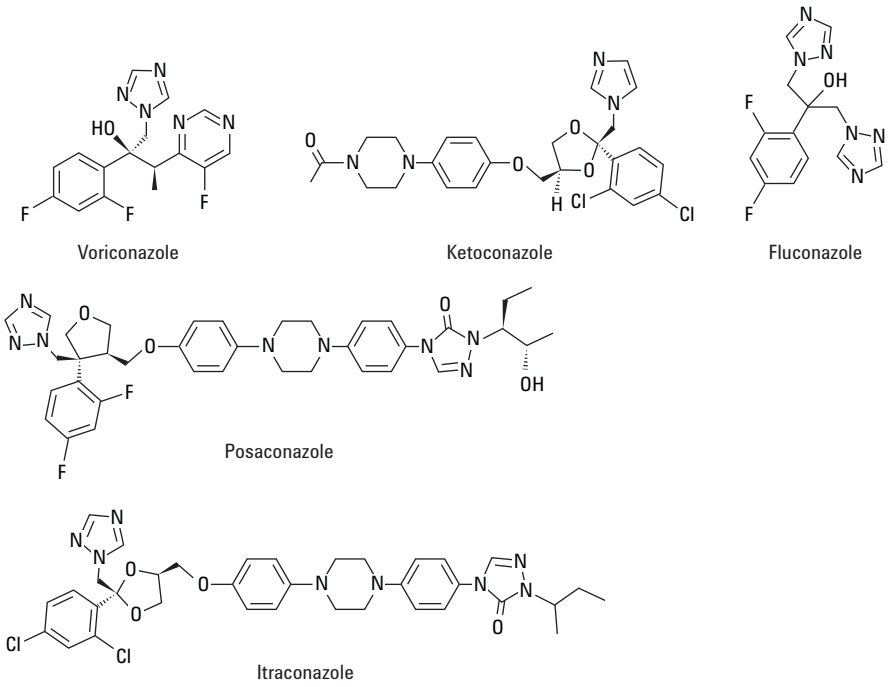

Figure 1. Chemical structures of the five antifungal analytes.

Table 2. MRM transitions.

Compound	Precursor ion	Product ion	Dwell	Fragmentor	Collision energy	CAV
Itraconazole	705.3	432.2	20	231	33	3
Itraconazole Q	705.3	392.2	20	215	36	3
Posaconazole Q	701.8	683.4	20	205	29	3
Posaconazole	701.8	127.0	20	205	60	3
Ketoconazole-d8	539.1	82.0	15	155	53	3
Ketoconazole	531.1	489.1	15	187	33	3
Ketoconazole Q	531.1	82.1	15	187	49	3
Voriconazole-d3	353.2	284.1	15	99	13	3
Voriconazole	350.2	281.1	15	99	13	3
Voriconazole Q	350.2	127	15	99	37	3
Fluconazole-d4	311.1	242.1	15	124	13	3
Fluconazole	307.1	238.1	15	99	13	3
Fluconazole Q	307.1	220.1	15	99	17	3

Q = Quantifier ion

Results and Discussion

Samples were prepared by spiking antifungal analytes into drug-free human serum, followed by addition of an internal standard mix, a protein crash with acetonitrile, and then dilution of the samples 10-fold with 2 % ammonium hydroxide in water. Samples were then analyzed through SPE/MS/MS using the RapidFire/MS/MS system and a reversed-phase C18 cartridge at 14 seconds per sample (Figure 2). This RapidFire/MS/MS methodology is capable of throughputs greater than 240 samples per hour, providing a high-throughput and very efficient mode of analysis. Carryover was assessed by analyzing the AUC of the blank calculated as % of the mean peak area of the 0.2 µg/mL samples. No significant carryover (< 5.0 %) was determined for all of the antifungals (Figure 2). When measuring higher concentrations of antifungals (> 25 µg/mL), we recommend using one blank injection (methanol) between wells.

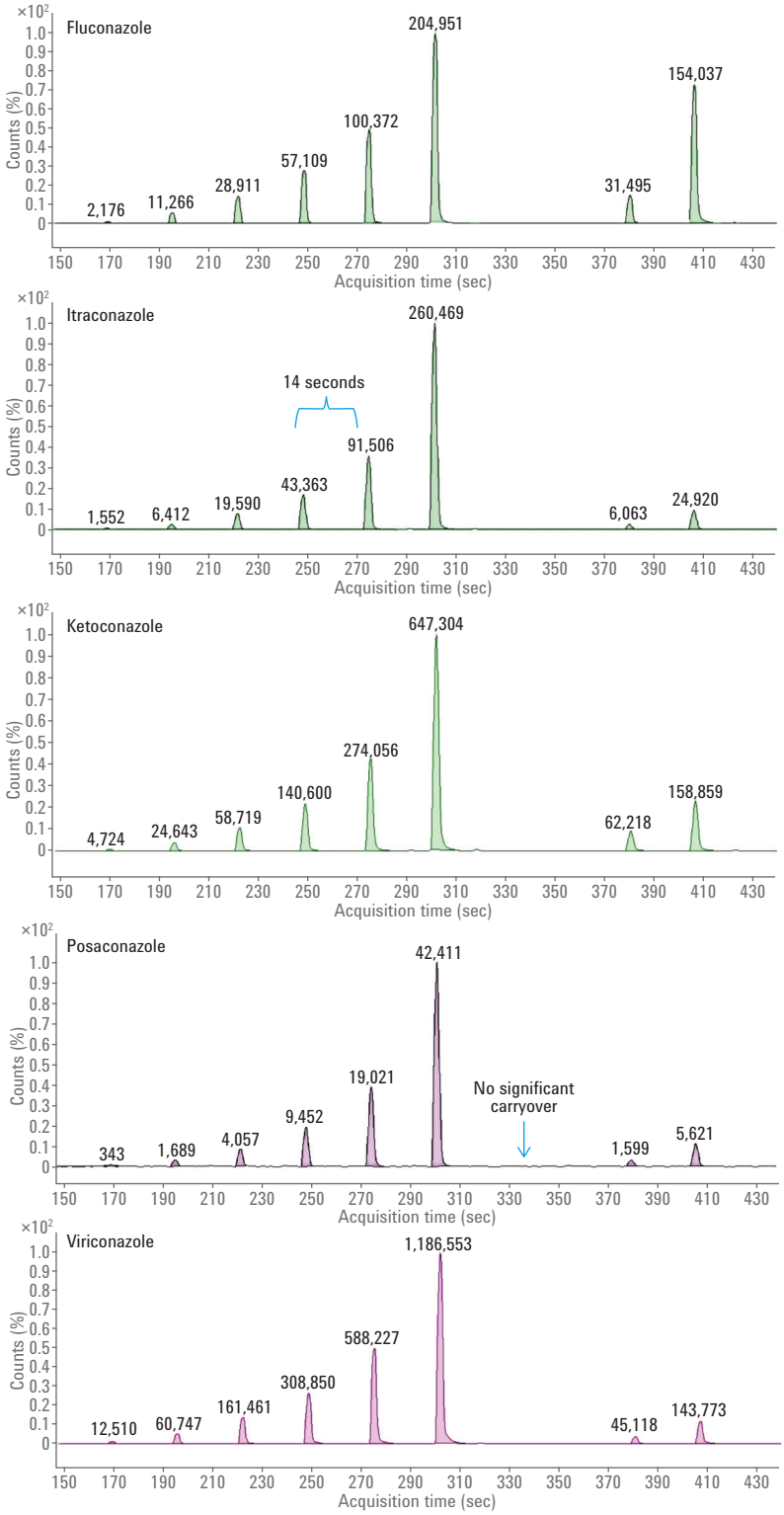

Figure 2. Representative calibration curves and QC data for each of the five antifungal analytes showing the injection-to-injection interval of 14 seconds. Carryover assessment using a matrix blank immediately after the highest calibrator for all analytes showed that no significant carryover was observed for any of the analytes.

Standard curves consisting of each antifungal spiked into serum had excellent linearity within the measured range (0.2–25 µg/mL) with an R^2 value greater than 0.995 (Figure 3). QC standards for each antifungal were run over a series of days to establish both intra- and interday precision and accuracy values. The accuracies determined were within 10 %, and coefficient of variation values were all less than 10 % for concentrations within the measured range (Table 3).

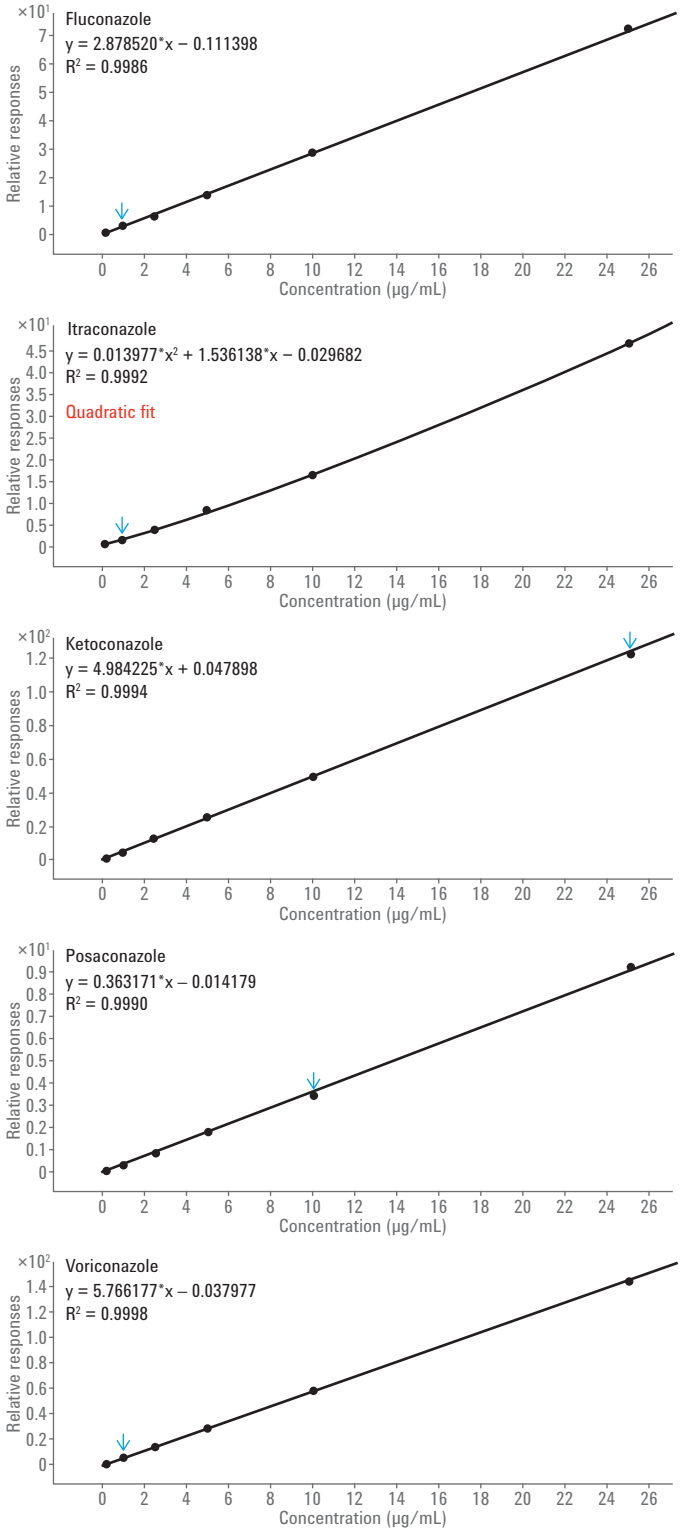

Figure 3. Representative calibration curves showing a linear range from 0.2–25 µg/mL for each of the five antifungal analytes.

The reproducibility of the method was evaluated by measuring > 2,000 sequential injections of all five antifungals spiked into serum. The instrument response was stable for each of the five analytes, with the coefficient of variation ranging from 2.3–6.9 %, showing the robustness of the RapidFire system, SPE cartridge lifetime, and consistency of quantitation for the analytes in the panel. The data for ketoconazole can be found in Figure 4, where the precision over > 2,000 injections was 4.4 %.

This procedure, consisting of a protein crash followed by dilute and shoot sample preparation and quick analysis on RapidFire/MS/MS, provides a very efficient method of measuring antifungals in human serum compared to traditional HPLC methods.

Table 3. Intraday and interday accuracy and precision data for the QC standards.

Concentration (µg/mL)	Interday % accuracy (n = 6)	Interday % precision (n = 6)	Intraday % accuracy (n = 6)	Intraday % precision (n = 6)
Voriconazole				
1.5	94.5	4.86	92.8	8.77
4.8	95.9	6.24	102.5	8.61
Itraonazole				
1.3	99.3	7.25	103.2	4.01
4.8	96.2	3.00	95.8	9.91
Fluconazole				
6.0	101.1	4.57	97.8	8.74
30	100.5	2.62	104.0	5.64
Ketoconazole				
4.0	94.5	4.04	102.5	8.34
17	103.2	2.76	99.2	5.74
Posaconazole				
1.3	107.3	10.00	101.5	7.73
5.0	101.5	5.02	99.8	9.73

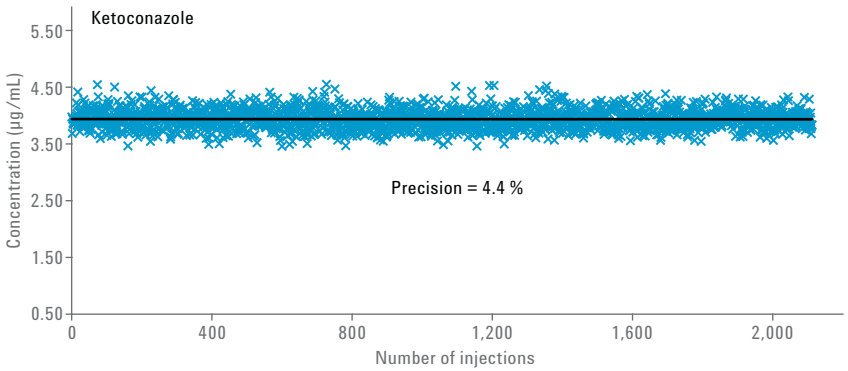

Figure 4. Reproducibility evaluation using sequential injections of ketoconazole.

Conclusions

Five antifungal drugs including voriconazole, ketoconazole, fluconazole, itraconazole, and posaconazole were quickly, accurately, and precisely measured in serum using a simple protein precipitation protocol and the Agilent RapidFire/MS system. Samples were analyzed at 14 seconds per sample, providing a high-throughput method capable of analyzing more than 240 samples per hour. This methodology provides comparable results to HPLC and LC/MS/MS, but at > 10x the speed and efficiency of these traditional methods. Therefore, this method provides a very efficient mode for measuring these five antifungal analytes in serum compared to traditional analytical methods.

References

1. Verdier, M-C.; *et al*. Liquid chromatography-tandem mass spectrometry method for simultaneous quantification of four triazole antifungal agents in human plasma. *Clin. Chem. Lab. Med*. Oct **2010**, *48(10)*, pp 1515-22.

2. Czasopismo, B.; *et al*. Rapid HPLC–MS/MS method for simultaneous quantitation of four routinely administered triazole antifungals in human plasma. *Clinica Chimica Acta* **2012**, *413*, pp 1-2, 240-245.

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